Unique translational positioning of nucleosomes on synthetic DNAs

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ABSTRACT
A computational study was previously carried out to analyze DNA sequences that are known to position histone octamers at single translational sites. A conserved pattern of intrinsic DNA curvature was uncovered that was proposed to direct the formation of nucleosomes to unique positions. The pattern consists of two regions of curved DNA separated by preferred lengths of non-curved DNA. In the present study, 11 synthetic DNAs were constructed which contain two regions of curved DNA of the form [(A₅-T₅)(G/C)₅]₄ separated by non-curved regions of variable length. Translational mapping experiments of in vitro reconstituted mononucleosomes using exonuclease III, micrococcal nuclease and restriction enzymes demonstrated that two of the fragments positioned nucleosomes at a single site while the remaining fragments positioned octamers at multiple sites spaced at 10 base intervals. The synthetic molecules that positioned nucleosomes at a single site contain non-curved central regions of the same lengths that were seen in natural nucleosome positioning sequences. Hydroxyl radical and DNase I digests of the synthetic DNAs in reconstituted nucleosomes showed that the synthetic curved element on one side of the nucleosomal dyad assumed a rotational orientation where narrow minor grooves of the A-tracts faced the histone surface with all molecules. In contrast, the curved element on the other side of the nucleosome displayed variable rotational orientations between molecules which appeared to be related to the positioning effect. These results suggest that asymmetry between the two halves of nucleosomal DNA may facilitate translational positioning.

INTRODUCTION
The first step in the packaging of DNA into a chromosome is the formation of a complex between 146 bp of DNA and an octamer of histone proteins, resulting in a particle known as a nucleosome. In a mature nucleosome, the DNA makes two left-handed turns around the spherical histone octamer which results in the DNA being bent into a conformation that is highly strained. Some sequences can better accommodate this strain than others. For example, conformationally flexible molecules such as poly(dA·T) are known to form nucleosomes of greater stability than conformationally rigid molecules such as poly(dA)-poly(dT) (1). Similarly, sequences that are anisotropically flexible have been shown to orient themselves on the surface of the histone octamer such that their preferred axis of movement coincides with the direction of bending imposed on DNA in the mature nucleosome (2–4). Intrinsically curved DNA molecules have been shown to form more stable nucleosomes than non-curved molecules (5), presumably because the bending of these molecules around the histone octamer requires less energy than the same process for a non-curved molecule. However, there is some question as to whether sequence-directed curvature in solution and bending around proteins is in the same direction (6, 7).

When DNA is bent around the histone octamer, the minor and major grooves on the inside face of the bend are forced to compress and the grooves on the outside face widen to a corresponding degree. This process is facilitated by sequences that possess statically narrow grooves or can readily accept groove compression if these grooves are oriented in the nucleosome such that they face the protein surface. Studies of bulk DNA (6) and nucleosome positioning DNA (8) have identified preferred locations for oligonucleotides along the nucleosome. Since the translational position along nucleosomal DNA defines the angular orientation of the grooves with respect to the protein surface, these preferences presumably reflect differences in the ability of a given sequence to accept minor or major groove compression (6) or to tolerate decreased base stacking at positions of extreme DNA bending (8). In spite of the considerable amount that is known about DNA in a nucleosome, the sequence requirements that lead to translational positioning of histone octamers at unique sites remain poorly understood. With respect to this point, attempts at producing a synthetic sequence that positions nucleosomes at a single site have failed.

The vast majority of natural DNA molecules do not preferentially form nucleosomes at a particular location along their length. However, many sequences have been identified that direct the deposition of histone octamers to single sites (9). These sequences were obtained from a wide range of eukaryotes and their viruses and occur in the vicinity of promoters, enhancers and in non-transcribed DNA that is packaged into constitutive heterochromatin. We have previously shown that natural DNAs that position nucleosomes at single sites possess several conserved

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features (10). These sequences were shown to be enriched in oligo(A)-tracts which assume a 10 base period on one half of nucleosomal DNA, decrease in frequency around the nucleosomal dyad and renew their 10 base period in a new phase on the second half of the nucleosome. For the present study, we constructed a set of 11 synthetic molecules containing two regions of DNA curvature separated by a variable length of non-curved DNA. Some of these constructs contain central regions of the same preferred lengths seen in natural nucleosome positioning sequences and some do not (10). Mononucleosomes were formed on these molecules through in vitro assembly reactions and the complexes were analyzed through chemical and enzymatic reactions. The results of this study revealed that multiple features of DNA are required for the positioning of the histone octamer at unique sites.

