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DNase I footprinting of the nucleosome in whole nuclei

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ABSTRACT

DNase I was used to footprint the 147 bp DNA fragment of the nucleosome in whole chicken erythrocyte nuclei. It was found that the higher-order structure imposes an additional protection on nucleosomes at sites close to the entry and exit points of the linker DNA, around the dyad axis (site S 0). The observed protection is extended up to 20 bp on either side of S 0. It is partial (~50%) and most probably reflects a full protection of different regions in alternatively oriented nucleosomes. These are the same regions which interact with linker histones. The results strongly support the findings by simulation of DNase I digests of unlabelled oligonucleosome fragments in the 30 nm fibre that in all nucleosomes sites S −5 to S −3 and S +3 to S +5 ara on the outside of the fibre exposed to DNase I.

The 30 nm chromatin fibre is the first level of transcriptionally dormant chromatin. Understanding its structure and the mechanism of its folding and unfolding is of principal importance for understanding the epigenetic regulation of the differentiated cell. However, its structure has been a subject of discussion for long time and various models have been proposed. They all conform with a helix of about seven nucleosomes per turn and ~11 nm pitch, but differ on the orientation and tilt of the nucleosomes and the path of the linker DNA since most of the experimental data is of low resolution. For reviews see [1–3]. Recent attempts to obtain high-resolution structural data by reconstitution of the fiber on repeats of unique DNA sequence produced contradicting results; flat ribbon vs. continuous helix [4,5]. These results were considered from different points of view in three very recent reviews by Tremethick [6], Kornberg and Lorch [7], and Wu et al. [8]. One of the basic questions that can discriminate among a straight vs. folded linker as well as a helix vs. flat ribbon is the mutual orientation of the nucleosomes in the fiber, that can be assessed by the exposure of different nucleosome cutting sites to the bulky DNase I molecule, which cannot penetrate the fibre and digest only the parts of DNA exposed on its surface.

DNase I has been successfully used to footprint the exposure of DNA in end-labelled bulk core particles [9], reconstituted H1 on chromatosomes [10], H1-depleted and non-depleted chromatosomes in solution [11,12], as well as in core particles and chromatosomes reconstituted on unique DNA sequence fragments [13,14]. For reviews see [1–3]. Digestion of whole nuclei with DNase I and fractionation of the resulting unlabelled DNA fragments on agarose gel produces a dinucleosome repeat pattern [15–17], which was originally explained as a result of protection of every second nucleosome in the fibre. It was later shown that the dinucleosome repeat does not necessary mean that every second nucleosome is protected, but only that every second nucleosome has different sites exposed to the enzyme [18,19].

Computer simulations of the digested patterns have shown that sites S ±8 and 0 in the plane of the dyad axis are fully protected and that either sites S −7, −6, +1 and +2 or sites S −1, −2, +6 and +7 are also protected inside the fibre [18,20,21] (cutting sites in the nucleosome core particle are labelled from S −8 to S +8, with S 0 in the middle (see Fig. 3A). Since the simulation results depend on the assumed cutting probabilities for different cutting sites in the nucleosome one has to verify, the validity of these assumptions by direct 5′-end-labelling experiments of DNase I digested nucleosomes.

In order to check the positions of the protected sites, whole nuclei were digested with DNase I, followed by digestion with micrococcal nuclease (MNase), isolation and end-labelling the 147 bp core particle DNA fragment. The resulting fragments were fractionated on a denaturing DNA gel. Calculations of the rate constants for DNase I cutting at sites S −5 to S +6 show that the sites S ±2 and S ±6, which are close to the linker entry/exit points of DNA around the dyad axis of the nucleosome, are less accessible for cutting and the opposite sites S −5 to S −3 and S +3 to S +5 are exposed to the enzyme.

