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DNA and protein determinants of nucleosome positioning on sea urchin 5S rRNA gene sequences *in vitro*

(chromatin/DNA–protein interaction/histone octamer)

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ABSTRACT **DNA and protein determinants of nucleosome positioning have been examined after *in vitro* reconstitutions of native or modified histone octamers onto tandem repeats of 207- and 172-base-pair DNA sequences containing the *Lytechinus variegatus* 5S rRNA gene and onto monomeric sequences derived from these by digestion with various restriction endonucleases. In all cases, a major nucleosome position as well as a number of minor positions have been observed, which indicates that the generation of multiple positions is an inherent property of the 5S rRNA gene sequence. Interestingly, all positions observed differ by multiples of 10 base pairs. Data obtained under different reconstitution conditions demonstrate that the observed distributions of nucleosomes on these DNA templates are equilibrium distributions. This study has also examined the positioning of histone octamers from which histone "tails" had been removed by tryptic digestion. Results indicate that the histone tails are not determinants of nucleosome positioning. Although our results suggest that the mechanical properties of the 5S rDNA are the fundamental factors determining nucleosome positioning, they are insufficient to direct all nucleosomes into a single location.**

It is well established that nucleosomes can be positioned over specific DNA sequences *in vivo* and *in vitro* (1). Although it has been demonstrated that the flanking boundaries established by neighboring site-specific proteins can influence nucleosome positioning *in vivo* (2), reconstitution of nucleosomes onto short DNA templates *in vitro* indicates that nucleosome positioning can also occur when such boundaries do not exist (3, 4). In many cases, results strongly suggest that the fundamental determinants of nucleosome positioning reside in DNA sequence-dependent mechanical properties such as bending and flexibility (5–9).

An example of sequence-dependent nucleosome positioning that has generated considerable interest is nucleosome formation on sea urchin 5S rRNA gene sequences. Simpson and Stafford (3) reported, using the DNase I footprinting technique, that nucleosomes are precisely positioned after *in vitro* reconstitution onto a short fragment containing the sea urchin 5S rRNA gene. Similar results have been reported during investigations of the effects of sequence alteration on nucleosome positioning (10) and carcinogen–nucleosome interaction *in vitro* (11). However, using restriction enzyme mapping of nucleosome-bound DNA sequences, Hansen *et al.* (12) observed multiple nucleosome positions on tandemly repeated sequences of the same 5S rRNA gene fragment, with the most preferred position occupying a sequence different from that indicated by DNase I digestion of the monomer fragment. To determine whether multiple positions are an exclusive property of tandemly repeated templates, we have examined the distribution of native and trypsinized nucleosomes present, after salt dialysis reconstitution, on tandemly repeated DNA templates and several monomeric templates derived therefrom by restriction endonuclease digestion. Our results indicate that multiple translational nucleosome positioning frames are an inherent property of the 5S rDNA and are determined primarily by the mechanical properties of this unique DNA sequence.

EXPERIMENTAL PROCEDURES

Preparation of DNA Templates. Monomer fragments and tandemly repeated DNA templates containing the *Lytechinus variegatus* 5S rRNA gene sequence were derived from plasmid p5S207-12 or p5S172-12 (13). Plasmids were purified from *Escherichia coli* HB101 by the alkaline lysis method (14) followed by CsCl gradient banding. Oligonucleosome templates consisting of 12 repeats of a 207-base-pair (bp) (207-12 template) or a 172-bp (172-12 template) DNA sequence were prepared by *Hha* I digestion of plasmid p5S207-12 or p5S172-12, followed by exclusion chromatography on Ultrogel A2 (15). *Eco*RI and *Msp* I monomer templates were prepared by restriction endonuclease digestion of the tandemly repeated 207-12 oligonucleosome template, followed by purification through 2% low-melting agarose gels (16). *Rsa* I and *Xmn* I monomer templates were prepared by restriction endonuclease digestion of the intact plasmid p5S207-12 and subsequent purification through Ultrogel A2 (15).

Preparation of Native and Trypsinized Histone Octamers. Native nucleosome core particles were purified from chicken erythrocytes as described (17). Trypsinized core particles were prepared from native core particles by digestion with immobilized trypsin, followed by purification through sucrose gradients (17). Native and trypsinized histone octamers were obtained from their respective nucleosome core particles by hydroxylapatite column chromatography (18).