MATERIALS AND METHODS

DNA preparation and analysis

Standard molecular biological techniques were used for DNA manipulations (11). Synthetic DNAs were step-wise assembled from oligonucleotides purchased from Integrated DNA Technologies Inc. and cloned into the polylinker of pUC18 to create clone 47 (Fig. 1A). Insertions and deletions were performed on this parent synthetic clone to form the 10 constructs shown in Figure 1. Construct 53 contains a segment of the polylinker of Bluescript while the remaining insertions were made with synthetic oligonucleotides. Sequences were determined with a sequenase kit from USB Inc. Radioactive DNA was prepared by PCR using primers (25mers) that are centered at 19 bp upstream and 33 bp downstream from the EcoRI and HindIII sites in pUC18 respectively. DNA was uniformly labeled with [α-32P]dATP or uniquely end-labeled by using one 32P-labeled primer in the PCR. DNA fragments were purified by electrophoresis through agarose gels and appropriate bands eluted from gels by the crush–soak method. A 195 bp EcoRI fragment from the 55 rDNA of Lytechinus variegatus was used in the studies shown in Table 1 (13). For the electrophoretic studies in Figure 1, end-labeled fragments were electrophoresed on the indicated percent (30:1) polyacrylamide (PA) gels at 4–5

Enzymatic analysis of reconstituted nucleosomes

After nucleosome assembly, reaction mixtures in 50 mM NaCl, 3 mM MgCl2, 1 mM β-mercaptoethanol, 10 mM Tris–HCl (pH 8.0) and 50 µg/ml albumin were digested with exonuclease III (Exo III) or DNase I as detailed in the figure legends (18,19). Alternatively, the reconstitutes were digested with restriction enzymes in buffers recommended by the manufacturers for 1 h at room temperature. Aliquots of the completed reactions were run on nucleoprotein gels to confirm that >95% of labeled DNA was still present in mononucleosomes. Digestions were performed in parallel with digestions of free DNA which had been taken through the steps of the reconstitution reactions in the absence of oligonucleosomes. Upon completion of the digestions, the reactions were made 0.1% SDS, 0.35 M NaCl, 30 mM EDTA and extracted with phenol/chloroform and then again with chloroform. The DNA was precipitated with ethanol and electrophoresed on 8% (19:1) PA gels in 1× TBE and 7.5 M urea. Microccoral nuclease digestions were performed on ∼3 µg/ml reconstituted nucleosomes in 1 mM CaCl2 with 2 U/ml for 10 min at room temperature. The DNA was purified as above, precipitated with ethanol and electrophoresed on agarose gels, where fragments of length 146 ± 15 bp were recovered and digested with restriction enzymes as detailed in the text.

Chemical analysis of reconstituted nucleosomes

Reconstituted nucleosomes were digested with hydroxyl radicals (20). The reaction was initiated by mixing equal volumes of a 0.4 mM solution of ammonium iron(II) sulfate, 0.8 mM EDTA and 10 mM sodium ascorbate (pH 8.0). This solution was immediately added to the reconstituted nucleosomes in an amount equivalent to 1/3 of the volume of the reconstitute. Aliquots of the completed reactions were run on nucleoprotein gels to confirm that >95% of labeled DNA was still present in mononucleosomes. The reaction was terminated by making the solution 20 mM thiourea and 80 mM EDTA. Samples were extracted with phenol/chloroform and then with chloroform and the DNA precipitated with ethanol and electrophoresed on 8% PA gels in 7.5 M urea. Gels were calibrated with MspI digests of pUC18 and G and G/A products of Maxam–Gilbert sequencing reactions (21).

RESULTS

Sequence and structure of the DNA fragments

The sequences of 10 DNA fragments used in this study are shown in Figure 1. Two 45 bp synthetic oligonucleotides were inserted into the polylinker of pUC18 to produce a recombinant parent plasmid. Insertions and deletions were then made in the region separating the two synthetic bending elements. The 10 plasmids were then used as templates in PCR to produce end-labeled or
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relative to the sequences in the –3 series. The extremes in the two

12%, all fragments in the +1 series displayed increased mobilities be seen in Figure 1 B. As the gel concentration increases from 6 to

percent gels, the three-dimensional shape of the DNA between the two ends has increasing influence on gel mobility (22) and this can be seen in Figure 1 B. As the gel concentration increases from 6 to 12%, all fragments in the +1 series displayed increased mobilities relative to the sequences in the –3 series. The extremes in the two groups are fragments 61 and 47 respectively. Although electro-

phoresis theory offers no straightforward interpretation of gel mobilities of fragments with such complex shapes, we attempted to correlate the electrophoretic data with predicted structures of uniformly labeled fragments. The PCR fragments ranged in length from 178 to 235 bp and contained 83–140 bp between the first A in tract 1 and the last A in tract 8. The fragments were named according to the phase relationship between the upstream and downstream A-tracts. For example, fragment 20 contains 20 bp from the center of tract 4 to the center of tract 5. The two major groups of sequences are the +0 to +1 series (20, 41, 51 and 61) and the –3 series (47, 57, 67 and 77). The –3 series matches the sequence phase we previously reported as the average for natural nucleosome positioning sequences (10).

