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Histone contributions to the structure of DNA in the nucleosome

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ABSTRACT **We describe the application of the hydroxyl radical footprinting technique to examine the contribution of the core histone tails and of histones H3 and H4 to the structure of DNA in the nucleosome. We first establish that, as was previously determined for a nucleosome containing a unique sequence of DNA, mixed-sequence nucleosomes contain two distinct regions of DNA structure. The central three turns of DNA in the nucleosome have a helical periodicity of ≈10.7 base pairs per turn, while flanking regions have a periodicity of ≈10.0 base pairs per turn. Removal of the histone tails does not change the hydroxyl radical cleavage pattern in either mixed- or unique-sequence nucleosome samples. A tetramer of histones H3 and H4, $(H3/H4)_2$, organizes the central 120 base pairs of DNA identically to that found in the nucleosome. Moreover, "tailless" octamers and the $(H3/H4)_2$ tetramer recognize the same nucleosome positioning signals as the intact octamer.**

Many studies have investigated the role of both DNA and the histone proteins in the architecture of the nucleosome (1–4). The structure of DNA in the nucleosome is altered in two ways from that found when the DNA is free in solution. First, DNA in the nucleosome is highly bent (1). This leads to the preferential association of the histone core with inherently curved DNA molecules, contributing to nucleosome positioning with respect to DNA sequence (5–9). Second, the average helical periodicity of DNA is altered from ≈10.5 base pairs (bp) per turn when free in solution to an *average* of ≈10.2 bp per turn when in the nucleosome (5, 10–12). In addition, high-resolution analysis using the hydroxyl radical DNA cleavage reagent has revealed that two distinct regions of DNA with different helical periodicities exist in a nucleosome containing part of the 5S ribosomal RNA gene of *Xenopus borealis* (12). These changes in DNA structure upon incorporation into a nucleosome are expected to influence both the translational positioning of DNA with respect to the histone core and the way nucleosomal DNA interacts with other DNA-binding proteins.

The central role of histones H3 and H4 in nucleosome structure and assembly, *in vivo* and *in vitro*, is supported by both physical (for a review, see ref. 13) and biochemical (14) evidence. Histones H3 and H4 form a tetramer, $(H3/H4)_2$, which binds to DNA and directs the subsequent association of histones H2A and H2B (15–17). Alterations in the histone components within the nucleosome are associated with many biological processes. Deficiency of histones H2A and H2B within chromatin leads to an increase in accessibility to RNA polymerases (18–20). Modification of the highly conserved N-terminal basic domains of the histone proteins (tails) is also correlated with increased transcription (21, 22). Acetylation of the histone tails influences the organization of nucleosomal DNA in some unknown way (23, 24). In spite of these observations, the influence of the core histone tails on the actual structure of DNA within a nucleosome and the extent to which they prevent the interaction of other proteins with nucleosomal DNA remains ill-defined.

In this study we have confirmed the generality of our previous conclusions regarding the application of the hydroxyl radical footprinting technique to a nucleosome including part of the 5S ribosomal RNA gene by analogous experiments with mixed-sequence nucleosome core particles (12). In addition, we have begun to define the histone domains in the nucleosome that are responsible for organizing DNA on the nucleosome surface as well as those involved in recognizing DNA sequence-directed nucleosome positioning signals. First, the role of the core histone tails in organizing DNA was examined. Exchange of the trypsinized core histones to the 5S DNA fragment confirmed a recent report (25) that the histone tails play no role in determining nucleosome position and have no effect on the helical periodicity of DNA in the nucleosome. Second, reconstitution of 5S DNA fragments with histones H3 and H4 indicated that the discontinuity in helical periodicity of DNA across the dyad axis of the nucleosome (12) is directed solely by the $(H3/H4)_2$ tetramer and that these histones by themselves can recognize the nucleosome positioning signals present in the 5S DNA fragment.