Nucleosome Reconstitutions. Reconstitution of oligonucleosomes and nucleosome monomers was carried out using the method of step-wise salt dialysis (12, 19). Template DNA in TE buffer (10 mM Tris·HCl/0.25 mM Na_2 EDTA, pH 7.8) was made to 2.0 M NaCl and mixed with histone octamers. Samples were then dialyzed over a 24-hr period against progressively lower NaCl concentrations and finally into TE buffer. DNA concentrations were 40–50 μg/ml. A ratio of 0.9 mol of octamer per mol of DNA repeat was used in all of the reconstitutions to minimize the possibility of association of more than one octamer with each repeat of the sequence.

Determination of Nucleosome Positioning. Nucleosome positions were determined as described (12). Briefly, nucleosome monomers and oligonucleosomes were digested into

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nucleosome core particles with micrococcal nuclease. To prevent salt-dependent sliding of nucleosomes, digestions were always carried out in 10 mM Tris·HCl/1 mM $CaCl_2$ buffer. After histones were removed from the DNA by Pronase digestion in the presence of SDS, the nucleosome-bound DNA was electrophoresed through 6% polyacrylamide gels, purified by the method of Maxam and Gilbert (20), and then digested with restriction enzymes that cut within the repeat. Restriction digests were electrophoresed on 6% polyacrylamide gels, stained with ethidium bromide (1 μg/ml), and photographed under UV illumination. The sizes of the fragments in each restriction digest were obtained from densitometer tracings of the photograph negatives and used to deduce the positions of bound nucleosomes. The relative amounts of nucleosome positions were obtained from the areas of the corresponding fragment peaks, after correction for differential ethidium bromide staining.

RESULTS

The rationale for determining nucleosome positions on specific DNA sequences by restriction endonuclease mapping of nucleosome-bound DNA is as follows. If all of the nucleosomes occupied the same position on a DNA template, and these nucleosomes were trimmed to 146-bp core particles by micrococcal nuclease digestion, two characteristic fragments would be generated by digestion of the isolated core particle DNA with each restriction enzyme that cuts within the nucleosome-bound DNA. On the other hand, no further cuts would be made by those enzymes, whose cleavage sites lie outside the nucleosome-bound DNA. However, if multiple positions are present, a number of fragments would be generated by each restriction digest. Furthermore, the relative intensities of these bands will reflect directly the quantitative distribution of nucleosomes on the DNA template. In contrast, if the nucleosomes were completely randomly distributed on the DNA templates, a smear of DNA would be seen after restriction enzyme digestion. By using this approach, we have been able to determine the number and relative affinity of nucleosome positions present on a number of different permutations of the sea urchin 5S rRNA gene sequence.

Since a major goal of this work was to determine whether the multiple positions we observed previously are present only on the tandemly repeated 207-12 template, we have determined nucleosome positioning on a related oligonucleosome template containing much shorter linker DNA lengths (172-12) as well as on four different monomer fragments

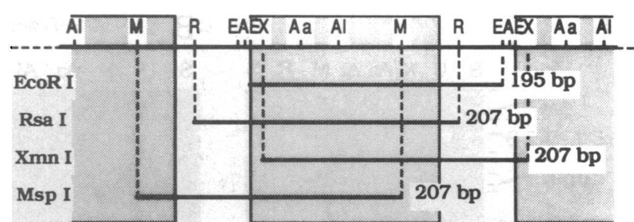

FIG. 1. DNA templates used for mononucleosome reconstitutions. See the text for details of preparation. Shaded areas represent the major nucleosome position on the sea urchin 5S DNA sequence (position 1–146). A, *Ava* I; E, *Eco*RI; X, *Xmn* I; Aa, *Aat* II; Al, *Alu* I; M, *Msp* I; R, *Rsa* I.

derived from restriction nuclease digestion of the tandemly repeated 207-12 source. As shown in Fig. 1, the 195-bp fragment derived from *Eco*RI digestion of the 207-12 template and the 207-bp *Rsa* I-derived monomer template contain sequences 1–146; however, they differ in the sequences that flank each side of this important positioning region. In contrast, neither the 207-bp *Xmn* I template nor the 207-bp *Msp* I template contains the complete 146-bp sequence of the major nucleosome position observed for the 207-12 oligonucleosome template (12). All four monomer fragments, however, selectively contain some of the alternate minor positions reported for the repeated 207-12 DNA.

