DNase I Site Mapping and Micrococcal Nuclease Digestion of Pachytene Chromatin Reveal Novel Structural Features*

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A comparison of the DNase I digestion products of the 32P-5'-end-labeled pachytene nucleosome core particles (containing histones H2A, TH2A, X2, H2B, TH2B, H3, and H4) and liver nucleosome core particles (containing somatic histones H2A, H2B, H3, and H4) revealed that the cleavage sites that are 30, 40, and 110 nucleotides away from the 5'-end are significantly more accessible in the pachytene core particles than in the liver core particles. These cleavage sites correspond to the region wherein H2B interacts with the nucleosome core DNA. These results, therefore, suggest that the histone-DNA interaction at these sites in the pachytene core particles is weaker, possibly because of the presence of the histone variant TH2B interacting at similar topological positions in the nucleosome core as that of its somatic counterpart H2B. Such a loosened structure may also be maintained even in the native pachytene chromatin since micrococcal nuclease digestion of pachytene nuclei resulted in a higher ratio of subnucleosomes (SN4 + SN7) to mononucleosomes than that observed in liver chromatin.

Extensive studies are now being carried out on the structure of eukaryotic chromosomes both at the level of interphase chromatin and at the level of metaphase chromosomes. The structure of the meiotic chromosomes, on the other hand, has received very little attention. The major events that take place during the meiotic prophase of male germ cells are (a) the initiation of pairing of the two parental homologous chromosomes at the zygotene stage and (b) subsequent genetic recombination between the paired chromosomes at the pachytene stage. Based on the extensive studies on Lilium and some representative study on the mouse, Stern and Hotta (1) have proposed the following sequence of molecular events that facilitate genetic recombination. First, single-stranded nicks are created by a meiotic specific endonuclease which are followed by single-stranded DNA exchange. Subsequently, the gaps are filled up by DNA repair mechanisms. Since the DNA in eukaryotic cells is in the form of nucleohistone complex, it is important to analyze the structural features of meiotic prophase chromatin which in turn could influence the meiotic processes.

The chromatin of rat pachytene spermatocytes contains, in addition to the somatic histones, additional testis-specific histone variants, H1t, TH2A, and TH2B and increased amounts of somatic histone variants H1a and X2 (2–6). The functional significance of these histone variants is not clear at present and it is generally believed that they somehow facilitate the meiotic events. Since the histones are now thought to be purely structural proteins, it is quite logical to assume that the influence of the histone variants should be at the structural level. From such structural alterations, if any, deductions can be made regarding their possible biological significance.

In this direction, our preliminary studies had shown that the testis-specific histones H1t, TH2A, and TH2B do take part in the nucleosome type of organization of rat testis chromatin (7). Subsequently, we have shown that the nucleosome core particles isolated from pachytene spermatocyte nuclei are less compact than liver core particles as revealed by circular dichroism and thermal denaturation studies (8). Assuming that the histone variants TH2A and TH2B do interact with the nucleosome core DNA at similar topological positions as histones H2A and H2B (9), we had proposed that the presence of TH2A and TH2B at the two ends of the core particle is responsible for the less compact nature of pachytene core particles. Here, we provide further biochemical evidence in support of such a hypothesis by analyzing the DNase I-sensitive sites of 32P-5'-end-labeled nucleosome core particles. Furthermore, we have also shown that such a less compact nucleosome core particle structure may be maintained even in the native chromatin as revealed by the kinetics of generation of subnucleosomal fragments by micrococcal nuclease.

MATERIALS AND METHODS

Male albino rats of this institute strain (body weight, 150 ± 10 g) were used in all the studies. Total nuclei were isolated from liver and testes according to the method described by Rao et al. (7). Pachytene stage nuclei were purified from the total testes nuclear preparation by employing the "STAPUT" technique of Meistrich (10) with minor modifications as described by Rao et al. (8). Pancreatic DNase I and micrococcal nuclease were purchased from Sigma, and Worthington Biochemicals, respectively. [γ-32P]ATP was prepared from carrier-free [32P]orthophosphate (Bhabha Atomic Research Center, Bombay, India) and ADP by the method of Walseth and Johnson (11).