MATERIALS AND METHODS

DNA Fragments. Radiolabeled DNA fragments contained the *X. borealis* somatic 5S RNA gene. A 583-bp *Hha* I–*Eco*RI fragment, a 214-bp *Dde* I–*Eco*RI fragment, and a 152-bp *Rsa* I–*Eco*RI fragment derived from plasmid pXP-10 (26) were used for nucleosome reconstitution after radiolabeling of the coding strand at the *Eco*RI site 78 bp upstream from the initiation site for transcription of the 5S gene. The axis of dyad symmetry of the resulting positioned nucleosome passes through the DNA ≈75 bp from the *Eco*RI site (27).

Nucleosome Reconstitution and Footprinting. Nucleosome core particles (0.2 mg of DNA per ml) in 35 mM NaCl/10 mM Tris·HCl, pH 8.0/1 mM EDTA/1 mM dithiothreitol were prepared (28) and portions were treated with trypsin from bovine pancreas (L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated; Sigma) at 6 μg/ml for 7 min at 25°C to remove the core histone tails. The reaction was stopped by adding trypsin inhibitor from hen egg white (Boehringer Mannheim) to 60 μg/ml and cooled on ice. Histones were analyzed in SDS/18% polyacrylamide gels (29).

Nucleosomes were reconstituted onto radiolabeled DNA fragments either by exchange with core particles (30) or by dialysis from high salt and urea with purified chicken erythrocyte histones (15, 31). Reconstituted nucleosomes, monitored by electrophoresis (32), were cleaved with DNase I or the hydroxyl radical as described (33) except that the final concentrations of the Fe(II) and H_2O_2 reagents in the cleavage reactions were 100 μM and 0.012%, respectively. Intact and trypsinized mixed-sequence nucleosome core particles (2 pmol) were incubated at 37°C for 30 min with 50 pmol of [γ-^{32}P]ATP and 5 units of bacteriophage T4 polynucleotide kinase (BRL) in 10 mM Tris·HCl, pH 8.0/2.5 mM $MgCl_2$ in a 20-μl volume and the reaction was terminated by the

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addition of 4 μl of 30 mM EDTA. The sample was cooled to 25°C, split into several aliquots, each of which was diluted to 35 μl with 10 mM Tris·HCl, pH 8.0/1 mM EDTA and then immediately treated with hydroxyl radicals. The cleavage reaction was terminated by the addition of glycerol to 5% (vol/vol) and cooling on ice. Treated nucleosome core particles were then immediately isolated from unincorporated label, cleavage reagents, and any free DNA in the sample by separation in a 4% polyacrylamide "nucleoprotein" gel (32). The labeled DNA in the core band was recovered and 146 bp in length fragments further isolated in a nondenaturing 5% polyacrylamide gel. The integrity of the core particles was not affected by exposure to hydroxyl radicals as monitored by nucleoprotein gel electrophoresis and by DNase I digestion (data not shown). Single-stranded DNA cleavage products were then visualized after electrophoresis in denaturing gels and data were quantitated as described (33).

RESULTS AND DISCUSSION

Hydroxyl Radical Cleavage of Mixed-Sequence Nucleosome Core Particles. A DNA fragment that contains the 5S RNA gene from *X. borealis* is known to assemble into a positioned nucleosome when reconstituted with histone proteins (27). The high-resolution hydroxyl radical footprinting technique (12) has shown that the region of DNA containing approximately the central 30 bp of DNA in the 5S nucleosome has a periodicity of ≈10.7 bp per turn, whereas DNA segments on either side of this region have periodicities of ≈10.0 bp per turn. This causes the phases of the two flanking regions of ≈10.0-bp-per-turn periodicity to be offset from one another by about 2 bp.

