Effects of Sequence Alterations in a DNA Segment Containing the 5 S RNA Gene from *Lytechinus variegatus* on Positioning of a Nucleosome Core Particle *in Vitro*

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Reassociation of a 260-base pair cloned fragment of *Lytechinus variegatus* DNA with core histones has been shown to give rise to a uniquely positioned nucleosome (Simpson, R. T., and Stafford, D. W. (1983) *Proc. Natl. Acad. Sci. U. S. A.* 80, 51–55). In an attempt to define the features that dictate the unique positioning of the nucleosome, we have constructed a number of mutants of this DNA sequence. The ability of these mutants to form positioned nucleosomes was analyzed by DNase I digestion of the DNA after reassociation with chicken erythrocyte core histones. While all the mutants were efficiently incorporated into core particles, not all of these modified sequences were capable of forming a positioned nucleosome. Of the 13 mutants examined, 7 fell into a class that gave rise to nucleosomes in which no unique positioning could be demonstrated. While no specific feature of the DNA sequences has been identified as the critical factor in allowing, or dictating, the formation of positioned nucleosomes, our results do indicate that the region 20–30 bases either side of the center of the core particle appears to contain the major elements necessary for positioning. Additionally, these studies clearly show that differences in the digestion of naked and core particle DNA are related to specific interactions of the DNA and histones rather than to an altered specificity of the enzyme induced by the presence of the proteins.

Although controversy still exists concerning the possibility of nucleosome positioning *in vivo*, the weight of evidence now seems to indicate that positioning does occur for certain DNA sequences (4–9). The mechanism(s) that lead to positioning remains unknown. For one set of positioned nucleosomes in a yeast plasmid, recent studies have suggested that local protein-DNA interactions lead to positioning *in vitro* (9). For probably three cases in *vitro* (6–8), it appears that the information sufficient for positioning resides in histones and DNA alone. Simpson and Stafford (7) associated a 260-bp fragment of DNA containing the 5 S RNA gene of *Lytechinus variegatus* with chicken erythrocyte core histones *in vitro* and showed that a positioned nucleosome was formed. A similar result was recently obtained by Ramsay et al. (8) with a 145-bp-length DNA fragment from *Escherichia coli*.

We have begun to define the features that lead to nucleosome positioning both *in vivo* (9) and, here, *in vitro*. For these *in vitro* studies, we have prepared a number of DNA fragments that are mutants of the 260-bp fragment used by Simpson and Stafford (7), bearing deletions or alterations of specific bases at defined sites. We have used these DNA fragments in reconstitution experiments with chicken erythrocyte core histones. From the results of these experiments, we have been able to define regions of the DNA sequence which appear necessary for positioning.

METHODS

Preparation of Core Particles—Core particles were isolated from chicken erythrocytes essentially according to the method of Lutter (10). Soluble large chromatin was produced by lightly digesting isolated nuclei with micrococcal nuclease. H6, H1, and non-histones were then removed by chromatography on Sepharose 4B in 0.65 M NaCl. Redigestion of this material with micrococcal nuclease yielded discretely sized particles from which core particles were isolated by Sephacyr S300 chromatography. Core particles were stored at 4 °C in 10 mM Tris/Cl, 1 mM EDTA, pH 8.0.

Reassociation—Reassociation was carried out by mixing a trace amount of labeled DNA with an excess of core particles, at a DNA concentration of 100 μg/ml. The mixture was then dialyzed against 0.8 M NaCl, 10 mM Tris/Cl, 1 mM EDTA, pH 8.0, for 5 h at room temperature, followed by dialysis against 0.1 M NaCl, 10 mM Tris/Cl, 1 mM EDTA, pH 8.0, for 16 h at 4 °C. Under these conditions, >90% reassociation was routinely achieved, as analyzed by low ionic strength gel electrophoresis on 6% polyacrylamide gels (11). For any given set of experiments, the degree of reassociation was the same for all the samples.