Isolation of Nucleosome Core Particles, 5'-End Labeling and DNase I Digestion—The nucleosome core particles were isolated from liver and pachytene nuclei essentially according to the method described by us earlier (7, 8) with minor modifications. Briefly, after carrying out micrococcal nuclease digestion of the nuclei to about 20% acid solubility, the solubilized chromatin released was made 0.1 M with respect to Na2HPO4, and the soluble fraction obtained after centrifugation at 10,000 × g for 10 min was loaded on a Bio-Gel A-5m column which had been equilibrated with 10 mM Tris-HCl, pH 7.4, 0.5 mM Na2EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The fractions were monitored at 260 nm, and the peak fractions that

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1The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; bp, base pairs.
contained the core particles having a DNA size of 146 bp (as judged by electrophoresis on a 6% polyacrylamide gel, 12), were used for further studies. The nucleosome core particles were labeled at their 5'-ends essentially according to the method of Simpson and Whitecock (13) using 5 units of T4 polynucleotide kinase (Bethesda Research Laboratories) and 1 A$_{260}$ of core particle and 50 µCi of [γ-32P]ATP in 50 mM Tris-HCl, pH 7.4, 10 mM MgCl$_2$, 5 mM dithiothreitol, 0.1 mM PMSF. The reaction was terminated by the addition of EDTA to 10 mM final concentration and the unreacted [γ-32P]ATP was separated from the core particles by gel filtration on a Bio-Gel A-5m column. For the kinetic studies, the 5'-end-labeled pachytene and liver core particles (1 A$_{260}$ unit each) were digested with 1.25 µg of pancreatic DNase I at 37°C in a total volume of 1 ml containing 50 mM Tris-HCl, pH 7.4, 10 mM MgCl$_2$, 0.1 mM PMSF. Duplicate aliquots of 20 µl were withdrawn at different time points onto Whatman No. 3 filter paper discs which were sequentially washed with 10% trichloroacetic acid, ethanol, and ether and counted in a LKB Rackbeta scintillation counter.

For DNase I site mapping, the nucleosome core particles, after labeling with [γ-32P]ATP, were directly used for digestion with DNase I. The 32P-5'-end-labeled liver and pachytene core particles (0.4 A$_{260}$ unit each) were digested with 0.5 µg of DNase I in a total volume of 1 ml containing 50 mM Tris-HCl, pH 7.4, 10 mM MgCl$_2$, 5 mM dithiothreitol, 0.1 mM PMSF. Aliquots (100 µl), taken at 0, 2, 5, and 10 min, were made 20 mM with respect to Na$_2$EDTA, pH 8.0. After adding sodium dodecyl sulfate to 1%, samples were digested with protease (100 µg/ml) for 2 h at 37°C. They were extracted once each with phenol:chloroform (1:1) and chloroform. After adding 25 µg of yeast tRNA as carrier to the aqueous phase, nucleic acids were precipitated with 2.5 volumes of ethanol. The precipitates obtained after 16 h were collected by centrifugation and were washed once with 80% ethanol. The DNA was analyzed on a 16% sequencing gel (40 x 0.8 cm) according to the method of Maniatis et al. (14). After the electrophoresis, the gel was covered with Saran wrap and subjected to autoradiography using Indu x-ray film. To quantitate the intensities of bands in the autoradiogram, all the lanes in the x-ray film were scanned in a LKB laser microdensitometer.

**RESULTS**

**DNase I Digestion of 5'-end-labeled Nucleosome Core Particles**—The nucleosome core particles isolated from pachytene and liver nuclei were first checked for their purity, histone composition, and integrity as nucleoprotein particles and were essentially of the same quality as described by us earlier (8). They were then subsequently labeled at their 5'-ends with [γ-32P]ATP. The 5'-end-labeled pachytene and liver core particles were digested with DNase I under identical conditions of enzyme to core particle ratio. The kinetics of digestion, as measured by the percentage of initial input counts that remained still precipitable with 10% trichloroacetic acid as shown in Fig. 1, revealed that a small but consistently higher percentage of 32P-DNA was released into trichloroacetic acid-soluble fraction in the case of pachytene core particles over liver core particles.