We wished to determine whether the details of nucleosomal DNA structure found in the hydroxyl radical study of a single unique-sequence nucleosome are common to all sequences of DNA wrapped in a nucleosome. To accomplish this goal, we repeated our analysis using nucleosome core particles containing mixed-sequence DNA that had been trimmed to a length of just 146 bp. This precludes the possibility of multiple translational positions that might exist when nucleosomes are reconstituted onto longer DNA fragments (9, 25). The cleavage pattern of trimmed core particles with DNase I (Fig. 1*A*) is identical to that reported earlier (34). The hydroxyl radical cleavage pattern (Fig. 1*A*, lanes 6 and 7) confirms the lack of steric hindrance to this small probe when compared with enzymatic probes (12, 34). Quantitative analysis of the hydroxyl radical cleavage pattern (Fig. 1*B*) reveals two regions with periodicities of 10.0 bp per turn (Fig. 1*C*) that are symmetrically juxtaposed to either side of a region in the center of the nucleosome core particle of greater helical periodicity (10.7 bp per turn). As in the 5S nucleosome, these flanking regions are out of phase with one another by about 0.2 turn of the DNA helix, or about 2 bp. Thus, a discontinuity in the helical periodicity of DNA in the nucleosome as the helix passes through the nucleosomal dyad axis seems to be a general property of nucleosomes and probably corresponds to the jog in the path of the DNA as the helix crosses the dyad symmetry axis that is observed in the crystal structure of mixed-sequence core particles (1, 3, 10, 35). Further, these results indicate that the pattern observed in the study of a unique-sequence nucleosome (12) represents the actual structure of DNA in a nucleosome and is not due to experimental artifact, as suggested (9).

Histone Contributions to the Organization of Nucleosomal DNA. The above experiments suggest that a collection of diverse DNA molecules can be assembled into relatively homogeneous nucleosomes. The results imply that the conformation of DNA is altered to allow precisely defined protein–DNA interactions to occur upon nucleosome formation. We have used the hydroxyl radical footprinting technique to assess the contribution of the histone tails and the $(H3/H4)_2$ tetramer to the precise organization of DNA in the nucleosome.

The histone tails (see Introduction) can be removed by mild treatment of nucleosome core particles with trypsin without significant damage to the central globular domain of the

