Digestion of insect chromatin with micrococcal nuclease, DNase I and DNase I combined with single-strand specific nuclease S1

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ABSTRACT

The chromatin of the lepidopteran Ephestia kuehniella was digested by micrococcal nuclease, DNase I and S1-nuclease combined with DNase I pretreatment. The resulting DNA fragments were analyzed by gel electrophoresis and compared with the DNA fragments of rat liver nuclei obtained by the same process. Extensive homology was revealed between insect and mammalian chromatin structure. The combined DNase I - S1-nuclease digestion yields double-stranded DNA fragments of lengths from 30 to 110 base-pairs. These DNA fragments are not obtained from nuclei predigested extensively with micrococcal nuclease. The results are discussed with respect to the internal structure of the chromatin subunit.

INTRODUCTION

The use of various nucleases as probes of chromatin structure together with other methods has lead to the nucleosomal model of the organization of the eucaryotic chromatin (1,2,3). These models are based partly upon the fact, that enzymatic degradation of DNA in situ or in isolated chromatin yields DNA fragments of discrete size classes, which are multiples of a monomer length of about 200 base-pairs (4,5,6). This basic DNA subunit has been found in all eukaryotic cells investigated with the appropriate biochemical methods (7,8). Differences however, exist in the DNA length belonging to one subunit in different species (7,8) or even between different cell types of the same organism (9,10). In spite of the variability of DNA length per subunit there seems to be a common inner structure of the nucleosome for all eukaryotes. The digestion of chromatin by endonucleases like DNase I and DNase II leads to a single-strand nicking of the DNA within the nucleosome at ten base-pair intervals (11,12,13) and this is identical for all eukaryotic organisms investigated.
I have investigated the chromatin of the silk gland nuclei of the meal moth by digesting isolated nuclei by micrococcal nuclease to determine the DNA repetition length of the chromatin subunits. As a probe for the internal nucleosomal structure the silk gland nuclei were digested by DNase I and the resulting DNA fragments were compared to those obtained from rat liver. In addition, I used the single-strand specific nuclease S1 as a further probe for the internal nucleosomal structure, in combination with DNase I. The S1 enzyme should recognize the single-strand nicks at the ten base-pair intervals produced by the DNase I pretreatment and one should obtain a series of double-stranded DNA fragments, which should correspond to the single-strand nicks produced by the DNase I.

MATERIAL AND METHODS

Isolation of nuclei: Highly polyploid nuclei were isolated from freshly prepared silk glands of 300 to 400 last instar larvae of the mediterranean meal moth, Ephestia kuehniella, kept in our laboratory.

The silk glands were homogenized in ice-cold isolation buffer (3mmol MgCl₂, 3mmol CaCl₂, 10mmol tris, pH 7.2, 0.25 mol sucrose, 1% NP 40) by pressing the suspension a few times through a narrow pipette (inner diameter about 200 μm). The sedimented nuclei were washed once in buffer containing 1% triton-X-100 and then twice in buffer without detergent. The nuclei were then resuspended in the desired digestion buffer to give a DNA concentration of about 100 µg/ml. The rat liver nuclei were isolated as described by HEWISH and BURGOYNE (4).

Digestion of nuclei:

Micrococcal nuclease. The standard reaction mixture consisted of 0.45 ml nuclei suspension in digestion buffer (1mmol CaCl₂, 0.5mmol tris, pH 7.2, 0.25 mol sucrose) and 25 units of micrococcal nuclease (Worthington, Biochemical Corp.). The digestion was carried out at 37°C for various periods of time ranging from 0-30 min. The reaction was stopped by adjusting the reaction mixture to a final concentration of 10mmol Na₂EDTA, 0.15mol NaCl and 1% sodium dodecyl sulphate. The lysed nuclei were then digested by proteinase K (Boehringer, Mannheim) at a concentration of 0.5 mg/ml reaction mixture.
for 2-3 hrs at $37^\circ C$. The DNA was extracted by the chloroform-
iso-amylalcohol procedure (11) and precipitated by two volumes
of ethanol overnight at $-20^\circ C$. In most experiments the con-
taminating RNA was removed by treatment of the redissolved
sample with RNase T1 (Boehringer, Mannheim) at a concentration
of 1500 U/ml for 2 hrs at $37^\circ C$.