The intrinsic shapes of the DNA fragments shown in Figure 1 A were expected to be complex. The A-tract regions have a sequence period of 10.0 bp but should have a solution pitch of 10.3–10.4 bp/turn (3). This would result in a left-handed curve in the vicinity of each block of four tracts. The angular orientation between the upstream and downstream curved elements should also vary with the length of the non-curved central region. Analysis of electrophoretic mobilities on increasing percent PA gels has been used to study complex DNA structures (22) and a summary of the molecules determined by a computer program for DNA curvature. The two projections representing each fragment were rotated by 60°. the molecules determined by a computer program for DNA curvature. The predicted structures of the 10 fragments shown in the right panels of Figure 1 B reveal similarities among sequences with a common phase designation while no two sequences of different phase designation share such similarities. In addition, the extremes in predicted structure coincide with those seen in the gel studies. Fragments 47 and 61 can be qualitatively described as S-shaped, with the arms of the S projecting either in front of or behind the plane of the page, respectively.

The exchange reaction (16) was used in this study to reconstitute mononucleosomes. Figure 2 shows a nucleoprotein gel of reconstituted fragments 61 and 67 using both limited histone inputs and moderate histone inputs in the presence of free competitor DNA. Both approaches revealed that fragment 67 has a higher affinity for histone octamers than fragment 61. The protein–DNA complexes were judged to be authentic mononucleosomes since each co-migrated with mononucleosomes from cellular chromatin and each yielded a protected fragment of ~145 bp in micrococcal nuclease digests (data not shown). Since

Figure 1. Sequence and structure of the DNA fragments. (A) The top line shows the 5’ common sequence which contains A-tracts 1–4. The next 10 lines provide the sequences immediately 3’ of tract 4, which vary in sequence depending on the fragment. The 10 fragments are designated by the number shown to the left of the variable region, which refers to the distance in bp from the center of tract 4 to the center of tract 5. Phase designations are also given (see text). The bottom line shows the sequence immediately 3’ of the variable region. This region is common to all fragments and contains A-tracks 5–8. (B) (Left) The indicated fragments were electrophoresed on 6, 8, 10 and 12% PA gels at 4°C. The mean R_L values (R_L = apparent length/actual length) were 1.39, 1.56, 1.77 and 1.81 respectively. Deviations from the means are plotted on the y-axis. (Right) Two-dimensional projections of the helical axis were drawn using a computer program for DNA curvature. The two projections representing each fragment were rotated by 60°.
Table 1. Relative energies of reconstitution of synthetic clones

<table>
<thead>
<tr>
<th>DNA fragment</th>
<th>Relative energy of reconstitution (cal/mol)</th>
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<tbody>
<tr>
<td>Genomic DNA</td>
<td>3600</td>
</tr>
<tr>
<td>5S rDNA</td>
<td>1500</td>
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<tr>
<td>20</td>
<td>700</td>
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<td>900</td>
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<td>47</td>
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<td>67</td>
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Wheat genomic DNA is 200 ± 20 bp and 5S rDNA is from L. variegatus (13). The reproducibility of these experiments was within the range 200 cal/mol.

Translational positioning of nucleosomes on synthetic fragments

Exo III is a highly processive 3' exonuclease that degrades most sequences at similar rates (24). Exceptions include runs of G(24) or T(25), which are degraded at reduced rates. Exo III pauses at the borders of free DNA and protein–DNA complexes and for this reason has been used extensively to map nucleosome positions on DNA (26). In the present study, Exo III pauses are considered to represent authentic nucleosome boundaries only after they meet three criteria (27). First, a pause in nucleosomal DNA must be significantly more persistent than in naked DNA. Second, a second pause must result in a number of bands that do not appear in naked digests. It was concluded that fragment 61 possesses five nucleosome positions spaced at 10 bp intervals, which are indicated by the overlapping rectangles. Multiple translational positions separated by 10 bp suggest that the five translational positions seen with fragment 61 share a common rotational orientation (3). Likewise, multiple positions were noted with all other fragments in the +0 to +1 series.