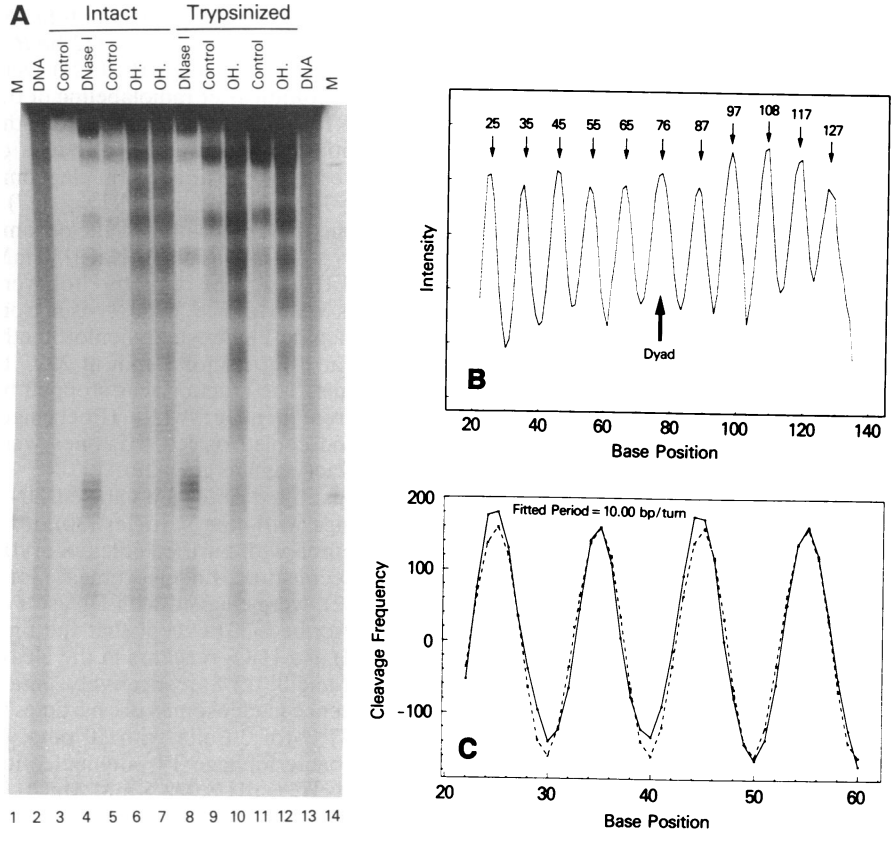

FIG. 1. Hydroxyl radical footprints of mixed-sequence nucleosome core particles before and after treatment with trypsin. (*A*) Autoradiograph of fragments generated by hydroxyl radical and DNase I cleavage of intact (lanes 3–7) and trypsinized (lanes 8–12) nucleosome core particles. Cleavage reagent is indicated above the lane; controls were samples treated in the same way as others except that no cleavage reagent was added. Lanes M represent size standards of 54 and 123 bp. Lanes marked "DNA" represent hydroxyl radical cleavage of naked DNA purified from intact core particles. (*B*) Plots of hydroxyl radical cleavage frequency at each nucleotide of the DNA within the mixed-sequence nucleosome core particles. Each data point represents a three-bond running average of individual peak optical density from the autoradiograph (see *Materials and Methods*). Approximate positions of maximum cleavage frequency are indicated. Numbers indicate the absolute length of the single-stranded DNA fragments. (*C*) Least-squares fit of a sine function to a portion of the cleavage frequency data shown in *B*. Shown is a plot of the original data set (solid line) and the fitted curve (dashed line). The period of the fitted function is indicated.

proteins (ref. 36 and Fig. 2*C*). Surprisingly, hydroxyl radical cleavage of trypsinized mixed-sequence core particles indicates that removal of the tail regions has no effect on the structure of DNA in a nucleosome (Fig. 1*A*, compare lanes 6 and 7 with lanes 10 and 12). This observation was confirmed by quantitative analysis (data not shown). One technical limitation to the above experiment is that changes in DNA structure near the extreme ends of the nucleosomal DNA following removal of the histone tails would not be detectable in our assay, because the longest hydroxyl radical cleavage products are occluded by the intense full-length band and the shortest products are not efficiently recovered. To alleviate this problem, histones from intact and trypsinized nucleosome core particles were reconstituted onto 5S DNA fragments of various lengths in an attempt to control for DNA end effects that may contribute to multiple translational positions (25). Hydroxyl radical cleavage of these reconstitutes (Fig. 2*A*), followed by quantitative analysis (Fig. 2*B*), indicates that no contribution to the periodicity of nucleosomal DNA by the histone tails can be detected throughout the entire length of nucleosomal DNA. This conclusion is consistent with physical measurements of the stability of the nucleosome with or without the histone tails (28) and with the lack of effect of the histone tails on the constraint of DNA to thermal unwinding (38). We suggest that the change in linking number in arrays of nucleosomes following acetylation of the tails (23) is not due to a change in helical periodicity of DNA in the nucleosome, but rather to a change in interaction between nucleosomes or a change in writhe of DNA within a nucleosome (24, 39).

Previous work has shown that the $(H3/H4)_2$ tetramer plays a central role in the structure of the nucleosome. The histone octamer is organized into a tripartite structure in which two H2A/H2B dimers associate with a central $(H3/H4)_2$ tetramer (1, 40). The tetramer is known to be the first histone protein unit to associate with DNA in reconstitutions *in vitro* and during chromatin assembly *in vivo* (17, 41, 42). The $(H3/H4)_2$ tetramer can also supercoil DNA (43, 44), and histones H3 and H4 are absolutely required in order to observe nucleosome-like products following nuclease digestion of *in vitro* reconstitutes (15, 44, 45).