Micrococcal Nuclease Digestion of Mononucleosomes. The DNA products obtained from micrococcal nuclease digestion of reconstituted *Eco*RI, *Xmn* I, and *Msp* I mononucleosomes are shown in Fig. 2. As was observed previously for the 207-12 oligonucleosomes (12), digestion of either the *Eco*RI or *Rsa* I mononucleosomes at equivalent enzyme concentrations yields a single stable fragment with apparent length of 153 bp when measured against pBR322/*Hha* I or pBR322/*Msp* I fragments as standards. This differs from the value of 146 bp traditionally associated with the nucleosome core particle. To distinguish whether this anomaly is caused by a specific feature of the 5S DNA or is due to micrococcal nuclease-dependent sequence specificity, a set of size standards composed of fragments derived from the 5S DNA itself was used to quantitate micrococcal nuclease products. With such calibration, the nucleosomal DNA is found to migrate at exactly 146 bp, indicating that the core particle DNA derived from nucleosomes positioned on the sea urchin 5S rDNA sequence exhibits aberrant migration on polyacrylamide gels. Given that all of the 207-bp monomer templates themselves exhibit unusual migrations on polyacrylamide gels (not shown), these results indicate that the portion(s) of the 5S rDNA sequences associated with anamolous electrophoretic

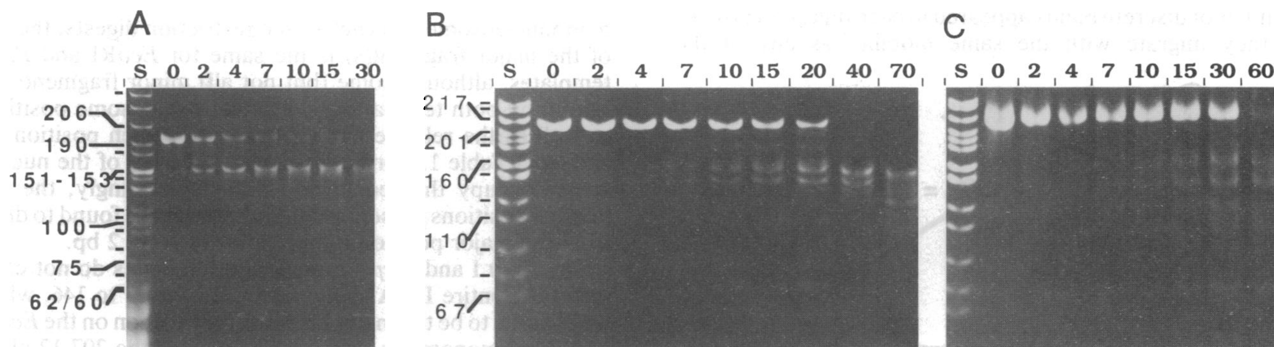

FIG. 2. Micrococcal nuclease digestion of the reconstituted nucleosomes on the *Eco*RI (*A*), *Xmn* I (*B*), and *Msp* I (*C*) monomeric templates. Reconstituted nucleosomes were digested with micrococcal nuclease; resulting DNA fragments were electrophoresed on 6% polyacrylamide gels after proteins were depleted by 2% SDS. Digestions were carried out at a nucleosome concentration of 0.5 mg/ml (DNA weight) and enzyme concentrations of 41 units/ml (for *Eco*RI mononucleosomes) or 4.5 units/ml (for *Xmn* I and *Msp* I mononucleosomes). The numbers on top of each lane indicate the time of digestion in minutes. The DNA standard (S) used in *A* is pBR322/*Hha* I, whereas that in *B* and *C* is pBR322/*Msp* I. Sizes are shown in bp.