Fig. 2 shows the autoradiogram when the DNase I digests of 5'-end-labeled liver and pachytene core particles were analyzed on a high resolution polyacrylamide gel. The autoradiogram shows the ladder of fragments generated by DNase I at the sensitive sites located at periodic intervals of nucleotides on both the strands. The overall pattern obtained with 5'-end-labeled liver core particles is comparable with that obtained by Lutter (15). One common interesting feature of these results is the relatively inaccessible nature of the sites at positions 30, 60, and 110 nucleotides from the 5'-end. Although a gross similarity of the distribution of DNase I sensitive sites is apparent between pachytene and liver core particles and therefore suggests an overall similarity of structural features associated with these two core particles, significant differences do exist at some of the sites in the pachytene core particles. It can be seen from Fig. 2 that the sites 30, 40, and 110 nucleotides away from the 5'-end are significantly more accessible in the pachytene core particles than in the liver core particles. Although at the outset it seems that the higher intensities at these bands might have resulted from a higher loading of radioactivity in the pachytene lanes, when the peak areas of these sites (obtained by cutting and weighing of the tracing of the densitometric scan) were expressed as a percentage of total radioactivity, the values obtained for the sites at 30, 40, and 110 nucleotides from the 5'-end in the pachytene core particles were found to be significantly higher than those obtained in the liver core particles (Table I). The average number of cuts per strand calculated as per the method of Lutter (15) were found to be 0.75 (2 min), 0.98 (5 min), 1.04 (10 min), and 1.29 (20 min) for liver core particles and 0.78 (2 min), 0.96 (5 min), 1.11 (10 min), and 1.36 (20 min) for pachytene core particles. The close similarity of the average number of cutting sites between the liver and pachytene core particles suggests that the differences observed at sites 30, 40, and 110 were contributed by the inherent accessibility of these sites and not due to differences in the extent of digestion by DNase I. According to the model of Schick et al. (9) and Nelson et al. (16) for the topological distribution of the core histones along the 146 bp of DNA, histone H2B interacts with the core DNA at sites between 30, 40, 100, and 120 nucleotides away from the 5'-end. It is, therefore, possible that the presence of testis-specific histone variant (TH2B) in the pachytene core particles, probably occupying similar topological position, might be responsible for the increased sensitivity of these sites to DNase I attack.
Rate of Generation of Subnucleosomes from Pachytene and Liver Nuclei following Micrococcal Nuclease Digestion—The results presented above on the DNase I sensitive sites on pachytene and liver core particles confirm our earlier conclusions based on circular dichroism and thermal denaturation profiles that the two ends of the pachytene core particles are loosened possibly because of weaker histone-DNA interactions. However, a question arises as to whether such a loosened structure is present even in the native chromatin or it is manifested only in the isolated nucleosome core particles. In order to answer this question, we have analyzed the rate of generation of subnucleosome fragments from the native chromatin following micrococcal nuclease digestion.