The assembly of histones H3 and H4 onto the DNA fragment containing the 5S RNA gene at a preferred position (see below) offered an opportunity to investigate the precise

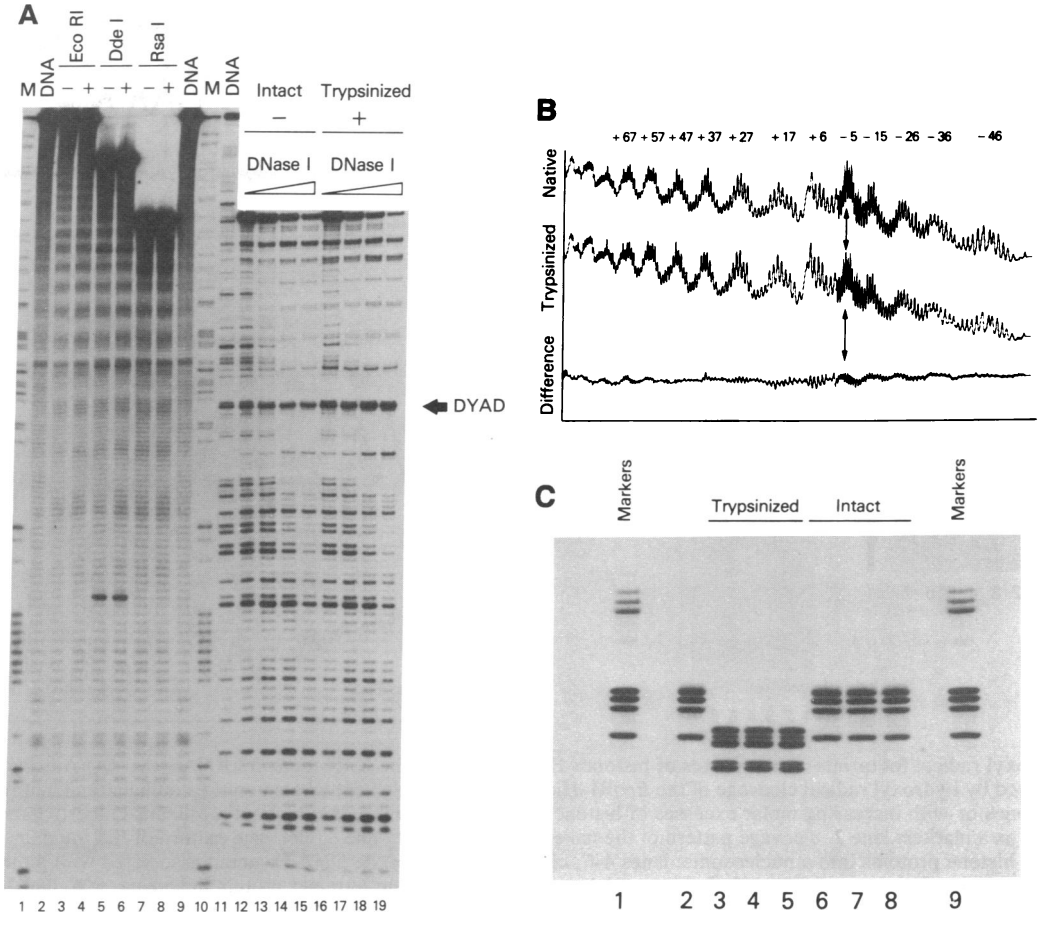

FIG. 2. Hydroxyl radical footprints of intact and trypsinized nucleosome core particles that include part of the *X. borealis* somatic 5S RNA gene. (*A*) Autoradiographs of 5S DNA restriction fragments that were labeled on the coding strand (see *Materials and Methods*), reconstituted with intact (−) or trypsinized (+) histone octamers, and subjected to hydroxyl radical cleavage (lanes 3–8). Lanes 2 and 9 represent hydroxyl radical cleavage of naked DNA, and lanes 1 and 10 show products of guanine (G)-specific sequencing reactions used as markers (37). As a control, intact (−) and trypsinized (+) nucleosomes were reconstituted onto the *Eco*RI–*Rsa* I fragment and cleaved with increasing amounts of DNase I as indicated. The location of the dyad axis of the nucleosome (27) is indicated. (*B*) Densitometric analysis of the hydroxyl radical cleavage pattern of native and trypsinized core particles reconstituted so as to include part of the 5S RNA gene. A plot of the difference between these two scans is also shown. The positions of the dyad axis and the peaks in the hydroxyl radical cleavage pattern are indicated. Peaks are numbered relative to the start site of transcription of the 5S RNA gene at position +1. Two scans were joined at position +1. (*C*) Chicken erythrocyte histones before (lane 2) and after (lanes 3–5) trypsin treatment of the nucleosome core particles used in this work. Lanes 6–8 show untreated samples; lanes 3 and 6, no inhibitor; lanes 4 and 7, with inhibitor added; lanes 5 and 8, with inhibitor added and stored for 2 weeks; lanes 1 and 9, markers that include the linker histones.