DNase I-digestion. The digestion was either carried out as
described by NOLL (11) for rat liver nuclei or at a one
hundred times lower DNase I concentration, but with digestion
time extended to 20 min for the silk gland nuclei, which is
not applicable for rat liver nuclei because of the high endo-
genous endonuclease activity (4,12). The actual digestion
time and DNase concentrations are given below the appropriate
figures. DNase I was purchased from Serva, Heidelberg.

Combined DNase I-S1 nuclease digestion. If silk gland
nuclei were digested by the combined method the DNase I
digestion was carried out first as described above, then the
samples were chilled in ice-water and the nuclei sedimented by
short centrifugation, washed once in ice-cold S1-nuclease
digestion buffer ($0.03mol \text{NaCH}_2\text{COO}, 0.05mol \text{NaCl}, \text{1mmol ZnSO}_4$
$5\% v/v$ glycerol, $\text{pH}$ adjusted to 4.6 with acetic acid, (16))
and then resuspended in 0.45 ml S1-digestion buffer. The
samples were then brought to $37^\circ C$ and S1 nuclease was added to
give a concentration of about 20 units/ml. The S1 digestion
was carried out at $37^\circ C$ over various periods, indicated below
the appropriate figures. The reaction was stopped and the DNA
was extracted as described above. For rat liver nuclei this
digestion procedure gives no satisfactory results, therefore
rat liver nuclei were digested by DNase I and S1 nuclease
simultaneously in S1 digestion buffer at $37^\circ C$ for only 2 min
with an 10 fold higher concentration of the S1 enzyme, which
is 200 units/ml reaction mixture. (The S1 nuclease was pre-
pared according to the method described by VOGT (16) and the
enzyme activity was determined using heat-denatured HeLa-cell
DNA by measuring the acid solubility increase per unit of
time.)

Electrophoresis of the DNA fragments

The precipitated DNA was collected by centrifugation, washed
twice in 70% ethanol, dried under vacuum and then dissolved in 50-100 µl diluted electrophoresis buffer (17) containing 20% sucrose. Samples were applied onto 2%, 3%, 6% or 10% polyacrylamide slab gels or in some experiments cylindrical gels (diameter 6 mm) with a migrating distance of 12 cm. (The 2% polyacrylamide gels were sustained with 0.5% agarose). The electrophoresis was carried out at a constant current of 60 mA per slab gel or 3 mA per cylindrical gel. During electrophoresis the slab gels were cooled by running tap water (about 15-18°C). The electrophoresis was finished when the bromphenol-blue dye marker has migrated about 11 cm of the total length of 12 cm of the gel. For 10% gels this was usually reached after about 5 hrs. Electrophoresis under denaturing conditions was carried out on polyacrylamide slab gels containing 6 mol urea (12,18). The DNA samples were denatured by solution in diluted electrophoresis buffer containing 10 mol urea and heating the samples to 60°C for 10 min. After the electrophoresis the DNA in the gels was stained either by ethidium-bromide (2 µg/ml) or by 0.005% "stains-all"-formamide (Serva, Heidelberg).

Densitometrical measurements were done from the negatives by a JOYCE-LOEBL microdensitometer.

RESULTS

1. Micrococcal nuclease digestion. An uncomplete digestion of DNA in isolated silk gland nuclei of the meal moth yields a series of DNA fragments, with increasing molecular weights typical for the nucleosomal organization of the chromatin. The electrophoretic mobility of these DNA fragments from the silk gland nuclei is exactly the same as those derived from rat liver nuclei (Fig.1), so that one can assume the same DNA length per chromatin subunit which has been determined as appr. 200 base-pairs (5,14). If the digestion is extended all the DNA of the silk gland chromatin gets degraded to fragments smaller than the monomer length, with a major band of DNA fragments of 140 base-pairs in length (Fig.1). The length of these DNA fragments, derived from extended digestion, has been determined by electrophoresis under denaturing conditions using denatured DNA fragments produced by DNase I digestion of rat
Figure 1. A) Comparison of DNA fragments from rat liver nuclei and meal moth silk gland nuclei generated by short micrococcal nuclease digestion. 25 u/ml, 2 min at 37°C. Electrophoresis was carried out on a 2% polyacrylamide -0.5% agarose slab gel. Digestion: 25 u/ml, 2 min at 37°C.