Materials and methods

Nuclei. Chicken blood was collected in PBS (0.15 M NaCl, 0.015 M phosphate, pH 7.6) containing 50 mg/l heparin. Cells were never exposed to EDTA. The cells were lysed in 50 mM Tris pH 7.5, 6 mM $MgCl_2$, 0.25 M sucrose and 1% Triton X-100, followed by several washes in the same buffer without Triton as in Ref. [3].

Nucleosome footprints. Nucleosome footprints were obtained as previously described in Ref. [12], but the nuclei were digested with DNase I before isolation of nucleosomes. Briefly, nuclei at concen-

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tration 1.5 mg DNA/ml in 20 mM Tris–HCl pH 7.5, 15 mM NaCl, 10 mM $MgCl_2$, 0.2 mM EGTA, and 0.2 mM PMSF (phenylmethylsulfonyl fluoride, Sigma) were digested with DNase I at 20 U/mg DNA at 37 °C for 10, 20, and 30 min. Samples were kept on ice, pelleted in a microfuge at 4 °C for 10 s, and resuspended in 250 μl 20 mM Tris–HCl pH 7.5, 15 mM NaCl, 1.2 mM $CaCl_2$, 100 μg/ml actin, 0.2 mM PMSF before digestion with micrococcal nuclease (36 U/mg DNA) at 37 °C for 5 min. Digestion was terminated with 20 mM EDTA (final concentration), nuclei were pelleted in a microfuge for 10 s, and the supernatants, usually more than 60% of the starting material were loaded onto 6–40% linear sucrose gradients. The DNase I digested oligonucleosomes from the sucrose gradients were either stored at −70 °C, or dialysed against 5 mM Tris pH 7.5, 15 mM NaCl, 0.1 mM EDTA and 0.1 mM PMSF overnight. Trypsin (4 μg /mg DNA) was added and proteolysis was kept at 20 °C until the bands of H1 and H5 histones were converted into smaller fragments (usually up to 5 min.). The extent of digestion was assessed by 18% polyacrylamide gel electrophoresis as previously described [12]. Digestion was terminated with trypsin inhibitor (Sigma) (40μg /mg DNA) and 1 mM PMSF (final concentration). The solution was made up to 1 mM $CaCl_2$ and digested with micrococcal nuclease (70 U/mg DNA) until most of the material was converted into core-particle length DNA. DNA was extracted with phenol–chloroform and with chloroform, precipitated with ethanol and fractionated on a 4% agarose gel in glycine buffer. The gel slice containing the 147 bp fragment was excised, and the DNA was electroeluted into a dialysis bag. 5′-end-labelling of the 147 bp fragments was performed with [^{32}P]ATP (Amersham) and T4 polynucleotide kinase (Pharmacia) at 37 °C for 1 h, as described in [12]. The labelled samples were fractionated on 8% polyacrylamide sequencing gels and exposed to X-ray films for several lengths of time.

Densitometry. Densitometry of autoradiographs was carried out as in [12]. The autoradiographs were photographed with a Cannon Ci-20PM CCD camera and transferred to a Macintosh (Mac OS X) computer using Image Grabber (John Blishin & Co Ltd., UK). Tracks were subtracted from the background and digitised using NIH Image software [20]. The scale in nucleotides (nt) was calibrated from interpolations of restriction digest markers as well as from counting the 1 nt bands in the autoradiographs. The images were expanded into Gaussian curves at 10 nt approximation. Resolution of the curves into the sum of Gaussian functions and all further manipulations were done with a Macintosh (Mac OS) computer using Mathematica 5 software (Wolfram Research, Inc.). For calculation of the first-order rate constants, autoradiographs were calibrated with a radioactive step wedge as described previously [12].