role of the tetramer in the organization of DNA within the nucleosome by hydroxyl radical cleavage. The stoichiometry of the association of histones H3 and H4 with the 5S gene-containing fragment suggests that only a single tetramer of these proteins is initially assembled onto DNA (data not shown; Fig. 3 *A* and *B*, lane 4). Densitometric analysis of this autoradiograph indicates that the ≈2-bp discontinuity in the helical periodicity of the DNA as it crosses the dyad axis of the nucleosome (Fig. 3*C*, octamer plot) is also present in the complex of DNA and histones H3 and H4 (Fig. 3*C*, tetramer plot). A plot of the difference between the cleavage patterns of these two complexes (Fig. 3*C*, difference plot) shows that the structure of the central 12 turns of DNA in each of these complexes is virtually identical. The difference plot indicates that the patterns begin to diverge beyond about 60 bp away from the dyad axis of the nucleosome, just beyond the peak in cleavage at position +59 (Fig. 3*C*). Thus ≈120 bp of DNA in the tetramer–DNA particle is organized in an identical fashion to that found in the complete nucleosome.

Additional interactions might extend beyond the central 120 bp of DNA in the tetramer complex (Fig. 3 *A* and *B*, lane 4). The cleavage pattern of the tetramer complex just outside of the central 120-bp region is clearly different from that in the nucleosome and might define a domain of "looser" interaction between the tetramer and the DNA. Alternatively, this pattern could be due to a subpopulation of complexes that are translationally shifted from the bulk population in the reconstituted sample or that have a second tetramer bound in a close-packed arrangement (see below) even at the lowest protein/DNA ratios studied. Thus we can define only the limits of the interaction of the tetramer complex with DNA over a range of about 15 bp, as shown in Fig. 3*C* (horizontal bar).

At protein/DNA ratios high enough to allow more than one tetramer to bind to the labeled DNA fragment (Fig. 3 *A* and