All 10 sequences share either identical downstream Exo III borders or downstream borders separated by integral multiples of 10 bp. Among +0 to +1 sequences, upstream borders are identical or separated by integral multiples of one helical turn (10 bp). Upstream borders among –3 sequences are identical or separated by integral multiples of one turn but are located approximately half a turn (6 bp) downstream of the nearest +1 fragment border. These results imply that all 10 fragments share a common rotational orientation along tracts 5–8 and that the +0 to +1 fragments share a common rotational orientation along tracts 1–4 that is nearly directly out of phase with the rotational orientation assumed by –3 sequences. Fragments 53 and 56 displayed upstream borders not shared with any of the other eight fragments. These sequences presumably posses a rotational orientation along tracts 1–4 that is different from both +0 to +1 and –3 fragments. DNase I and OH• analysis of reconstituted nucleosomes presented below confirm these predictions.

Certain complications have been encountered in previous Exo III mapping studies, including read-in pauses (27), premature pauses (28), failure of the enzyme to pause at nucleosome borders (25) and unpaired pauses resulting from octamers overhanging one end of a DNA fragment (29). Therefore, MNase protection assays were employed in order to independently analyze the reconstitutes (30–31). In the studies presented in Figure 4, reconstituted nucleosomes were digested with moderate levels of MNase and the protected DNA was resolved on an agarose gel in order to recover 146 ± 15 bp fragments. The higher resolution afforded by purifying the core DNA from PA gels was precluded by the curvature present in these constructs. The 146 ± 15 bp DNA recovered from agarose was digested with internal restriction enzymes common to all fragments and run on native (Fig. 4) or denaturing PA gels (not shown). Translational positions of nucleosomes on the reconstituted fragments were
then inferred from the lengths and intensities of the resulting bands. Figure 4A shows a schematic diagram that illustrates the cutting site of KpnI in the synthetic fragments. The broad bands from the gel-purified fragments resulted from the limited resolution afforded by agarose gels. Because MNase displays modest sequence specificity, digests of naked DNA can produce populations of discreet fragment sizes (32). Fragments that result from naked DNA digests are indicated in the figure with filled circles. Lane 9 contains aliquots of the corresponding fragments recovered from the agarose gel. Lanes 6–9 contain aliquots of the fragments recovered from the gel-purified fragments that were subsequently digested with KpnI. Because MNase digests produce the same fragment in clone 77 that is protected from Exo III digestion, fragments expected if MNase digests 67 and 77 digested with MNase and KpnI.

Figure 4. Micrococcal nuclease mapping of reconstituted nucleosomes. (A) A schematic diagram that illustrates the cutting site for KpnI in the synthetic molecules. Uniformly labeled reconstituted synthetic molecules were digested with 2 U MNase/ml for 10 min at room temperature. The labeled DNA was recovered and run on an agarose gel where 146 ± 15 bp fragments were isolated and digested with KpnI. (B) An autoradiograph of a native 8% PA gel. The left-most lane is a marker containing an MspI digest of pUC18. Lanes 2–5 are aliquots of fragments 51, 57, 61 and 77 recovered from the agarose gel. Lanes 6–9 contain aliquots of the corresponding fragments recovered from the agarose gel that were subsequently digested with KpnI. Fragments expected if MNase digests produce the same fragment in clone 77 that is protected from Exo III digestion are indicated by arrows. Lane 10 contains unreconstituted fragment 77 digested with MNase and KpnI.

Restriction enzymes display reduced cutting in nucleosomal DNA (33) and this feature has been utilized to study translational positioning of nucleosomes. HaeIII and MspI were chosen for this analysis because of their multiple recognition sites in the vicinity of both sets of tracts. Figure 5 presents a summary of four separate HaeIII and MspI digests of mononucleosomal synthetic DNAs. The results of the Exo III and MNase mapping experiments suggest that MspI site 1 should be largely accessible in –3 fragments while +0 to +1 fragments should display less efficient cutting at this site. Figures 5B and C show that the –3 molecules were effectively cleaved at MspI site 1 while +0 to +1 constructs displayed less efficient cutting at this site. Based on the Exo III maps, one would expect cutting at MspI site 1 to follow the order 77 = 67 > 57, 47, 56 > 61, 53, 51, 41, 20. Assuming that restriction sites located on the edge of nucleosomal DNA are cut more efficiently than internal sites (34), cutting at site 2 was also expected to follow this order. The experiments confirmed these predictions. Figure 5C also provides a summary of HaeIII digests of reconstituted nucleosomes. The results of Exo III digests (Fig. 3) suggested that reconstituted constructs 67 and 77 should be efficiently cleaved at HaeIII site 1 while all other fragments should display relatively inefficient cutting at this site. HaeIII sites 2 and 3 were expected to be occupied by histone octamers with all fragments and thus resist cleavage. The summary of HaeIII digests presented in Figure 5C are consistent with these predictions.
Figure 5. Restriction enzyme sensitivity of reconstituted DNAs. (A) Schematic diagrams of *MspI* and *HaeIII* cutting sites in the synthetic fragments with the A-tracts indicated by dark blocks. (B) Each of the synthetic DNAs labeled on the bottom strand were reconstituted into mononucleosomes, digested with *MspI* and the purified DNA run on a native 8% PA gel. The bands that result from *MspI* digestion are numbered according to the designation given in (A). (C) A summary of four separate *MspI* digests of synthetic nucleosomal DNA labeled on either the top or bottom strand. Cutting at each of the three *MspI* sites is quantified by the bar graph. The open region at the top of each bar represents one standard deviation. The left-most bar quantifies cutting at site 1 where constructs were labeled on the top strand. The central bar represents cutting at site 2 of bottom labeled constructs and the right-most bar cutting at site 3 with bottom labeled constructs. Constructs 20 and 53 do not posses a site 3. Panel (C) is a summary of *HaeIII* digests of reconstituted nucleosomes. The bars represent the three cutting sites as described for *MspI*.