Results and discussion

To obtain a direct footprint of the additional protection imposed by the higher-order chromatin structure on the nucleosome: (i) whole nuclei were digested with DNase I, (ii) nucleosomes were isolated by micrococcal nuclease digestion and sucrose gradient fractionation, (iii) histone tails were removed by partial trypsin digestion, (iv) nucleosomal DNA was trimmed to core particle size and (v) gel purified and 5'-end-labelled DNA fragments were examined on a denaturing polyacrylamide gel. A footprint is shown in Fig. 1. It shows the accessibilities to the enzyme of sites S −5 to +6. The evaluation of the accessibility of site S −6 is unreliable because the 10 bp fragments are partially lost during the precipitation procedures [12]. The digestion pattern shown in Fig. 1 is similar to the patterns obtained when chromatosomes are digested in solution [12]. The very weak cuts at sites S 0, ±1 and ±4 show that there was no sliding of the nucleosomes along the DNA during the procedures between DNase I digestion and the trimming of the isolated nucleosomes with MNase down to 147 bp length. Thus the frame of reference of the DNase I digestion pattern reflects

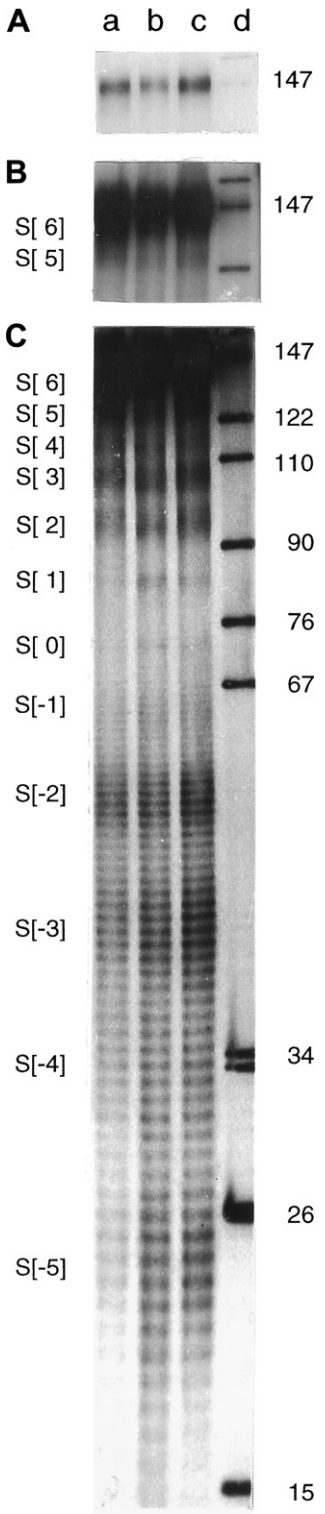

Fig. 1. Autoradiograph of a single-stranded gel of 5′-end labelled DNA from the 147 bp fragment obtained when nucleosomes were digested with DNase I in whole nuclei. Lanes a–c: nuclei digested with DNase I at 20 U/mg DNA at 37 °C for 10, 20, and 30 min. Lane d: restriction fragment marker pAT153/Hpa II. (A) Very short exposure showing only the 147 nt band. (B) Intermediate exposure which shows that the band of site S[+6] is weaker than S[+5]. (C) Longer exposure that shows all bands from S[−5] to the 147 nt.