B) Comparison of denatured DNA fragments of rat liver and meal moth silk gland nuclei generated by extended micrococcal nuclease digestion on a 10% polyacrylamide gel under den. conditions. 25 u/ml, 10 min at 37°C. The micrococcal nuclease digest DNA fragments are sized relative to a DNase I digest of rat liver nuclei (left sample, the most intensive band is accepted as the 80 bases fragment (11)). In the graph the sizes of the single-stranded DNA fragments are plotted against the electrophoretic migration. • • DNA fragments from a DNase I rat liver nuclear digest. Arrow indicates the position of the most intensive band from the micrococcal nuclease digest. In all photographs of gels the electrophoretic migration is from top to bottom.
liver chromatin as calibration standards (7,11) (Fig.1).

2. DNase I digestion. Mild digestion of isolated silk gland nuclei of the meal moth reveals an internal structural arrangement of the DNA within the nucleosome identical with that of rat liver (11), duck erythrocyte (8,19), HeLa-cells (8), yeast (8) and Neurospora (7). The electrophoretic analysis of denatured DNA extracted from DNase I treated silk gland nuclei shows clearly that the DNA is nicked with a regular spacing of multiples of 10 nucleotides (Fig.2). The single-stranded DNA fragments have exactly the same electrophoretic mobility as those obtained from rat liver nuclei (Fig.2), which indicates that the insect chromatin has the same internal chromatin structure as shown for other organisms (7,8,11,19).

3. Combined DNase I -S1 nuclease digestion. The single-strand specific nuclease S1 was used to obtain more information upon the cleavage sites of the DNase I within the chromatin. This single strand-specific nuclease might be able to recognize the single-strand breaks generated by the DNase I and therefore to produce double-strand breaks just at the same sites, where the pancreatic DNase has nicked the DNA before. Thus one should obtain double-stranded fragments of DNA of characteristic lengths, which should correspond to the DNase I single-stranded fragments. The electrophoretic analysis of the DNA extracted from nuclei, which were first digested by DNase I and then shortly by the S1 enzyme is shown in Fig.2. It is clearly visible, that distinct classes of double-stranded DNA fragments are present. At least 8 well-defined bands are visible. These bands do not appear, when nuclei are treated with S1 nuclease alone. They also do not appear in that intensity, when nuclei are digested by DNase I alone, but including washing and incubation in S1 digestion buffer without enzyme. In the experiments with the DNase I alone some of the bands are present, but very faintly stained and always on a high background smear (Fig.2).

The length of the DNase I -S1 nuclease generated DNA fragments was determined electrophoretically under denaturing conditions and calibrating the slab gel with DNase I generated single-stranded DNA fragments obtained from rat liver nuclei.
Figure 2. A) Denatured DNA fragments from meal moth chromatin digested with DNase I and DNase I -S1 nuclease, compared to the DNase I generated DNA fragments of rat liver nuclei. Chromatin of the silk glands was digested with DNase I (3 units/ml) for 20 min at 25°C alone or in addition with S1 nuclease (20 units /ml for 4 min at 37°C). RNA was digested with RNase T1 and DNA fragments were analyzed on a 10% polyacrylamide gel containing 6 mol urea.

B) Native DNA fragments from meal moth chromatin digested as described in 2A. The double-stranded DNA-fragments were analyzed on a 10% polyacrylamide slab gel without containing urea. All other conditions are identical between A and B.

The most intensive band was assumed as the 80 nucleotide band (7,11) (Fig.2). From this evidence arises, that the double-stranded DNA fragments produced by the combined DNase I -S1 digestion have lengths, which are consistent with the 10-nucleotide spacing of the DNase I digestion. No additional band appear. The relative intensities of the bands are different between the DNase I alone and the DNase I -S1
nuclease-treated nuclei. While in DNase I digests the 80-
nucleotide band is always more intensive than the other bands, it are the 70- and the 80-nucleotide band which are most intensive after the combined DNase I -S1 digestion (Fig.2). Furthermore the 60-nucleotide and the 120-nucleotide band, which are always weak but present in the DNase I-digestion of silk gland nuclei, disappear nearly completely after the S1-digestion.

The same digestion procedure applied upon nuclei pre-
digested extensively with micrococcal nuclease (40-50 %
acid-soluble material) does not lead to these DNA fragments
(Fig.3). The typical "micrococcal nuclease limit digest

Figure 3. Comparison of double-stranded DNA fragments
genenerated by a) DNase I -S1 digestion of native silk gland
chromatin (as in Fig.2). b) DNase I -S1 digestion of silk
 gland chromatin predigested with micrococcal nuclease (to 40 -
50% acid-soluble material). c) DNA fragment pattern generated
 by micrococcal nuclease digestion followed by DNase I without
S1 nuclease digestion and d) DNA fragment pattern generated
by micrococcal nuclease digestion alone. Reaction conditions
are the same as described in Fig.2. DNA fragments were
analyzed on 10% polyacrylamide gel under native conditions.
pattern" remains unaffected by the second digestion with DNase I -S1 nuclease. Only the DNA fragments shorter than 70 base-pairs appear to be present in a low concentration (Fig.3).