Pachytene and liver nuclei were digested with micrococcal nuclease to release the digested chromatin fragments. The DNA extracted from such digests of pachytene and liver nuclei were analyzed on a 6% polyacrylamide nondenaturing gel. It can be seen from Fig. 3, A and B that progressively distinct subnucleosomes were generated. A comparison of the DNA pattern obtained from pachytene (Fig. 3A) and liver (Fig. 3B) chromatin clearly reveals that the nucleosome fragmentation from the native chromatin into distinct subnucleosomes is significantly higher in pachytene chromatin than in the liver chromatin. The two major populations of subnucleosomal DNA observed in these studies, marked as SN4 and SN7, correspond to the similar fragments described by Nelson et al. (16). They have indeed shown that SN4 and SN7 particles are complementary to each other and are generated in equimolar amounts as a result of an asymmetric micrococcal nuclease cleavage at approximately 30 nucleotides away from the 5'-end of 146-bp DNA containing core particle. They have further shown that the particle SN4 contains a pair of histones H2A and H2B associated with 30–40 bp of DNA, while the particle SN7 contains (H3,H4), H2A, and H2B with 95–115 bp of DNA. A quantitative analysis was done from the densitometric scan by computing the areas of the peaks belonging to subnucleosomal and mononucleosomal DNA fragments, and the results were plotted as the ratio of subnucleosomes to mononucleosomes obtained as a function of percentages of DNA acid solubility. Such an analysis gave a pattern shown in Fig. 4, and it can be seen that the percentage of subnucleosomal particles obtained in pachytene chromatin digests was considerably higher than those obtained in liver chromatin digests. It is also evident from Fig. 4 that the percentage of the ratio of subnucleosomal DNA to mononucleosomal DNA reaches a near plateau at about 40% in pachytene chromatin. On the other hand, in the case of liver chromatin, this percentage shows a gradual increase up to only about 24% even when 40% of the chromatin DNA became acid soluble following micrococcal nuclease digestion. Based on the experiments of Nelson et al. (16), these results therefore, can be taken as suggesting that the sites 30 and 40 nucleotides away from the 5'-end of the pachytene core particle are having weaker histone-DNA interactions. Thus, these results further corroborate the results obtained with DNase I site mapping and suggest that a weaker histone-DNA interaction at the H2B (TH2B) interacting site may be prevalent even in the native chromatin.

**DISCUSSION**

We have used in the present as well as in our earlier studies (8), rat liver chromatin as a control to evaluate the structural
Structure of Pachytene Chromatin

FIG. 3. Analysis of nucleosomes and subnucleosomes released from pachytene and liver nuclei after micrococcal nuclease digestion. Pachytene and liver nuclei were digested with 15 units of micrococcal nuclease/10 A260 units of nuclei/ml. Aliquots were withdrawn in duplicates at different time intervals into 1 M NaCl, 1 M perchloric acid to measure acid-soluble DNA. Parallel aliquots were withdrawn and the digestion arrested with 10 mM EDTA. DNA was extracted from the digests, analyzed on a 6% polyacrylamide gel, and the DNA fragments visualized by ethidium bromide staining. The number under each lane represents the percentage digestion.

A, pachytene; B, liver. SN4 and SN7 represent subnucleosomal particles 4 and 7, respectively. MNC, mononucleosome core DNA region; S, HinfI digest of pBR322 DNA.

features of meiotic prophase pachytene chromatin of rat testes. First, because liver chromatin is representative of interphase chromatin and hence the structural alterations observed in pachytene chromatin should be functionally meaningful with respect to the meiotic events. Second, liver core particle has only somatic histones H2A, H2B, H3, and H4 and therefore the structural deviations in pachytene core particle can be attributed to the presence of histone variants TH2A, X2 (H2A variant), and TH2B. As reported earlier (8) the extent of replacement of the somatic histones H2A by X2 and TH2A and H2B by TH2B in pachytene core particles is about 60%. Based on the circular dichroism and thermal denaturation studies we had earlier shown that the nucleosome core particles from pachytene chromatin are less compact than the liver core particles (8). From the thermal denaturation studies we had inferred that the histone-DNA interaction at the H2A-H2B binding domain at the two 5'-ends is weaker in the pachytene core particles. The results presented in this communication on the relative accessibility of DNase I sensitive sites have provided strong evidence in support of such conclusions. In addition, these results have further revealed the exact sites on the pachytene core particles which show weaker histone-DNA interactions. According to the linearized model proposed by Schick et al. (9) for the symmetrical arrangement of histone molecules on the two strands of nucleosome core DNA, one molecule of histone H2B has been implicated to interact with the DNA at a site between 20 and 40 nucleotides from the 5'-end and the other molecule of H2B at a site between 100 and 120 nucleotides from the 5'-end. Thus, it becomes immediately apparent that the sites at which enhanced sensitivity towards DNase I digestion was observed in pachytene core particles, in the present study, are the sites at which H2B and probably TH2B interact with the nucleosome core DNA. This type of enhanced sensitivity towards DNase I ensues only when the level of shielding imparted by the electrostatic interaction of histones with the DNA is low. Therefore, it is very likely that the presence of TH2B (which has replaced somatic H2B to 60% (8)) in the pachytene core particle may be responsible for the weaker histone-DNA interaction at sites 30–40 and 110 nucleotides from the 5'-end.