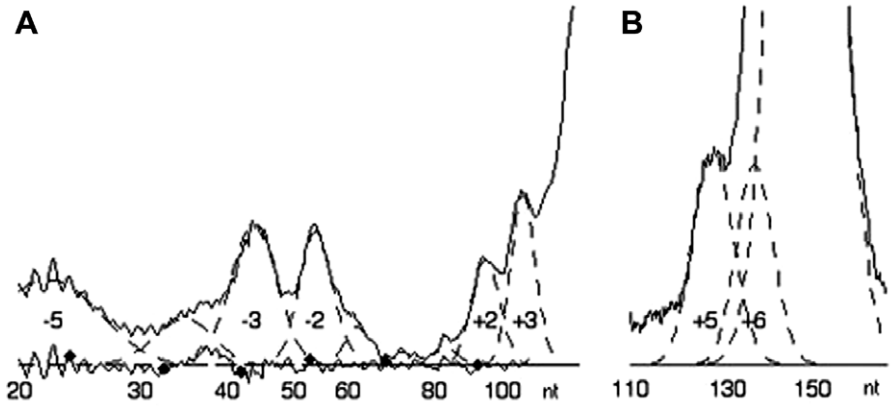

Fig. 2. Densitometer traces of the radiographs of the DNase I footprint (solid lines). (A) Long exposure, 20–120 nt region. (B) Short exposure 110–170 nt region. Dashed lines represent the resolution into sum of Gaussian curves. ♦ Difference curve between the densitometer curve and the sum of Gaussian functions. Numbers −5, −4,...+6 denote bands derived from cutting at sites S −5 to S +6, respectively. Upper curves shows traces of nucleosomes digested in solution to a comparable degree; in (A) the S −2 band is seen to be the strongest band in the pattern (from Ref. [12], Fig. 3k) and in (B) S +6 band is about twice as intense than S +5 band (from Ref. [12], Fig. 6B).

the exposure of the nucleosomes to the enzyme in the higher-order structure inside the nucleus. The apparent difference is that the band from site S −2 is of similar magnitude or slightly weaker than the band S −3, whereas in chromatosome digested in solution [12] it is the strongest band (compare with the upper curve in Fig. 2A). A very weak cutting can be detected at site S 0, which can be explained by a possible absence of linker histones and/or higher-order structure in less than 5% of the nucleosomes. Since both 5'-ends of DNA are labelled, this technique always gives symmetrically averaged pictures. If all nucleosomes are uniformly protected the footprint would reflect the true protection, but if they are alternately protected the footprint would give an average of the different patterns.

Densitometer traces of the autoradiographs were expanded into Gaussian components for each cutting site (as shown in Fig. 2), to allow quantitative evaluation of the extent of digestion. The proportion of digestion (Pn) for different time-points has been corrected for digestion at sites closer to the labelled end, and first-order rate constants were calculated from semi-logarithmic plots as in [9,12] (not shown). The rate constants for the well-resolved strong bands of sites S −5 to S −2 are within ±5% error. For the less well-resolved bands of sites S +2 and S +3, they are within ±10%. The error for S +6 is around ±20% because of the overlap with the strong 147 nt band. The rate constants for the very weak bands S −1, 0 and +1 give errors well above ±20% because of uncertainties regarding the background level and are not considered further. All constants are reduced considerably in comparison to the rate constants for DNase I digestion of chromatosomes in solution (Table 1). Most probably, this is due to the very high concentration of chromatin in the nucleus, which slows down the diffusion of the enzyme. Thus, the only reliable comparison that can be made between footprints of nucleosome in solution and in the nucleus is the comparison of the ratios of the corresponding constants. Examination of Table 1 shows that whilst in solution the rate constant for S −2 is the highest, followed by the constants for S −3 and S +6, in the nucleus both constants for S −2 and S +6 are considerably smaller than the rate constant for S −3. There are two groups of the ratios of rate constants in the nucleus to the corresponding constants in solution. For sites S −5, −4, −3, +3 and +5 these ratios are very similar (mean value 0.204 ± 0.012), whilst the ratios for S −2, +2 and +6 are about half of that value (mean 0.114 ± 0.023). These results suggest that if sites S −5 to −3 and S +3 to +5 are fully exposed to the enzyme, then sites S −2, +2 and +6 are about half exposed (Fig. 3B), or half of them are either exposed, or protected in each nucleosome (Fig. 3C and D) and

Table 1
Rate constants for DNase I digestion of different sites of the nucleosomal DNA in the nucleus and in solution

Site	Fragment lengths nt[a]	$K_1 \times 10^6$ chromatosome in nucleus	$K_2 \times 10^6$ chromatosome in solution[b]	K_1/K_2
−5	22.5	3.27	17	0.19
−4	33.5	1.10	5	0.22
−3	43.2	4.95	26	0.19
−2	52	2.85	29	0.098
−1	62.1	0.5	2.4	—[c]
0	72.3	0.16	0.1	—[c]
1	82.5	0.26	0.9	—[c]
2	93.8	1.0	7	0.14
3	106.1	2.13	10	0.21
4	—	—	—	—
5	125	2.3	11	0.21
6	136	2.9	28	0.104

[a] Converted from Ref. [30].
[b] From Ref. [12].
[c] Because of the significant errors these ratios have not been calculated.

