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ABERRANT DNASE I DIGESTION KINETICS OF NUCLEOSOMAL CORE PARTICLES FROM SEA URCHIN SPERM

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The pancreatic deoxyribonuclease (DNase I) digestion rates at the susceptible sites on nucleosomal core particles from blastula, gastrula and sperm cells of the sea urchin, Parechinus angulosus, have been determined. Although there are differences in their isohistone composition, the rates of digestion are similar for both embryonic stages. The rates of digestion for sperm core particles are 3-5 times lower than for embryo core particles at the more, and up to 2.5 times lower at the less susceptible sites. An explanation for these differences could be sought in the sperm isohistones H2B which are characterized by N-terminal extensions of 20-25 amino acid residues.

Canonical nucleosomal core particles consist of 146 b.p. of DNA wrapped around a histone octamer of two of each histone H2A, H2B, H3 and H4 as a superhelix of 1.75 turns (1). The highly conserved histones H3 and H4 form a tetramer, $(H3\text{-}H4)_2$, which can form a nucleosome-like particle with DNA, while the less conserved histones H2A and H2B are proposed to form heterotypic dimers which associate with opposite faces of the H3-H4 tetramer and with DNA (2). The order of the binding of the histones along the nucleosomal core particle DNA has been determined by DNA-protein crosslinking experiments (21-22). This order has been shown to be evolutionarily conserved in core particles isolated from various species containing different isohistones (24).

It is conceivable that the isohistones (for reviews see 3-4) may bind to DNA differently. Sea urchin embryos and sperm have a distinct set of isohistones (5-10). We are using these well characterized nucleosomal core particles from sperm and early embryo of the sea urchin Parechinus angulosus, which contain isohistones in varying proportions, to study the effect of these proteins on nucleosome structure. Using DNase I, we show that the susceptibility to DNase I of the core particle attached DNA is significantly different for sperm nucleosomal core particles as compared to those from embryos. We propose that the longer H2B isohistone variants in sperm are possibly, at least in part, responsible for these differences in DNA-protein interactions.

EXPERIMENTAL PROCEDURES

Sea urchin embryo cultures

Sea urchin embryos from Parechinus angulosus were grown as described previously (5). 7-hour-old and and 21 hour-old embryos (early blastula and gastrula respectively in the particular species) were used to isolate chromatin.

Isolation of nuclei

All operations were performed at 4°C. Nuclei were isolated essentially according to (11) except that the $MgCl_2$-containing buffers were replaced with buffers containing 60 mM KCl, 15mM NaCl, 0.15 mM spermine, 0.5mM

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ermidine, 5mM β-mercaptoethanol, 15mM TRIS-HCl (pH7.4), 0.2 mM EDTA, 0.2 mM EGTA and 0.2 mM PMSF (12). The tergent Triton X-100 was replaced with Nonidet LE. Nuclei were resuspended in the above buffer with 0.25 M crose at a DNA concentration of 5mg/ml.

eparation of nucleosomal core particles

Core particles were prepared essentially according to (13) with modifications. $CaCl_2$ was added to a spension of nuclei to a final free concentration of 1 mM from a 100 mM stock solution. Micrococcal nuclease .1.31.1, Sigma) was added to 20 units/mg DNA and the nuclei were digested at 37°C for 3 1/2 minutes for astula and gastrula chromatin and 20 minutes for sperm chromatin. The reaction was stopped by adding EDTA a final concentration of 5 mM and cooling on ice. The chromatin was pelleted and extracted with 600 mM NaCl, mM TRIS-HCl (pH 7.5), 1.0 mM EDTA and 0.2 mM PMSF for blastula and gastrula chromatin and 1.1 M NaCl in the me buffer for sperm chromatin, for 3-4 hours at 4°C. The insoluble material was pelleted at 4000xg for 5 nutes. The supernatant contains 85-95% of the nuclear DNA as soluble chromatin. The histone content of this action and the cores prepared from it was monitored by SDS gel electrophoresis (14).