Since both the H2B interacting sites, namely 30–40 nucleotides and 100–120 nucleotides from the 5'-end, are showing higher accessibility towards DNase I attack, it can be inferred that histone TH2B replaces histone H2B at both these sites. However, it cannot be concluded at present whether such a replacement occurs within a single pachytene core particle or the nucleosome core particles isolated are a heterogenous mixture of core particles containing TH2B at the site inter-
acting at 30–40 nucleotides and some others containing TH2B at the site interacting at 100–120 nucleotides.

Micrococcal nuclease, in addition to its exonuclease attack on the linker DNA subsequent to initial endonucleolytic attack, also cuts DNA within the core particles at sites spaced about 10.5 nucleotides apart (17). At a given specific site within the nucleosome core, only one strand will be in touch with the surface of the histone octamer. As a preliminary cleavage, the other strand which is free gets cut by micrococcal nuclease, following which the corresponding site on the protected strand gets cut (secondary cleavage) leading to a double-stranded cleavage. Nevertheless, the rates of secondary cleavage are pretty high and the same for all the sites. The higher release of subnucleosomes (SN4 and SN7) from pachytene core particle (Figs. 3 and 4) may probably result either from enhanced primary or secondary cleavages. These results do, however, suggest that a weaker histone-DNA interaction at the H2B (TH2B) interacting sites may be maintained even in the native chromatin.

Based on the x-ray crystallographic studies of nucleosome core particle at 7 Å resolution, Richmond et al. (18) have proposed that the H2A-H2B dimers add on to either side of the exposed face of the H3-H4 tetramer, binding to the DNA of the last half turn of the superhelix. Since, the two H2B binding sites are 80 nucleotides apart, which actually spans one superhelical turn, it can be visualized that the H2A-H2B dimers stabilize the inner folding of the core DNA induced by histones H3 and H4. Therefore, it is possible to envisage two levels of stabilization of the nucleosome, one being stabilized by the H2A-H2B dimers, while the other being stabilised by histone H1 at the chromatosome level. The tripartite structure of the histone octamer within the nucleosome core particle is also supported by the recent x-ray crystallographic studies on the histone octamer at 3.3 Å by Burlingame et al. (19).

What then is the biological significance of such a loosened histone-DNA interaction at the H2A-H2B binding domain thus destabilizing the inner folding of the core DNA within the pachytene core particle? It may also be mentioned here that Baer and Rhodes (20) have recently shown that transcriptionally active nucleosome core particles are deficient in one pair of histones H2A and H2B which facilitate the binding of RNA polymerase II and subsequent transcription. These results, therefore, stress the importance of histones H2A-H2B binding domain of the nucleosome core particles in determining their functional competence. As mentioned earlier in the introduction, one of the important biochemical events in the meiotic prophase chromatin is genetic recombination involving DNA strand exchange between the two parental homologous chromosomes. Since the DNA in the eukaryotic cell is packaged in the form of nucleohistone complex, it is quite likely that such a loosened histone-DNA interaction may facilitate “disentanglement” of the DNA strand of one of the parental chromosomes to undergo exchange with the DNA strand of the other chromosome. Thus, the appearance of the testis-specific histone TH2B and probably TH2A in pachytene spermatocytes and the consequent replacement of the corresponding somatic histones, up to 60%, may render the pachytene spermatocyte chromatin competent to undergo genetic recombination. We would like to stress here that since the replacement is substantial and also since our unpublished observations have shown that the variant histones TH2A and TH2B are randomly distributed along the pachytene spermatocyte chromatin, the entire length of pachytene chromatin may be competent to undergo recombination. However, the actual sites of recombination, which are few in number, may be determined by other mechanisms. This interpretation may not be an overexaggeration as Stern and Hotta (21) have similarly interpreted the vast excess of endonucleolytic cleavage sites observed over the actual chiasma frequencies.

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