Labeling—The cloned mutant fragments were excised from the parent plasmid by digestion with the restriction enzyme EcoRI and purified by gel electrophoresis and electroelution (12). 3' End labeling was performed by filling in the EcoRI tails with the large fragment of DNA polymerase I and [α-32P]dATP, followed by a chase with an excess of dATP and dTTP. 5' End labeling was achieved by dephos-
DNA Sequence and Nucleosome Positioning

Preparation of 5 S Mutants—The plasmid pLV405-10 contains 10 copies of the 260-bp 5 S rRNA gene fragment joined head to tail at the EcoRI site of pBR322. It was used as the starting material for the generation of the altered sequences. For preparation of the deletions about the AluI site (base 69), the plasmid was digested with AluI and subsequently trimmed with Bal31. The brief treatment with the exonuclease yielded a population of fragments 0–30 bp shorter than the starting material. To ensure that these fragments were blunt ended, they were filled up with large fragments of DNA polymerase I. Fragments in the size range of 290–290 bp were purified by polyacrylamide gel electrophoresis, ligated together, and digested with EcoRI to yield monomeric units. To reduce background positives, this material was digested with AluI to preclude the cloning of sequences that still contained an AluI site. The resulting material was cloned using pAT153 as the vector. Positive clones containing inserts of the desired length were selected for further analysis and ultimately subjected to sequence analysis to determine the exact size and position of the deletions. From this pool, clones containing the 5 S sequence with deletions of 1, 5, 6, 7, 10, 15, and 17 bp around the AluI site were obtained. These are referred to as d1, d5, d6, d7, d10, d15, and d17 in the remainder of the paper. The locations of the deletions are shown in Fig. 1B.

A mutant containing a 6-bp substitution abutting the AluI site was generated as follows. The plasmid containing the 10 copies of the 5 S gene was digested with AluIII sites at 63 and AluI (site at 69) removing a 6-bp fragment. The ends were polished with the large fragment of DNA polymerase I and the sample was then treated with DNA ligase in the presence of an excess of Smal linkers (CCCGGG). Monomeric units were obtained by EcoRI digestion and these fragments were cloned in pAT153. Positive clones with inserts of approximately 260 bp which contained a Smal site were subjected to limited sequence analysis to determine their exact structure. Clone s1 was shown to contain the original 5 S sequence except for the substitution of the sequence CCCGG in place of the sequence AAATAG between position 63 and 70 (Fig. 1B).

RESULTS

The rationale for using DNase I digestion as an analytical method for assessing nucleosome positioning is as follows. When DNA is wrapped around a histone octomer, accessibility to the nucleic acid for nucleases is limited. Specifically, for DNase I, which attacks DNA through the minor groove, hisone-bound DNA will only be cut when this motif is exposed to solvent components and not when it is closely apposed to the protein core of the nucleosome. If a nucleosome is positioned specifically on the DNA sequence, the digestion patterns for DNA external to the core particle should be like that for protein-free DNA. In contrast, in the core particle region, the digestion patterns for the complex and DNA alone

Fig. 1. Structure of the mutants. A, a schematic representation of the 260-bp, d0, fragment showing the position of the 5 S rRNA gene and the major restriction endonuclease sites: EcoRI (E), AluI (A), HpaII (H), and MnlI (M). The site of the 3' end labeling with 32P is indicated by the asterisk. The positions of the mutations in the fragments d1, d5, d6, d7, d10, d15, d17, s1, and a1 are shown relative to the sequence of the parent fragment, d0, between bases 50 and 90. Deleted bases are represented as --- and substituted bases are underlined. The sequence of d0 is taken from Lu et al. (14). C, schematic representation of the parent fragment 80 and the mutant sequences h1, h2, and h3 showing the sites of action of the relevant restriction enzymes: EcoRI (E), AluI (A), HpaII (H), and MnlI (M). The orientation of the region between the two HpaII sites is indicated by the arrow.
should differ with the core particle displaying the characteristic alternating cut sites and noncut regions with a periodicity of about 10 bp. This pattern must extend over a range of about 120 bp; the regions near the ends of the 146-bp core particle segment are somewhat less well defined in nucleosomes assembled with longer DNA fragments (8) than in isolated core particles per se. On the other hand, if a nucleosome forms randomly on a DNA fragment, a cutting pattern similar to that obtained for the digestion of free DNA would be expected for the DNA complex over the entire fragment length, since any given DNA sequence would occur, statistically, in an accessible position as often as any other DNA sequence. However, for fragments the length of those we study here, on statistical grounds it would be expected that the central region of the DNA fragment would be present in a nucleosome at a higher frequency than the ends of the fragment; therefore, the digestion pattern in this region might show some differences from that of the naked DNA. It is likely that there may exist intermediate situations between truly random histone-DNA interactions and uniquely positioned core particles, such as multiple possible positions. For the purposes of this report, we will adopt the criteria above as necessary for defining a positioned nucleosome and class random or possibly multiply positioned core particles together as nonpositioned.