The two blocks of A-tracts in all fragments shown in Figure 1 are oriented in a head-to-tail manner and thus are not related by an axis of dyad symmetry that characterizes the histone octamer. In order to determine whether this orientation played any role in the positioning effect, fragment 67 was reconstructed such that the four upstream A5 -tracts, 1–4, were replaced with four T5 -tracts without changing the sequences between adjacent tracts.

Figure 6. Exonuclease III analysis of fragment 67-I. A schematic diagram; top and bottom labeled strands are as in Figure 3. The three lanes shown for each strand correspond to digestion times of 8, 16 and 24 min on reconstituted nucleosomes. The position indicated by the dark rectangle corresponds to the single position seen with fragment 67 (see Fig. 3).

Exo III analysis of nucleosomes reconstituted on this inverted 67 fragment (67-I) are presented in Figure 6. The major nucleosome position seen with 67 (Fig. 3) was also seen with this construct. However, in four of four separate experiments, fragment 67-I also displayed several additional Exo III pauses. In support of the Exo III experiments, restriction enzyme protection assays of the type shown in Figure 5 revealed that 67-I displayed significantly reduced cutting efficiencies at *MspI* sites 1 and 2 when compared with fragment 67 (data not shown). In addition, fragment 67-I displayed energies of reconstitution that were significantly greater than those seen with 61 and nearly as high as those seen with 67 (data not shown). These results imply that both phase and the symmetrical arrangement of the two blocks of tracts are involved in the formation of stable translational positions.

**Rotational orientation of DNA in nucleosomes reconstituted onto synthetic fragments**

The rotational orientation of a DNA molecule on a protein refers to the angular orientation of the axis of individual base pairs with respect to the protein surface. Reconstituted nucleosomes were digested with DNase I (19) and hydroxyl radicals (35) in order to determine the rotational orientation of the synthetic DNAs in mature nucleosomes. Both probes cut the DNA backbone after entering the minor groove and thus minimal cutting occurs when the minor groove faces the histone octamer. In naked DNA, the synthetic curved elements were expected to produce a sinusoidal pattern of OH• cutting along their lengths, with each A-tract displaying a local minimum of cutting frequency due to its compressed minor groove. This pattern was expected to be mirrored in location and accentuated in intensity in those regions of nucleosomal DNA where minor grooves of A-tracts face the histone surface (36). This relationship held for most, but not all curved regions in the synthetic molecules studied in this report.

Figure 7A shows OH• cleavage reactions performed on naked and mononucleosomal DNAs 61 and 67. With 61, the bases of lowest OH• cleavage efficiency in naked DNA along both tracts 1–4 and tracts 5–8 are also minima of cleavage in reconstituted nucleosomes (see Fig. 8 for a detailed map of DNase and OH• cleavage sites). With 67, minima of cleavage of naked and nucleosomal DNA are centered on the same bases along both tracts 1–4 and tracts 5–8 are also minima of cleavage in reconstituted nucleosomes (see Fig. 8 for a detailed map of DNase and OH• cleavage sites). With 67, minima of cleavage of naked and nucleosomal DNA are centered on the same bases along both tracts 1–4 and tracts 5–8 are also minima of cleavage in reconstituted nucleosomes (see Fig. 8 for a detailed map of DNase and OH• cleavage sites). With 67, minima of cleavage of naked and nucleosomal DNA are centered on the same bases along both tracts 1–4 and tracts 5–8 are also minima of cleavage in reconstituted nucleosomes (see Fig. 8 for a detailed map of DNase and OH• cleavage sites). With 67, minima of cleavage of naked and nucleosomal DNA are centered on the same bases along both tracts 1–4 and tracts 5–8 are also minima of cleavage in reconstituted nucleosomes (see Fig. 8 for a detailed map of DNase and OH• cleavage sites). With 67, minima of cleavage of naked and nucleosomal DNA are centered on the same bases along both tracts 1–4 and tracts 5–8 are also minima of cleavage in reconstituted nucleosomes (see Fig. 8 for a detailed map of DNase and OH• cleavage sites).
naked DNA along tracts 1–4 are not rotationally oriented such that they face the histone surface in mature nucleosomes.