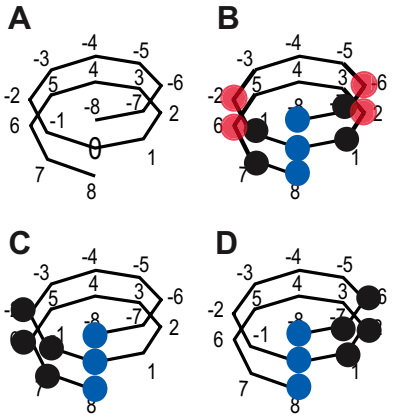

Fig. 3. (A) Numbering of the nucleosome cutting sites according to [31]. (B) Symmetrical protection by the higher-order structure, full protection of sites S 0, ±1 and ±7 (black circles) and partial protection of S ±2 and ±6 (red circles). (C,D) Two nucleosomes that are alternately protected either at sites S −1, −2, +6 and +7, or at sites S −7, −6, +1 and +2. The protection of sites S 0 and ±8 (blue circles) was shown previously [14] and is shown here for completeness. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

confirm the predicted alternating orientation of consecutive nucleosomes [20].

Attempts to obtain a footprint of the 167 nt fragment of the chromatosome failed because the zero time point of digestion always contained single-stranded micrococcal nuclease nicks at site S +7, which were comparable in magnitude to, or even stronger than, the cuts by DNase I (not shown). These single-stranded nicks suggest that the protection is probably of the alternating kind. It is also possible, however, that because of its smaller size MNase cuts at sites that are inaccessible to the bulky DNase I molecule.

These results show that the higher-order structure gives additional protection at sites S −2, S +2 and S +6 and possibly at site S −6, which are partially protected by H1/H5 histones in solution digests [12]. We have shown previously that to explain the DNase I digestion patterns of unlabelled polynucleosomes, especially the disappearance of the strong 140 nt band, it has to be assumed that at least three of the six sites S ±6, ±7 and ±8 have to be protected by the higher-order structure [20]. The significance of these results is that they show that in chicken erythrocytes, the bulk of the nucleosomes have their sites S −5 to −3 and S +3 to +5 exposed to DNase I (i.e. on the outside of the fibre) and that all the protected sites (i.e. those within the fibre) are up to ±20 nt from the dyad axis (S 0). One can speculate that if all the nucleosomes in the fibre are uniformly or alternately oriented, their mutual orientations have to be defined by the nucleosome–nucleosome contacts and not by the DNA linker length. These results are in agreement and enhance previously published results, using different experimental techniques (X-ray and neutron scattering in solution, scanning transmission electron microscopy and cryoelectron microscopy), which showed that both the linker DNA and the linker histones are inside the fibre [22–28].

These results relate differently to the recently published structures of reconstituted fibres on repeats of multiples of positioning DNA sequences. Whilst Richmond's group [4] has shown a two-start flat ribbon, in which the whole nucleosomes and the linker DNA are exposed to the enzyme, the reconstitutes of Rhodes' group [5] show a helical fibre with uniformly tilted nucleosomes and internal linker DNA in agreement with our results.

Thus, the results from DNase I digestion of end-labelled 147 bp core particle DNA fragment in whole nuclei are in a very good agreement with the results from unlabelled oligonucleosomes in whole nuclei as well as in solution [20,29] and the two techniques complement each other.

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