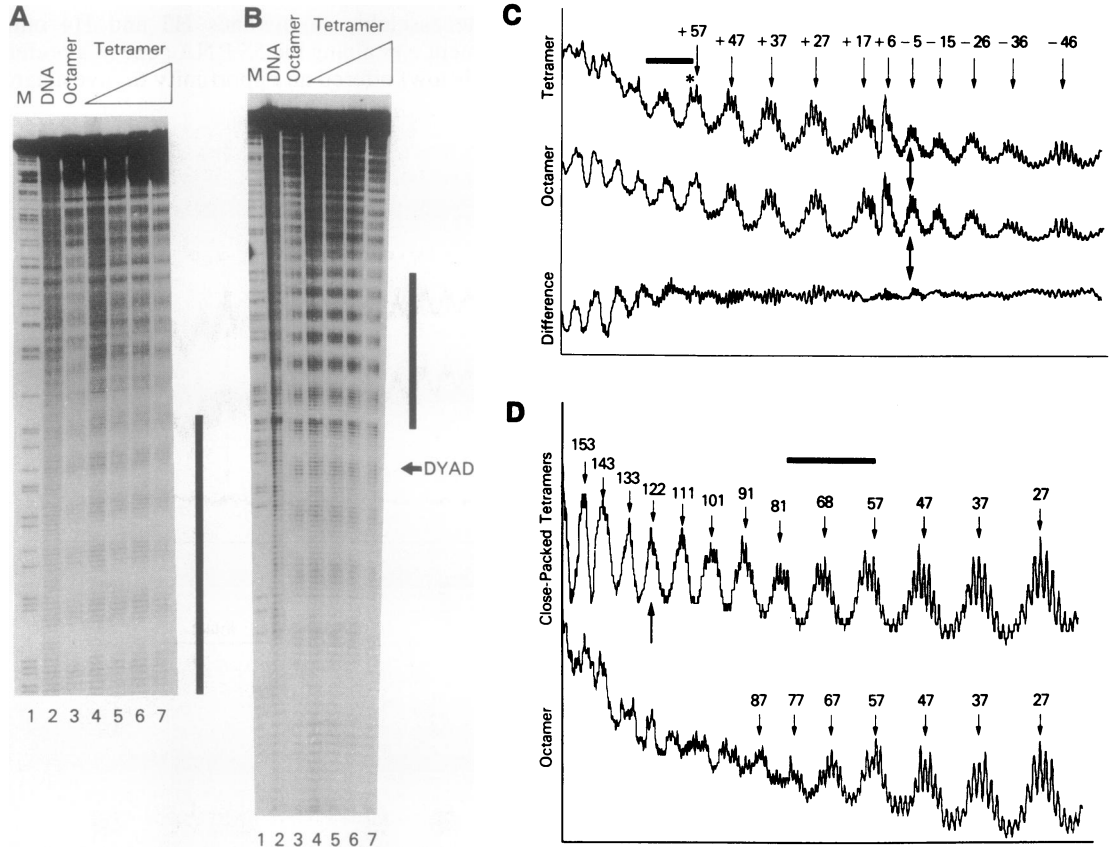

FIG. 3. Hydroxyl radical footprints of complexes of histones H3 and H4 with the *X. borealis* 5S RNA gene. (*A*) Autoradiograph of DNA fragments generated by hydroxyl radical cleavage of the *Eco*RI–*Hin*dIII 5S DNA fragment labeled on the coding strand and reconstituted with all four core histones or with increasing molar excesses of histones H3 and H4. Lane 1, products of a Maxam–Gilbert G-specific sequencing reaction included as a marker; lane 2, cleavage pattern of the naked DNA fragment; lane 3, cleavage pattern of the fragment when associated with all four core histone proteins into a nucleosome; lanes 4–7, cleavage pattern of the DNA fragment associated with an increasing amount of the $(H3/H4)_2$ tetramer. The approximate ratio (wt/wt) of labeled DNA fragment to tetramer protein in lanes 4, 5, 6, and 7 was 1:0.4, 1:0.6, 1:0.8, and 1:1.1, respectively. This corresponds to about one tetramer complex for every 200, 145, 100, and 70 bp of DNA, respectively. The position of a putative nucleosome positioning element (12) is indicated (vertical bar). (*B*) Autoradiograph of the same samples as in *A* but subjected to electrophoresis for a shorter period of time to expose shorter fragments. The position of the nucleosome dyad axis of symmetry is indicated. (*C*) Densitometric analysis of hydroxyl radical cleavage pattern of the complexes of $(H3/H4)_2$ tetramer and of the histone octamer with 5S DNA. The tetramer plot represents the cleavage pattern of the 1:1 $(H3/H4)_2$ tetramer/DNA complex as shown in *A* and *B*, lane 4. The octamer plot shows a cleavage pattern of the 5S nucleosome as shown in *A* and *B*, lane 3. A difference plot (octamer − tetramer) is also shown. The position of the peaks in hydroxyl radical cleavage with respect to position +1 of the 5S gene and the dyad axis of the nucleosome are indicated. Horizontal bar indicates the region in which the octamer pattern diverges from the tetramer pattern. Asterisk indicates the position of the first reproducible difference between the two patterns. (*D*) Densitometer analysis of the hydroxyl radical cleavage pattern of the octamer– and close-packed $(H3/H4)_2$ tetramer–5S DNA complexes. The hydroxyl radical cleavage pattern of 5S DNA reconstituted into a close-packed tetramer complex at a DNA/tetramer ratio (wt/wt) of 1:1.1 (one tetramer per 70 bp of DNA; *A* and *B*, lane 7) and the cleavage pattern of 5S DNA reconstituted in a nucleosome at a DNA/histone octamer molar ratio of 1:1 are shown. Peaks in cleavage are indicated as before. Horizontal bar indicates the region where two tetramers are packed together, and the vertical arrow indicates the putative position of the dyad of the adjacent tetramer positioned downstream of the 5S RNA gene.