Aliquots of the supernatants, containing 4-6 mg DNA in 2-3 ml, were applied to linear, 5-20% (w/w) sucrose adients in 500 mM NaCl (1 M NaCl for sperm chromatin), 10 mM TRIS-HCl (pH 7.5), 1.0 mM EDTA and 0.2 mM PMSF d centrifuged at 83000xg for 16 hours at 4°C. To prepare H1-stripped, long chromatin, fractions larger than tra core size were pooled, dialysed overnight against 10 mM TRIS-HCl (pH 7.5), 50 mM NaCl, 0.2 mM EDTA, and 2 mM PMSF and concentrated by pressure ultrafiltration to 0.5-1.0 mg DNA/ml.

To determine the optimum micrococcal nuclease redigestion time to produce core particles, the DNA of which as flush, from long chromatin 100-200 ul aliquots (0.5-1.0 mg DNA/ml) were made 1 mM $CaCl_2$ and digested at a ncentration of 100 units/ml in pilot experiments. Aliquots of 10 ug DNA were removed, the DNA was extracted d then analyzed on an 8% denaturing polyacrylamide gel (200 mm x 230 mm x 1.8 mm). A bulk digestion was then erformed at the optimum time. The reaction was stopped by adding EDTA to 5 mM and cooling to 4°C. Core rticles were isolated as the 11S fraction from linear 5-20% (w/w) sucrose gradients in 50 mM NaCl, 10 mM TRIS-l (pH 7.5), 1.0 mM EDTA and 0.2 mM PMSF and centrifuged at 182000xg for 16 hours at 4°C. The monosome action was dialysed exhaustively against 10 mM TRIS-HCl (pH 7.5) and 1.0 mM EDTA and then concentrated by essure ultra-filtration to a concentration of 0.5-1.0 mg DNA/ml.

abelling of core particle DNA at the 5'-ends

Core particle DNA, at DNA concentrations of 25-50 ug/ml (i.e. 30-60 pmol of 5'-ends) was labelled at the 5'-d with ^{32}P according to (13) in 70mM TRIS-HCl (pH 7.6), 5 mM dithiothreitol and 10 mM $MgCl_2$ with 10 -20 pmol -^{32}P)-ATP (Amersham) (>5000 Ci/mmol) and 3-4 units T4 polynucleotide kinase (2.7.1.78, Amersham) for 1 hour 37°C. Under these conditions at least 25-35% of the core particles were labelled.

Nase I digestion of 5' -labelled core particles

5'-labelled core particles were digested with pancreatic nuclease (DNase I) (3.1.21.1, Sigma) in 70mM TRIS-l (pH7.6), 5 mM dithiothreitol and 10 mM $MgCl_2$ according to (8). Digestion products were analyzed on 8% enaturing polyacrylamide gels and digestion rate constants were determined according to (13).

ESULTS

he protein component of the nucleosomal core particles.

Removal of histone H1 from partially digested sea urchin sperm chromatin requires a higher NaCl concentration han is necessary for the preparation of H1 stripped chromatin from either sea urchin embryos or chicken rythrocytes (13,16). The optimal NaCl concentration for the given species was 1.1 M. At this NaCl oncentration, no H1 is present in nucleosomes as determined by gel electrophoresis (Fig.1). Also, the core articles subsequently prepared from the H1 stripped chromatin contain relatively equivalent amounts of the core istones (Fig. 1 and Table 1).

he DNA component of the nucleosomal core particles.