The combined DNase I -S1 nuclease digestion of rat liver nuclei under the same reaction conditions as applied to silk gland nuclei did not lead to well-defined double-stranded DNA fragments. This might be due to the high endogeneous endonuclease activity, which is present in rat liver nuclei (4,12). For a second incubation of rat liver nuclei (first incubation in DNase I) in the absence of S1 nuclease yields between 3 - 4% more acid-soluble material and in the presence of S1 nuclease there is an increase in acid-soluble material of only 5 - 6%. However, if both enzymes, S1 and DNase I, are applied simultaneously to rat liver nuclei for only two minutes, the same double-stranded DNA fragments are obtained as from the insect nuclei (Fig.4). But there is always a high background smear of DNA with continuously decreasing molecular weights.

Figure 4. DNA fragments from rat liver and meal moth chromatin digested with DNase I -S1 nuclease, analyzed electrophoretically under native conditions. A) DNA from rat liver nuclei, digested with DNase I (300 units/ml) and S1 nuclease (200 units/ml) simultaneously for 2 min at 37°C. B) DNA from silk gland nuclei digested as in Fig. 2.
DISCUSSION

Digestion of silk gland nuclei of the moth *Ephestia kuehniella* with different nucleases has revealed that the chromatin structure is similar to that of other eukaryotes (7,8,11,13). The chromatin repeating subunit, as tested by digestion of the isolated nuclei by micrococcal nuclease, is associated with approximately the same length of DNA as it is in rat liver chromatin. The DNase I digestion pattern indicates identical chromatin substructures in the insect chromatin and the rat liver chromatin.

This chromatin substructure is particular interesting, and several hypotheses have been formulated to explain the striking digestion pattern at multiples of ten bases. The DNA might be kinked (2,15) or bent (14) around the nucleosome, there might be one or more cleavage sites per nucleosome (20) and there might be even a heterogeneous population of nucleosomes (8,20). The introduction of the combined DNase I -S1 nuclease digestion should at least yield more information upon the DNase I cleavage sites within the nucleosomes.

For the interpretation of the results from the DNase I -S1 nuclease digestion it is important, if all or only part of the DNase I single-strand nicks are cleaved to double-strand breaks by the S1 enzyme. After denaturation of the DNase I -S1 generated double-stranded fragments, however, there seem to be no additional DNA bands and no increase in some of the single-stranded bands if compared to the double-strand banding pattern so that one can assume, that most of the single-strand nicks are cleaved to double-strand breaks. If this assumption is true, one can decide if the single-strand nicks produced by the DNase I are staggered on both DNA strands at intervals of ten bases on each strand according to one helical turn (11,14) or if other structural features like kinks are responsible for the single-strand nicking (2,15). In the first case, one should obtain double-stranded fragments, which are multiples of 5 base-pairs. This has never been observed, neither in the DNase I -S1 digestions nor in micrococcal "limit digest" experiments (8,13,19). The model of the "kinky" helix (2,15), however is compatible with the data presented,
if the kinking occurs every ten base-pairs (15) and if the kinks are potential cleavage sites.

Another possible model, which is compatible with the data presented, is the conception of only one cleavage site per nucleosome, which has been discussed to explain the "limit digest" obtained by extended micrococcal nuclease digestion (20). This model includes structural heterogeneity, which might also explain the very different intensities of the different DNA bands obtained after DNase I -S1 nuclease digestions. The more intensive bands of 80- and 70-base-pairs must be due either to preferential cleavage sites in one nucleosome, or if only one site per nucleosome is susceptible, to structural heterogeneity of nucleosomes, implying that there are certain structures more frequently present in the total chromatin. Another problem which makes the interpretation much more complicated, is the question, whether the DNase I cleavage sites are equally well distributed on "spacer" DNA as on "core" DNA. The results demonstrated in Fig.3 indicate, that this is not the case. Digestion of the DNA between the nucleosomes with micrococcal nuclease leads obviously to the destruction of most of the DNase I -S1 cleavage sites. Therefore one has to consider in addition that there may be a non-uniform spacing between the cores of the chromatin subunits (8).

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