B, lanes 5–7) several "close-packed" tetramers are found. At the highest protein/DNA ratio studied, the entire DNA fragment is bound to tetramers and yields a periodic cleavage pattern along its entire length (Fig. 3*B*, lane 7). The tetramer positioned over the beginning of the 5S gene is not influenced by subsequent binding of close-packed neighbors. Densitometric analysis of close-packed tetramers shows that the peaks associated with the first tetramer to bind to 5S DNA do not change as an additional tetramer binds to the DNA fragment, whereas the pattern changes drastically outside of this region (Fig. 3*D*). New peaks in cleavage are found approximately at positions +163, +153, +143, +133, +122, +111, +101, +91, and +81 (as indicated on the upper scan in Fig. 3*D*) and are highly suggestive of the pattern expected for a second tetramer bound in a close-packed arrangement against the first. The junction where two tetramers pack together at higher histone ratios is also clearly seen in this analysis (Fig. 3*D*, horizontal bar), and a comparison of the repeating patterns suggests that these two tetramers are related by a 180° rotation (about 5 bp) about the helical axis of DNA. This direct investigation of the organization of DNA into the histone H3/H4 tetramer is consistent with indirect observations on DNA structure within this particle derived from nuclease protection (15, 44) and linking-number-change (24, 43, 44) experiments.

Histone Contributions to Nucleosome Positioning. When histones are reconstituted onto particular DNA fragments *in vitro*, the octamer is found to adopt nonrandom rotational and translational positions with respect to the DNA helix (4–9). The core histone tails are not involved in recognizing these positioning signals in DNA, since both trypsinized and intact core histones adopt the same position after *in vitro* reconstitution of nucleosomes on the *Lytechinus variegatus* 5S RNA gene (25) or the *X. borealis* 5S RNA gene (this study).

Previous work has suggested that histones H3 and H4 have the essential role of nucleating formation of the nucleosome (15, 17) and are capable of some specific sequence recognition when they bind (46). We find that the $(H3/H4)_2$ tetramer is sufficient to recognize the same nucleosome positioning sequence on 5S DNA as the complete octamer of histones. This is clearly indicated by the obvious tetramer footprint centered over the start of the 5S RNA gene at low protein/DNA ratios, analysis of which indicates that the dyad axis of this particle is in exactly the same position as the complete octamer (Fig. 3 *A* and *B*, lane 4). The tetramer binds to this position to the exclusion of all other sites, even when longer DNA fragments (≈600 bp) containing the 5S RNA gene are used in the experiment (data not shown). The position of the first tetramer to bind is not influenced by the subsequent loading of additional tetramers onto the DNA (Fig. 3*D*). The clear protection over the start of the 5S RNA gene at low molar excesses of histones H3 and H4, at which only a tetramer should form, suggests that the essential features of DNA that direct nucleosome positioning will depend upon the organization of that DNA with histones H3 and H4. Therefore histones H2A and H2B need have no contribution to the positioning of the histone core relative to a DNA sequence. This conclusion is consistent with observations on *Lytechinus* 5S RNA genes (46). Thus, the loss of one or both of the H2A/H2B dimers during cellular processes such as transcription (18, 19, 47, 48) would not be expected to completely destroy the nucleosomal organization of chromatin.

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