The length of the core particle DNA was determined by 5'-end labelling purified DNA and subsequent lectrophoresis on denaturing gels with the appropriate labelled standards. The scans in Fig. 2 indicate that NA from both gastrula (A) and sperm (B) core particles is 145-146 base pairs long. However, whereas the core article associated DNA from embryo cells varies in length by only 2 bases from the average at half height, the NA associated with the sperm core particles is considerably more heterogeneous with respect to length. At half eight the variation in size amounts to 8 bases from the average in the case of sperm (Fig.2). Since both types f particles were produced by the enzyme under the same conditions, this difference may indeed be due to an ltered susceptibility of the chromatin from the two types of cells to the enzyme. It might seem that the ifference in nucleosomal core particle DNA length is significant and might affect the interpretation of the Nase I digestion kinetics. However, there is only a small fraction of the DNA which is longer in relation to he bulk of the DNA as shown by the scans in Fig. 2 which are overexposed autoradiograms. Also, it has been ossible to calculate rate constants which are proportionally comparable with published data for the various ites of DNase I digestion for both the core particle preparations (see Figs. 4 and 6). Thus, the nucleosomal ore particles from both these cell types, by the criteria mentioned above, are essentially similar. These ucleosomes only differ in their content of isohistones.

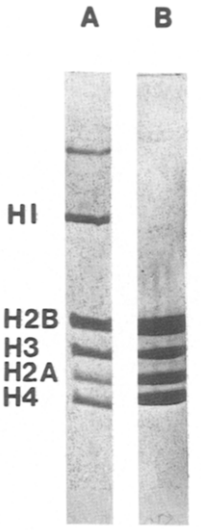

Figure 1
SDS-gelelectrophoresis of sperm histones.

(A) sea urchin sperm histones from chromatin partially digested with micrococcal nuclease
(B) histones present in nucleosomal core particles prepared from partially digested chromatin stripped of H1.

DNase I digestion kinetics of sea urchin nucleosomal core particles.

During the course of the pilot micrococcal nuclease digestion experiments we noticed a considerable difference in the susceptibility of chromatin from sperm and embryo cells towards this enzyme. The rate of core particle production from embryo cells was greater than from sperm cells, thus longer digestion times were necessary to digest H1-stripped chromatin from sperm than from embryos. This is in agreement with previous work (11, 28). To further investigate this observation, we have determined the rates of DNase I digestion of the nucleosomal DNA at each of the susceptible sites.

Nucleosomal core particles from blastula, gastrula and sperm chromatin were 5'-labelled and then digested with DNase I. The DNA products were purified and analysed on 8% denaturing polyacrylamide gels. The autoradiograms of these gels are shown in Figs. 3 and 4. The expected 10 base ladder was observed for blastula, gastrula and sperm core particles although for sperm there is another superimposed ladder (see below). The positions of the sites were identified by scanning one of the lanes of the autoradiograms with a densitometer (see Fig. 5 for sperm) and, because the gel resolution is such that single base differences can be distinguished, counting the bases separating the peaks of radioactivity. Sites were assigned and the DNase I digestion rates were determined according to Lutter (13) (Fig. 6A).

The digestion patterns of the DNA extracted from DNase I treated core particles from sperm chromatin as well as from blastula and gastrula clearly show a 10 base ladder which is indicative of core particle associated DNA

Table 1
Relative quantitation of histones in chromatin and nucleosomal core particle preparations. The gel lanes in Figure 1 were scanned using a densitometer and the areas under the peaks of the 4 core histones were determined. This is expressed as the amount of Coomassie Blue bound to each histone as a percentage of the total for the core histones.