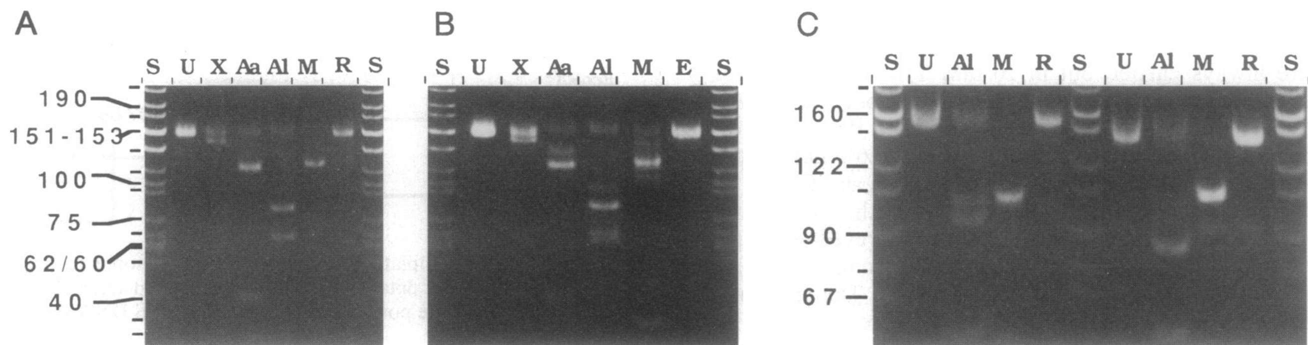

FIG. 3. Six percent polyacrylamide gel electrophoresis for restriction enzyme mapping of nucleosomal DNA obtained from *Eco*RI (*A*), *Rsa* I (*B*), *Xmn* I (*C*, lanes 2–5) mononucleosomes and the 136-bp subnucleosomal DNA from *Xmn* I mononucleosome reconstitute (*C*, lanes 7–10). The letters on top of each lane represent the restriction enzymes used (see Fig. 1; U, unrestricted nucleosomal DNA). DNA standards are p5S207-12/*Hha* I (*A* and *B*) and pBR322/*Msp* I (*C*). Sizes are shown in bp.

behavior are bound to the positioned nucleosomes after reconstitution.

In contrast to the single stable digestion product obtained with mononucleosomes derived from reconstitution of the *Eco*RI and *Rsa* I fragments, micrococcal nuclease digestion of the mononucleosomes reconstituted onto *Xmn* I and *Msp* I templates yields 146-bp DNA fragments and some fragments smaller than 146 bp (see Fig. 2 *B* and *C*), even when using 10-fold lower enzyme concentrations. The *Xmn* I monomers trim to fragments that are found, when properly calibrated, to be 146 bp and 136 bp in length, with many smaller fragments also present. In the case of *Msp* I mononucleosomes, we find no clear kinetic stop and a much larger fraction of the total products migrating at <146 bp.

Determination of Histone Octamer Positioning on Mononucleosomes. The core particle DNAs obtained by micrococcal nuclease trimming of reconstituted mononucleosomes were each subjected to redigestion by several different restriction endonucleases. Examples of gel electrophoresis patterns obtained are shown in Fig. 3. Fig. 4 depicts a scan of lane 6 in Fig. 3*B*. Pairs of bands are observed that sum to 146 bp, the length of the uncut DNA. Note that if one member of a pair is very small, it is difficult to observe on the gel, since pair members are present in equimolar quantities, but stain in proportion to the mass of each. Furthermore, with some positions the *Msp* I site is absent, leading to no further cleavage of the core particle DNA.

To test the possibility that some or all of the fragments observed in Fig. 3 resulted from the DNA sequence specificity of micrococcal nuclease, we isolated core particle size DNA from a partial digest of the *free* 207-12 DNA template and incubated it with the same restriction enzymes. Although a number of discrete bands appeared in each digest, in no case did they migrate with the same mobility as any of the

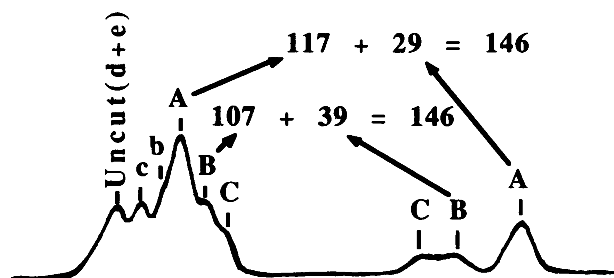

FIG. 4. Quantitation of restriction digests of core particle DNA. Shown is the densitometer scan of lane 6 of Fig. 3*B*. Numbers indicate fragment sizes derived from migrations of 5S DNA internal standards. Arrows indicate two pairs of bands that sum to 146 bp. Letters indicate nucleosome positions that correspond to observed fragment sizes (see Table 1).