Figure 7B shows an autoradiograph of a sequencing gel where bottom labeled mononucleosomes were analyzed by DNase I and OH• cleavage. Lanes 1–3 confirm that the naked DNase I digests of three different fragments produced the same pattern of bands in the region of tracts 5–8. The other seven fragments produced the same pattern of bands in this area of common sequence (data not shown). DNase I digests of each of the 10 reconstituted fragments shown in Figure 1 resulted in a 10 base period of cutting in the region of tracts 5–8. The preferential cut sites are centered on the same nucleotides in each of the constructs, which implies that all major nucleosome positions formed on all fragments share a common rotational orientation in the region of tracts 5–8. The location of the cut sites in the region of tracts 5–8 is centered in the middle of the (G/C)5 regions that separate each A5/7. This implies that the minor groove of each (G/C)5 is facing away from the protein surface while the minor groove of each A5/7 is facing toward the histone surface. With OH• digests of naked DNA, minimal cutting is seen in the 3′-half of the A-tracts, where the minor groove is expected to be compressed (37). The areas of minimal and maximal OH• cutting along tracts 5–8 in nucleosomal DNA for all 10 fragments are centered on the same bases as in naked DNA digests and coincide with the preferred DNase I cutting sites of nucleosomal DNA. These results suggest that all fragments share a common rotational orientation in the vicinity of tracts 5–8 where the center of minor groove compression seen in naked DNA faces the histone octamer in reconstituted nucleosomes.

Figure 7C (left) shows DNase I analysis of the rotational orientation of tracts 1–4 with synthetic molecules labeled on the top strand. Lanes 1–3 confirm that the naked DNase I digests of three different fragments produce the same pattern of cut sites in the region of tracts 1–4. The 10 synthetic sequences do not share the same pattern of cut sites in nucleosomal DNA. Sequences 47, 56, 57, 67 and 77 appear to share a common set of cut sites which include cuts at the 3′-end of A-tracts. The other constructs share common digestion patterns which do not include cuts at the 3′-end of A-tracts. From Figure 7C (right panel) it is evident that the synthetic DNAs display variable OH• cleavage patterns beginning at tracts 3 and 4 and continuing through the central region. The +0 to +1 sequences share a common OH• cleavage pattern which displays maximal cleavage at bases that are cleaved minimally in –3 fragments. Thus, +0 to +1 fragments appear to possess a rotational orientation along tracts 1–4 that is nearly directly out of phase with the rotational orientation adopted by –3 fragments. Constructs 53 and 56 display a rotational orientation along tracts 1–4 that is intermediate to that preferred by +1 and –3 constructs.

A summary of OH• and DNase I cutting sites along the synthetic molecules is presented in Figure 8. The fragments are grouped according to their phase designation. Cutting on the two strands is offset by ~2 bp in the 5′ direction at any particular site of cleavage. This pattern is expected based on the geometry of the minor groove, where the shortest distance across the groove is not parallel to the axis of individual base pairs (37). The DNase I cutting patterns for all sequences are vastly different between the two strands. This has been noted previously and is possibly due to the chiral nature of the enzyme, resulting in two different interactions with the cutting site (25). Since all constructs share a common rotational orientation in nucleosomes along tracts 5–8, it is expected that they should differ in rotational orientation along
Figure 8. Summary of DNase I and hydroxyl radical cleavage of synthetic clones. A summary of DNase I and OH\(^{-}\) cleavage sites along tracts 1–4 and 5–8 is shown. Cleavage on the top strand is shown above the A-rich strand and bottom strand cleavage is shown below the T-rich strand. The constructs are designated as in Figure 1 and are grouped such that –3 fragments, +1 fragments and intermediate fragments form three separate blocks. Strong DNase cleavage sites are represented by dark lines, weak DNase cleavage sites are indicated with light lines and sites of maximal OH\(^{-}\) cleavage are represented by open rectangles. Data is from several experiments of the type shown in Figure 7A–C.