histone	chromatin	core particles
H2B	38	38
H3	22	20
H2A	17	15
H4	23	27

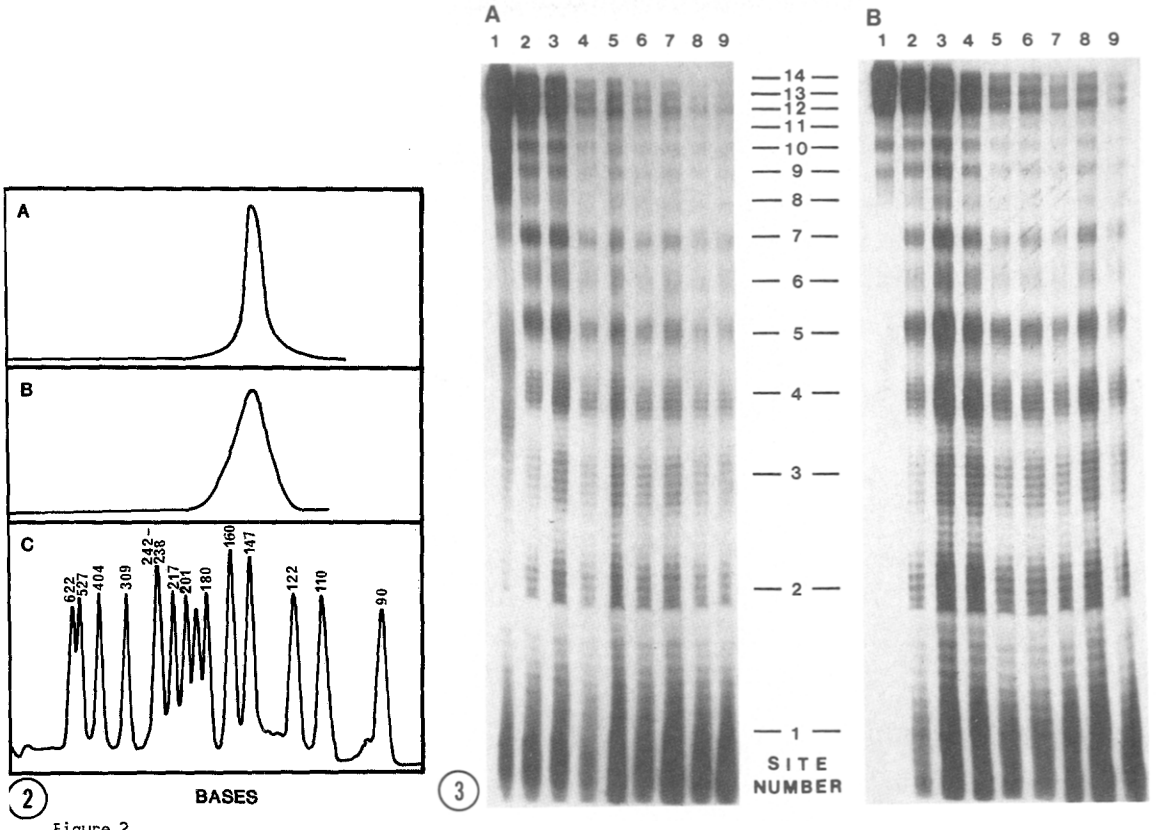

Figure 2
Sizing of DNA in nucleosomal core particles of sea urchin embryo and sperm chromatin.

H1-stripped long chromatin from gastrula and sperm cells was redigested to core particles using micrococcal nuclease. Optimal redigestion conditions were chosen and the DNA products thus produced were extracted, labelled at the 5'ends with ^{32}P and analyzed on 8% denaturing polyacrylamide gels. The scansof the autoradiographs were resolved into fractions with a suitable computer programme assuming the trailing edge to represent part of a normal distribution.
(A) 21 hour gastrula core particle
(B) Sperm core particle
(C) Standard - an Hpa II digest of pBR322.

Figure 3
Autoradiogram of products of digestion by DNase I of blastula and gastrula core particles.

The digests of blastula (A) and gastrula (B) core particles were analyzed on an 8% denaturing polyacrylamide gel. Lanes 1-9 represent digestion times 0, 10 seconds, 20 seconds, 40 seconds, 60 seconds, 100 seconds, 3 minutes, 6 minutes and 12 minutes. Site numbers are assigned according to (13) and the enzyme concentration was 5 units DNase I/ml.

igs. 3 and 4). However, a close inspection of the digest from sperm chromatin reveals a second 10 base ladder ffset by 5 bases that is superimposed on the typical ladder (Figs. 4 and 5). In Fig. 4, additional bands of adioactivity are observed between the bands at sites 1 and 2, 4 and 5, 5 and 6 and 9 and 10, respectively.