fragments generated from restriction enzyme digestion of the nucleosome-bound DNA (not shown). Thus, the observed presence of multiple bands is *not* an artifact of sequence-specific micrococcal nuclease cleavage but instead is due to multiple nucleosome positions present on the reconstituted templates. An additional potential concern of the micrococcal nuclease approach is whether the isolated core particle DNA samples used for restriction analysis represent a majority of the nucleosome population present prior to micrococcal nuclease digestion. In the case of the tandemly repeated 207-12 template, it has been observed routinely that >75–80% of the nucleosome-bound DNA can be recovered in the core particle band. For the 172-12 template, and the *Eco*RI and *Rsa* I templates used here, similar recoveries are obtained. However, in some experiments, we have restricted core particle DNA isolated from incomplete digests (where the recovered core particle band represents a much smaller percent of the original population) and obtained identical positioning results. Thus, for the tandemly repeated templates, and the *Eco*RI and *Rsa* I monomer templates, the positions determined reflect accurately the total population of nucleosome positions present originally after reconstitution. However, the susceptibility of the *Xmn* I and *Msp* I reconstitutes to overdigestion (Fig. 2 *B* and *C*) indicates that in these cases, it is possible that we may be observing the positions of only the most stable nucleosomes.

By correlating the lengths of the restriction nuclease-generated fragment pairs with the known cleavage sites, we can establish the positions occupied by the histone cores on the various mononucleosomes. In addition, the relative intensities of bands provide a semiquantitative comparison of position preferences. Consider first the *Eco*RI and *Rsa* I mononucleosomes: in each of the restriction digests, the size of the major fragment(s) is the same for *Eco*RI and *Rsa* I templates, although some (but not all) minor fragments are shared by both templates. The actual nucleosome positions as well as the relative amount present in each position are shown in Table 1. For both templates, ≈50% of the nucleosomes occupy the sequence 1–146. Interestingly, the less favored positions present in both templates are found to differ from the major position by multiples of 10 ± 2 bp.

The *Xmn* I and *Msp* I monomeric templates do not carry intact the entire DNA sequence from bases 1 to 146, which we observe to be the major nucleosome position on the *Eco*RI and *Rsa* I monomeric templates as well as the 207-12 oligonucleosome template (12). The greater susceptibility of *Xmn* I and *Msp* I mononucleosomes to overdigestion by micrococcal nuclease suggests that the nucleosomes reconstituted onto these two templates may be less stable than the nucleosomes found on templates that contain the preferred 1–146 nucleosome position (Fig. 3). In the case of *Xmn* I mononu-

Table 1. Nucleosome positions occupied by histone cores

Template	Protocol*	Major position	Minor positions
Monomer			
*Eco*RI	I	A	C, E > B > D
Rsa I	I	A	B, C, c > b > d, e
Xmn I	I	—	A′, B > C > D
Msp I	I	—	d/e > c
Multimer			
172-12	II	A	B, C > b, c, d, E, e
207-12	I	A	C > B, b, e > c, D, d, E, F, f, G, g, H, h
	II	A	Same as above
	III	A	Same as above

Abbreviations used for nucleosome positions are as follows. A = 1–146; B = 10–156; C = 20–166; D = 30–176; E = 40–186; F = 50–196; G = 60–206; H = 70–9′ (9′ is the 9th bp on the 5′ flanking sequence—i.e., the adjacent downstream repeat); b = −10 to 136 (−10 is the 10th bp from position 1 on the 3′ flanking sequence—i.e., the position 197 on the adjacent upstream repeat); c = −20 to 126; d = −30 to 116; e = −40 to 106; f = −50 to 96; g = −60 to 86; h = −70 to 76; A′ = 10–146. The error inherent in the assignment of these positions is ±2 bp.