When associated with histones by octamer transfer, all of the DNA fragments studied formed nucleosome core particles as judged by a decrease in their electrophoretic mobility on low ionic strength polyacrylamide gels to about half that of the parent DNA fragment. In each case, greater than 90% of the labeled DNA was associated with histones. Thus, none of the alterations in sequence preclude interaction of the DNA with histones to form core particles.

Figs. 2-7 compare the DNase I digestion patterns of 3' end-labeled fragments digested as either naked DNA or as core particles. In this and all other cases we have examined, the digestion patterns of protein-free DNA samples are essentially identical, except for the regions where alterations in sequence have been made. For the six fragments (d0, d1, d5, d6, d7, and d17) shown in Fig. 2, it is clear that there exists a wide spectrum of differences between the patterns for naked and complexed DNA. The digestion patterns for the complexes of the three fragments do, d7, and d17 are clearly very different from those for the corresponding naked DNA. The autoradiogram for d0, the parent 5 S gene fragment, shows a distinct region where major cutting sites alternate with protected regions with an average periodicity of about 10 bp each. Within this region, there exist major cutting sites for DNA alone that are protected in the core complex (shown by small circles) and sites that while weakly cut in naked DNA are strong sites in the complex (shown by small triangles). The region that demonstrates major differences between free and complexed DNA spans about 120 bases from base 30 to 150, in good agreement with the previous positioning of the core particle between bases 20 and 160 (7) and more recent studies of the position of the core particle formed on tandemly repeated sequences containing the 5 S gene.3

The digestion patterns of the core complexes of fragments d7 and d17 also differ markedly from their corresponding naked DNA patterns; both show the alternating pattern of cutting and protection seen for d0. However, it is also apparent that the patterns of d0, d7, and d17 differ significantly from one another as regards the positions of the major cutting sites within the sequence. This can be seen most clearly in

\[ \text{FIG. 2. The DNase I digestion patterns of the fragments d0, d1, d5, d6, d7, and d17 mapped from the left hand 3' end of the fragments}. \]

![DNase I digestion patterns of fragments](image)

the summary of major cutting sites shown in Fig. 3. The fragments are aligned by a best estimate of the position of the core particle. The numbers shown for the fragments are positions of nucleotides in the original fragment, not lengths. For the d7 fragment to the right of the deletion, cutting sites occur at about the same sequences as for the d0 complex. To the left of the deletion, cutting sites for the two complexes occur with similar spacing but at different sites. We would thus suggest that for the d7 and d0 fragments, the major positioning signal lies to the right of the deletion, since this
area interacts similarly with histones for the two DNA segments.

In contrast, for the d17 fragment, cutting sites to the left of the deletion occur at the same sequence as in the d0 histone complex. To the right of the deletion, cutting occurs at sites with similar spacing, but at different sequences. Thus, for the d17 DNA segment, it appears that the dominant positioning signal resides in the segment of the DNA to the left of the deletion.

The digestion patterns of d5 and d6 show fewer differences between complexed and naked DNA than do d0, d7, and d17, yet they are not totally consistent with a completely random arrangement of DNA and histones. The digestion pattern of d6 as a core particle complex is quite similar to that observed for d7, with many of the major cutting sites occurring at the same sequence in the two fragments. However, the d6 pattern also contains a number of additional strong cutting sites that are offset by 3–5 bases from those that correspond to the d7 major cutting sites and which appear to have a 10-bp periodicity. This digestion pattern can be explained best on the basis of the nucleosome occupying a small number (possibly as few as two) of distinct positions on the DNA, one of which may be the same as that for d7. The cutting patterns of the naked and complexed fragment d1 are very similar; this appears to reflect a nearly random interaction between the DNA and the core histones.