The competitive reconstitutions performed in this study (Fig. 2 and Table 1) suggest that the synthetic fragments have exceptionally high affinities for histone octamers that perhaps is expected for sequences that possess their degree of bending (5). Most striking is the variation in nucleosome stability between synthetic molecules. The +0 to +1 class of fragments form stable nucleosomes when compared with bulk DNA, but each of their multiple nucleosome positions are far less stable than the position that is preferred on fragments 67 and 77. It is likely that there are multiple features of sequences 67 and 77 that are required for their highly stable, unique positioning. Each of the two bent segments is likely to be required, since constructs containing only tracts 1–4 or 5–8 did not position nucleosomes at a single site (data not shown). The symmetrical arrangement of the two sets of tracts is also likely involved in translational positioning, since a number of positions were noted with 67-I that were not seen with 67 (Figs 3 and 6). The phase relationship also appears to be relevant for the positioning effect, since fragments 77 and 67 and, to a lesser extent, fragment 57 position a majority of their nucleosomes bordering on tract 1 while other fragments of similar length and sequence but different phases do not (Figs 3–5). A minimum length separating the two blocks of oligo(A)-tracts also appears to be important, since positioning is only seen in fragments 67 and 77, even though all –3 fragments share a common rotational orientation along both sets of tracts. Given the extensive homology of the central region between different constructs, the above observations suggest that the integral length separating the two regions of curvature is the driving force in producing different classes of nucleosomes on these constructs.

We do not know why the phase relationship is important for the positioning effect. One possibility relates to the structure of the molecules, since the intrinsic shape of these DNAs roughly correlates with the phase designation (Fig. 1B). It is conceivable that the shape of the –3 constructs efficiently directs the reconstitution reaction to a specific location along their length. For example, the (H3H4)\(_2\) tetramer has been shown to bind positively and negatively supercoiled DNA in two different conformations without a loss in binding affinity (38). Since the –3 and +0 to +1 fragments display different macromolecular shapes, the assembly of these fragments may proceed by different routes. The results of this study do not differentiate between the influence of DNA curvature and rotational signals on the assembly reaction. In addition, there has been controversy concerning whether DNA retains its intrinsic structure under the high salt conditions used in the reconstitution reaction (39,40 and references cited therein). If a specific curved structure was responsible for translational positioning in 67 and 77, the particular rotational orientation seen along these fragments could be a consequence and not a cause of the unusually stable translational position. On the other hand, the unusual rotational orientation displayed by tracts 1–4 in –3 sequences might be responsible for the positioning effect given a sufficiently long uncurved central region.

Given standard nucleosome 10.0 bp/turn in distal areas and 10.7 in the central three turns (36), sequences with a +2 phase designation should display the same rotational orientation on both sides of the nucleosomal dyad. The top DNA model in Figure 9 provides an example of this situation and closely resembles the rotational orientation seen in this study with the +0 to +1 fragments. The principle of forming maximal numbers of favorable histone–DNA contacts has been investigated as a possible cause of translational positioning. Patterton and Simpson constructed a +2 phase multimeric synthetic molecule in order to facilitate the placement of compressible minor grooves facing the histone octamer at multiple sites along the length of nucleosomal DNA (41). Similar to the +0 to +1 sequences described in this report, this construct displayed multiple translational positions.
However, the commonly held view (3,4) that increasing numbers of compressible minor grooves facing the histone surface results in an increase in stability of a nucleosome might not hold true when applied to highly curved fragments. The preferential formation of nucleosomes on kinetoplast DNA does not require complete overlap with the curved sequences nor does nucleosome stability increase as a function of increasing contact with the curved DNA (5). These results and similar studies with a circular intron segment from Caenorhabditis elegans (15) imply that the presence of large numbers of A-tracts in nucleosomal DNA does not necessarily result in increased instability when compared with nucleosomes that posses a small number of tracts, even if the two types of nucleosomes share a common rotational orientation along their A-tracks.

As illustrated at the bottom of Figure 9, –3 fragments assume a rotational orientation along tracts 1–4 that does not place compressed sections of their minor grooves facing the histone octamer. Curiously, the –3 sequences form nucleosomes of greater stability than the +0 to +1 sequences whose tracts 1–4 assume a more typical rotational orientation. Given the rotational orientation along tracts 5–8 in –3 constructs where the 5′-half of each A5 faces the histone octamer, the presumably unfavorable rotational orientation displayed by tracts 1–4 was the expected result, since these sequences apparently adopted standard pitches when packaged into nucleosomes. A similar distribution of A-tracks was seen in a tobacco satellite nucleosome positioning sequence, where four of six homopolymeric A-tracts are spaced at 10 bp intervals at one edge of the nucleosome in an arrangement nearly identical to that seen with tracts 1–4 in 67 and 77 (42). In addition, the translational positioning of these four tracts within the satellite nucleosome suggests that significant sections of their compressed minor grooves in naked DNA face away from the histone octamer. It was proposed that this set of tracts was responsible for translational positioning of nucleosomes on the satellite DNA while a second distinct region of curvature defined the rotational setting. Thus, it is conceivable that the unusual rotational orientation of tracts 1–4 in the –3 constructs facilitates translational positioning by a mechanism not related to providing an intrinsically narrow or anisotropically flexible groove to the histone surface. According to this view, tracts 1–4 and 5–8 play distinct roles in the formation of positioned nucleosomes on the –3 fragments where tracts 5–8 set the rotational orientation and tracts 1–4 facilitate translational positioning. Consistent with this proposal is the fact that tracts 5–8 assume the same rotational orientation in all sequences while tracts 1–4 assume the same translational position in 67 and 77 and in the major position in 57. Such a model raises the question as to why tracts 1–4 assume a dominant role in translational positioning even though both sets of tracts consist of [(A5·T5)(G/C)5]4. The two sets of tracts are oriented in a head-to-tail manner in all fragments except 67-I and thus are not related by an axis of dyad symmetry that characterizes the underlying histone octamer. It is interesting to note that 67-I is the only fragment in this study that displays Exo III pauses that are separated by non-integral multiples of a helical turn (Fig. 6). It is conceivable that the symmetrical arrangement of the sets of tracts in this molecule allows either tracts 1–4 or tracts 5–8 to determine rotational orientation and this effect could be detrimental to unique positioning.