*The protocols used for nucleosome reconstitutions are as follows. Protocol I: 1.5 M NaCl, 4 hr; 1.0 M, 4 hr; 0.75 M, 3 hr; 0.5 M, 3 hr; TE, >12 hr. Protocol II: 1.0 M, 6 hr; 0.6 M, 12 hr; TE, 6 hr. Protocol III: 1.0 M, 6 hr; 0.6 M, 12 hr; 0.3 M, 4 hr; TE, 4 hr.

cleosomes, two discrete populations of DNA fragments are found to be protected from micrococcal nuclease digestion: one of the two protected DNA fragments migrates as core particle size DNA, whereas the other is 10 bp shorter. These two fragments have been separated; restriction enzyme mapping of each reveals that those particles containing core particle size DNA occupy mainly the positions 10–155 and 20–165. Perhaps not surprisingly, the particles that retain only 136 bp of DNA after micrococcal digestion correspond to structures in which the nucleosome has occupied the sequence 10–146. Thus, histone core preference for the major position (1–146) is sufficiently strong to yield a stable 136-bp subnucleosomal particle lacking 10 bp of DNA at one end in about 30% of the reconstitutes. Nucleosomes reconstituted onto the *Msp* I monomeric template appear to be the least stable: only very weak protection of nucleosome core particle size DNA is observed. As a result, even though positioned nucleosomes are observed on this fragment, it is virtually impossible to make even semiquantitative conclusions of the distributions present on the *Msp* I template.

Positioning on Tandemly Repeated Templates. Nucleosome positions observed on the 172-12 oligonucleosome template, as well as on the 207-12 template reconstituted using three different kinetic pathways in dialysis, are shown in Table 1. Both templates consist of tandemly repeated sequences containing the preferred nucleosome position sequence; however, the 172-12 template has 35 bp less linker DNA between the repeats. Results indicate that the most favored position on both oligonucleosome templates is the same as the major position (position 1–146) found for the *Eco*RI and *Rsa* I mononucleosomes and is independent of dialysis protocol. The fact that identical nucleosome positioning can be achieved by salt dialysis reconstitution using different kinetic pathways indicates that the observed positioning patterns represent an equilibrium distribution of positioned nucleosomes. This conclusion is supported further by findings that the distribution of positions on the 207-12 template is independent of the histone/DNA input ratio (J.C.H. and K.v.H., unpublished data). Although multiple positions are observed in both cases, minor positions on the 172-12 template that are >40 bp away from either side of the major position are not detected. On the other hand, we observe minor positions on the 207-12 template as far as 70 bp from either side of the major position. As was observed for the *Rsa* I monomer (Table 1), the total fraction of nucleosomes occupying sequences 3′ to the major position is greater than the fraction occupying the sequences 5′ to the major position.

Positioning of Trypsinized Histone Cores. To understand better the regions of the histone octamer that function in sequence-dependent nucleosome positioning, histone octamers from which the "tail" segments of the histones have been removed by trypsin digestion (see ref. 17) have also been reconstituted onto each of the previously described DNA templates. The densitometer scans of restriction mapping of the nucleosomal DNA from the native (Fig. 5*B*) and trypsinized (Fig. 5*C*) reconstituted *Eco*RI mononucleosomes are shown in Fig. 5. In this example, as well as with all other templates (not shown), we observe absolutely identical po-

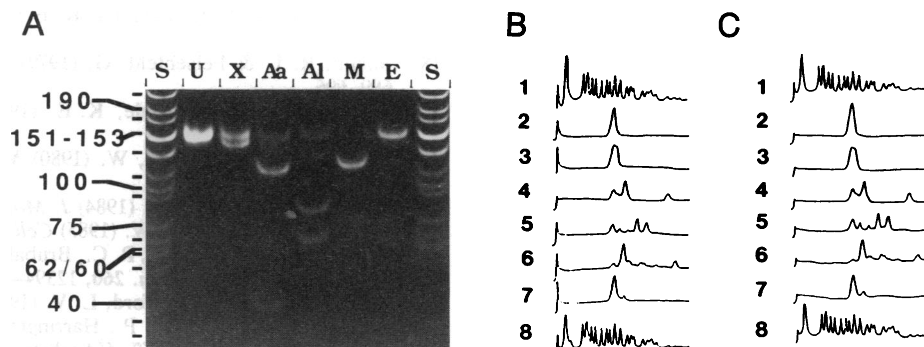

FIG. 5. Comparison of nucleosome positioning in native and trypsinized reconstitutions. (*A*) Six percent polyacrylamide gel electrophoresis showing restriction enzyme mapping patterns of the nucleosomal DNA purified from the reconstituted trypsinized nucleosomes on the *Eco*RI monomeric DNA template. Restriction enzymes used and DNA size standard are the same as in Fig. 3*A*. (*B*) Densitometer scans for the 6% polyacrylamide gel shown in Fig. 3*A*. (*C*) Densitometer scans for the 6% polyacrylamide gel shown in *A*. Sizes are shown in bp.

sitioning patterns for native and trypsinized histone octamers. These results indicate that, under these conditions, the histone tails are not determinants of nucleosome positioning.