The rate of digestion by DNase I of the comparable susceptible sites in the core particle associated DNA ould be a sensitive test to assess similarities or dissimilarities in a set of core particles derived from fferent types of chromatin. Differences in the overall shape of the protein component of such particles y be expected to either sterically hinder the DNase I from approaching a particular site or to induce various egrees of distortion of the DNA at such sites. In order to analyze the rates of digestion at the typical sites the 10 base ladder, it becomes necessary to consider the structural alternatives of the sperm particle ssociated DNA revealed by the existence of the two 10 base ladders offset by 5 bases.

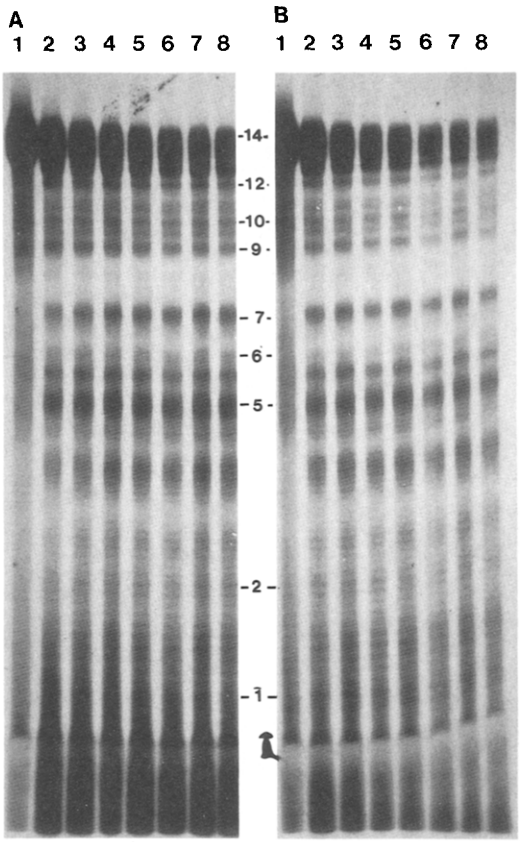

Figure 4
Autoradiogram of products of digestion by DNase I of sperm core particles.

Labelled sperm core particles were digested with DNase I, the DNA isolated and analysed on an 8% polyacrylamide gel. Lanes 1-8 represent digestion times of 0, 15, 30, 45, 60, 75, 90 and 105 seconds respectively with 500 units DNase I/ml (A) and 5000 units DNase I/ml (B). See text for site numbers (between lanes (A) 8 and (B) 1) which were assigned according to (13).

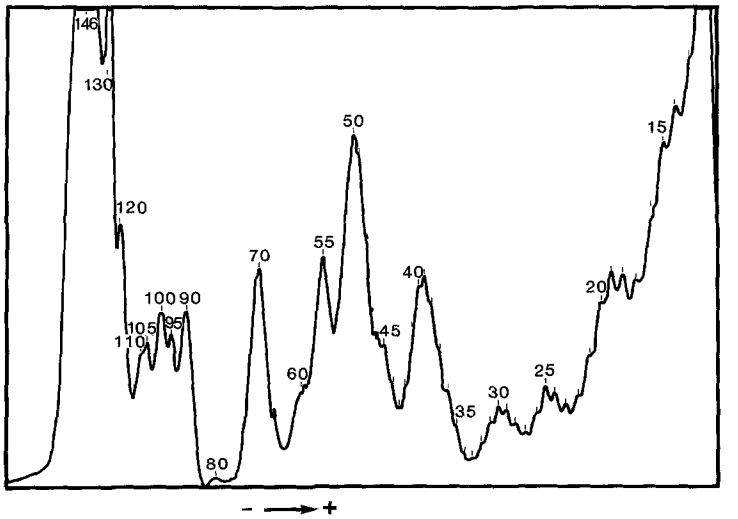

Figure 5
Partial DNase I digest of sperm core particle DNA.

Densitometer scan of the 30 second digestion time lane of the autoradiogram in Figure 4(B). Numerical values shown are for the number of bases from the 5' end of the DNA and were assigned by counting the bases between the peaks of radioactivity to determine the 10 base ladder and, from Fig. 2B, the length of the core particle DNA.