The above model suggests that the atypical rotational orientation displayed by –3 fragments along tracts 1–4 directly influences translational positioning. The bending requirement for DNA in a nucleosome is not uniform around the histone octamer. At 1.5 and 4–5 turns from the nucleosome dyad, regions of exceptionally high bending impose departures from ideal base stacking and result in a different balance between minor and major groove compression patterns than is seen elsewhere along the particle (43). These positions are also preferential sites for HIV integration in nucleosomal DNA (44). Given the variation in nucleosomal
DNA structure, the relative demand for intrinsic curvature and intrinsic flexiblity is expected to vary along nucleosomal DNA. For these reasons, the atypical rotational orientation displayed by the –3 fragments along tracts 1–4 might be restricted to particular locations. Supporting this view is the observation that tracts 1–4 are located in the same translational position along nucleosomal DNA in fragments 67 and 77 and in the major position on 57 while tracts 5–8 occupy different translational positions along these nucleosomal DNAs (Fig. 3). In these preferred positions, tracts 3 and 4 are located in a region that imposes high bending and an unusual balance between minor and major groove tracts 3 and 4 are located in a region that imposes high bending these nucleosomal DNAs (Fig. 3). In these preferred positions, tracts 3 and 4 are located in a region that imposes high bending and an unusual balance between minor and major groove tracts 3 and 4 are located in a region that imposes high bending these nucleosomal DNAs (Fig. 3). In these preferred positions, tracts 3 and 4 are located in a region that imposes high bending and an unusual balance between minor and major groove tracts 3 and 4 are located in a region that imposes high bending these nucleosomal DNAs (Fig. 3). In these preferred positions, tracts 3 and 4 are located in a region that imposes high bending and an unusual balance between minor and major groove compression on nucleosomal DNA (43). The rotational orientation along tracts 1–4 in –3 fragments contains at least one potentially favorable sequence feature that could contribute to its restriction to particular regions of nucleosomal DNA. At the star shown above the A-strand in Figure 9, the base pair axis is perpendicular to the histone surface and the T-strand lies above the A-strand. The preferential location of TT dinucleotides in this orientation is one of the most recognizable sequence characteristics of nucleosomal DNA (9,45) and potentially facilitates the packaging of DNA in highly bent conformations by allowing unstacking at TT (8). The inability of fragment 67-I to position a nucleosome at a single site is consistent with this view, since the A-strand should lie above the T-strand in this region of the reconstituted nucleosomes. However, it should be pointed out that the major position seen with 67-I corresponds to the single position of 67, which implies that another factor(s) is involved in unique positioning.

Sequences 67 and 77 were modeled after natural nucleosome positioning sequences in terms of two regions of bending and the length of the central region (10). Other studies of natural nucleosome positioning DNAs have also suggested that asymmetry between the two halves of nucleosomal DNA could be involved in translational positioning. The 5S rDNA nucleosome positioning sequence from L. variegatus shares common features with the tobacco satellite sequence mentioned above and with constructs 67 and 77. Nucleosomal DNA on one side of the 5S rDNA dyad assumes a rotational orientation where intrinsically narrow minor grooves directly face the histone surface while those on the other side of the dyad do not (see fig. 2 in 36). In addition, a comprehensive study of bulk dinucleosomal DNA has indicated that asymmetry between the two halves of a nucleosome is an average property of chicken DNA (46). The similarities between natural nucleosome positioning sequences and the positioning sequences described in this report should allow these synthetic molecules to serve as models for the further study of the role of DNA curvature in the control of nucleosome positioning in the cell.

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