DISCUSSION

The presence of multiple translational nucleosome positioning frames on each of several different restriction fragments containing the 5S rRNA gene indicates directly that multiple nucleosome positioning is an inherent property of this DNA sequence. Furthermore, these results indicate that multiple nucleosome positioning observed previously on tandemly repeated dodecamers of the 5S rDNA sequence (12) is not due to its repeated structure or the salt-dependent folding that it undergoes during reconstitution. Although multiple translational nucleosome positions have been observed previously on natural (21–23) and synthetic (9) DNA sequences, it is not yet clear whether this is a general property of all sequences that position nucleosomes. That the phenomenon is not restricted to chromatin reconstituted *in vitro* is also suggested by the early observation of Ponder and Crawford (24), who obtained evidence for multiple positioning of nucleosomes in animal viruses. Our observation that the nucleosome positions present on the 5S rDNA differ by multiples of 10 bp, together with the observed aberrant migration of isolated 5S core particle DNA on polyacrylamide gels, suggest that the mechanical properties of the DNA are responsible for nucleosome positioning. Consistent with this notion, computer simulations (details available on request) using refined dinucleotide wedge angles (25) indicate a 40° bend centered at sequence 65 and less pronounced bends near positions 20 and 160 of the 5S rDNA sequence. Interestingly, Shrader and Crothers (9) have also observed multiple nucleosome positions differing by 10 bp on sequences known to be composed of a series of DNA bends.

The finding that the multiple positions present on the 5S rDNA after salt dialysis reconstitution are equilibrium distributions permits us to estimate the energy differences between different nucleosome positions. We find that in most cases the major position (1–146) is occupied by about 50% of all nucleosomes. In contrast, the number of nucleosomes distributed into any particular minor position corresponds to about 5–20%. Thus, the free energy differences between major and minor positions are only of the order of 0.5–1.3 kcal/mol (1 kcal = 4.18 kJ).† This result does not mean the total binding energy for a nucleosome is small; it only compares differences between favored positions. Actually, the fact that we see no evidence for randomly positioned nucleosomes argues for rather large values of the binding energy compared with randomly chosen positions.

The results obtained with trypsinized histone octamers show that histone tails play no role whatsoever in determining nucleosome positioning, consistent with other evidence indicating that histone tails contribute very little to the overall stability of the nucleosome core particle (17). In addition, these results argue against changes in nucleosome positioning as the mechanism by which covalent modification of the histone tails might influence nuclear functions. Furthermore, the generation of a nucleosomal particle on the *Xmn* I monomer fragment in which only 136 bp interact with histones indicates that the regions of the histone octamer that contact the DNA entry and exit points are also not essential for formation of positioned nucleosomes. A similar finding utilizing reconstitution on a fragment of *E. coli* DNA was reported by Ramsay *et al.* (26). Since DNA exit and entry contacts are presumed to involve H2A, these results seemingly restrict the regions of the nucleosome core that are involved in selecting DNA positions to the globular portions of H4, H3, and possibly H2B. However, the finding of at least 12 different translational nucleosome positioning frames on the 207-12 template differing in binding energies by only 0.5–1.3 kcal/mol indicates that there is no single specific histone core–DNA sequence interaction required for formation of a positioned nucleosome. Rather, we suggest the following model: the inner portion $(H3/H4)_2$ of the histone core positions itself with respect to bends and/or flexibility of the DNA. While one position is favored in any sequence, alternative positions displaced by multiples of 10 bp, which allow the same "face" of the DNA to interact with the core, may be nearly as favorable. The histone tails may be freely modified, and it is even likely that H2A and H2B may be removed without disturbing the positioning.

†Values were calculated using the equation: $N_i/N_o = e^{-(E_i-E_o)/RT}$.

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