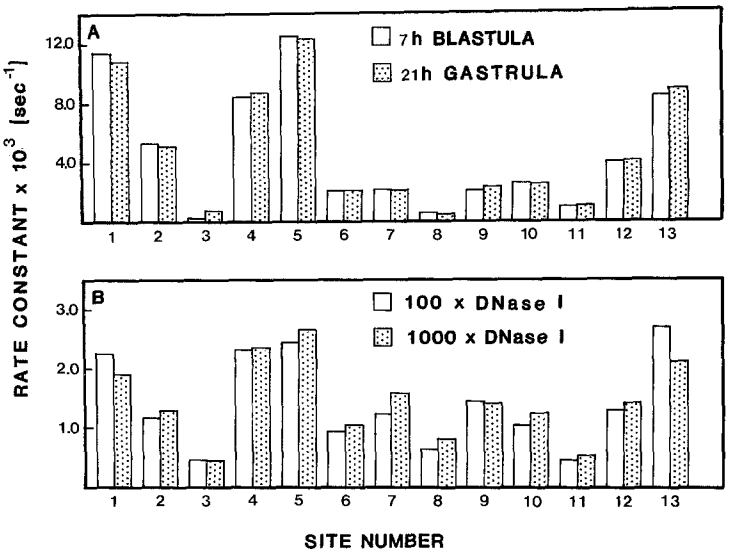

Figure 6
Bar charts of the DNase I digestion rate constants at each site of cleavage of (A) embryo and (B) sperm core particles.

In (B), core particles were digested with DNase I at 500 units DNase I/ml and 5000 units DNase I/ml (i.e. 100X and 1000X the enzyme concentration for (A)). Rate constants were calculated according to (13).

An explanation of the two superimposed ladders may be sought in the existence of some DNA strands being either shorter or longer at the 5' end by 5 bases. However, the experiments in Figure 2 identify the longest piece of DNA as 155 bases. Therefore, the ladder offset by 5 bases results from longer DNA. Consequently the radioactivity at these sites (i.e. sites at 15, 25, 45, 55, 95 and 105 bases from the 5' end (Fig. 5)) has been added to the radioactivity of the sites which are 5 bases shorter (i.e. sites at 10, 20, 40, 50, 90 and 100 bases from the 5' end (Fig. 5)) in order to calculate rate constants for the sites 1-13. The determined rates constants of digestion by DNase I of sperm core particles are different from those of blastula and gastrula core particles. The bar charts in Fig. 6 show the rate constants of DNase I digestion at the various sites for blastula and gastrula (Fig. 6A) and for sperm (Fig. 6B). For blastula and gastrula the rate constants vary from 1.18×10^{3} sec^{-1} (site 3) to 12.86×10^{3} sec^{-1} (site 5) whereas for sperm the rate constants vary from 0.47×10^{3} sec^{-1} (site 3) to 2.68×10^{3} sec^{-1} (site 5). Sperm core particle digestion rates are significantly lower. At sites 1, 2, 4, 5, 12 and 13 the rates are 3-5 times smaller, whereas at sites 3, 6, 7, 8, 9, 10 and 11, the rate constants are only up to 2.5 times smaller than for the corresponding sites in blastula and gastrula core particles. Although the digestion rates are different, the protected sites, 3, 8 and 11, are the same in sperm and embryo core particles.

In order to determine the rate constants of cleavage by DNase I at each site, in the sperm core particle it was necessary to increase the enzyme concentration 100 and 1000 fold. Also, the autoradiogram exposures (Figs. 3 and 4) are 5 times longer in the case of sperm. This increase did not alter the digestion kinetics (Figs. 3,4,6). This is typical of a first order reaction where the enzyme concentration is very much smaller than that of the substrate. It is concluded that the affinity of the enzyme, DNase I, for the substrate, sperm core particle DNA is reduced when compared to that for embryo core particles.

DISCUSSION

The sites of DNase I digestion on the nucleosomal core particle are on average 10.4 pairs apart (13 and 17). Various sites are cleaved at different rates (13,18 and 19). It has been suggested that the sites at which

there are greater rates of digestion may be the result of an increased exposure of these sites, whereas steric hindrance caused by the histones, at the other sites, results in slower rates of digestion (1 and 13). This would apply to the sites 3, 6, 8 and 11. The smaller differences in reaction rates (i.e. at sites 1, 2, 4, 5, 7, 9, 10, 12 and 13) have been interpreted as resulting from differences in the angular disposition of these sites on the surface of the core particle (20). From the former it could be argued that the N-terminal sequences of the core histones are at least partially responsible for the degree of DNase I susceptibility. Sequence variation within these regions could then alter, qualitatively and/or quantitatively, the DNase I sensitivity pattern. However, the experiments presented show that the DNase I digestion rates of blastula and gastrula embryo core particles are very similar though these core particles contain isohistones of the H2A and H2B group in different proportions (3, 4 and 5). The latter represents a subtype of the short H2B histones with a reduced number of basic residues in the N-terminal region. Thus, minor sequence variations in the N-terminal regions of the short H2B histones do not manifest themselves as altered DNase I susceptibilities on the nucleosomal core particle.

However, in sperm core particles, DNase I digestion kinetics show that the susceptibility of the DNA at the cleavage sites is substantially reduced as compared to blastula and gastrula core particles. The reason for this could be sought in the core proteins. Only minor differences exist between sperm and embryo histones H3, H4 and H2A (9,10). However, sperm histone H2B has an extension of 20-25 amino acids at the N-terminal (6,7 and 8). Therefore, it is possible that the substantial decrease in susceptibility of sperm core particles to DNase I digestion, is conferred by these long H2B's. Furthermore, we have observed two types of differences in susceptibility (Fig. 6). Firstly, the rate constants at frequently cut sites are 3-5 times smaller in sperm core particles and, secondly, the rate constants at the less preferred sites differ by a factor of up to 2.5. Thus, a decrease in the cutting frequency at the more preferred sites results in this decreased susceptibility. These sites are situated near the entrance and exit points of the DNA on the core particle surface (i.e. sites 1, 2, 12 and 13). The histones H2B have been localized around sites 3 and 11 by DNA-protein crosslinking experiments (21 and 22). The sperm core particles contain long histone H2B variants with 4-5 repeating pentapeptides each containing 2 basic amino acid residues (6, 7 and 8). It is possible that these extensions, in a suitable conformation, and on interacting with DNA, could reduce the digestion rates for the trimming of the core particle with micrococcal nuclease and could decrease the DNase I digestion rates at the these susceptible sites. It has been shown that there is an increased crosslinking of histone H2B to the core particle DNA in sea urchin sperm when compared to chicken erythrocyte and Drosophila embryo core particles It has been hypothesized that the N-terminal extensions of these H2B's are involved in the additional contacts with the DNA. In addition, our data suggest that a decrease in the DNase I digestion rates at the sites adjacent to where histone H2B binds on the nucleosomal core particle DNA could be due to these N-terminal pentapeptides. However, it is also possible that, in vivo, these histone H2B N-terminal extensions could be involved in chromatin higher structures (25). It is therefore also conceivable that the altered DNase I digestion kinetics could be as a result of displaced contacts onto the nucleosomal core particle after a disruption of these contacts within the chromatin higher structure.

The results presented show that sequence variation in the N-terminal region of the histone H2B, as realized in the short H2B isohistones from sea urchin embryos, does not alter the core particle structure sufficiently to become detectible by DNase I probing, though core particles with less heterogenous histone components from the pluteus larva stage have been shown to be less susceptible (23). However, the extension of the N-termini as in the long H2B isohistones, leads to a core particle with different DNase I susceptibility characteristics The presence of certain isohistones in nucleosomal core particles could possibly confer changes in the overal conformation which are reflected by differences in the accessibility of the DNA to nucleases and other enzymes

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