The digestion patterns of the fragments d0, d10, and d15 in free and complexed state are shown in Fig. 4. Similar to the results for fragments d5 and d6, the patterns for d10 and d15 are not totally random, yet are not consistent with uniquely positioned nucleosomes. While the cutting patterns of d1, d5, d10, and d15 are not consistent with the presence of a positioned nucleosome, their cutting patterns do contain a number of strong cutting sites for the complex that are not present in the naked DNA control (e.g., sites around position 100 and 110 for d1 in Fig. 2). We do not understand the origin of such sites in these nonpositioned nucleosomes. The presence of these sites might be explained on the basis of a tendency of some sequences to lie in an exposed position (for DNase I) in all or several of the possible positionings of the nucleosome. In general, however, the apparently randomly associated core particles give rise to fewer and less pronounced strong cutting sites than do the uniquely positioned particles.

There is a striking visual impression gained from inspection of the digestion patterns for the positioned and nonpositioned complexes. During the digestion, positioned core particles give rise to very intense bands separated by virtually uncut regions. In contrast, while there may be an indication of periodic cutting for the nonpositioned complexes, the quantitative differences between the regions which are cut more or less frequently never approach those observed for the positioned particles. Fig. 5 shows such differences for the d0 and d1 complexes presented as densitometric scans of the autoradiograms. The band at about position 100 was scaled to maximum intensity for the two samples. While the position of cutting for certain sites in the d1 complex is close to or identical with those in the d0 complex, the relative intensities differ markedly for the two samples. Further, the difference in intensities between cut sites and noncut (for d0) or less frequently cut (for d1) is apparent. Similar variations in quantitative cutting patterns are present for fragment d10.

Experiments using fragments labeled at the 5′ end of the upper strand, as the sequence is pictured in Fig. 1A, were less clear in demonstrating positioning or lack of positioning of the core particles (data not shown). Digestion of this strand when complexed in core particles proceeded 2–5 times faster than digestion of the complementary strand. While such a result is not in keeping with the known mode of action of the enzyme, it is in agreement with results reported by Simpson and Stafford (7) for the parent 5 S fragment and by Ramsay et al. (8) for the 145-bp E. coli DNA segment. The digestion patterns for the 5′ labeled, positioned particles (d0, d7, d17) were consistent with positioned nucleosomes. However, the pattern for the other fragments was not as random as it appeared for the 3′ end labeled fragments. In general, it seems that cutting of the upper strand, when in core particles, is influenced more by DNA sequence than is the cutting of the lower strand.

Fig. 4 also shows the digestion patterns for 3′ end labeled free DNA and the histone complexes of mutant sequences h1, h2, and h3. Together with the propositus, d0, fragment, the fragments h1, h2, and h3 show the distinct digestion pattern characteristic of a positioned nucleosome. Indeed, the cutting sites at positions from the label to 120 are at exactly (with a few minor variations) the same position in the sequence for all four particles. From the study of these particles and others with deletions or insertions at the HpaII site (data not shown), it can be inferred that alterations at or beyond nucleotide 120 in the original sequence have no effect on the positioning of the core particle. We do not wish to imply that any DNA sequence can be accommodated in this region of the core particle.

Since deletions at the AluI site were found to be capable of
FIG. 5 (top). Densitometric scans of the autoradiograms for the core particles d0 (upper tracing) and d1 (lower tracing). The tracings were scaled such that the major band at position 100 was of equal intensity for both samples.

FIG. 6 (bottom). The DNase I digestion patterns of the fragments d0 and s1 as either free DNA (D) or as core particles (C). Digestion conditions were as in Fig. 2. The substituted region is at the position indicated by the bracket on the right. Bases within the substituted region that are cut in both fragments are indicated by the open circle (○) while the position cut in d0 but not s1 is marked by the open triangle (△).

either destroying or reforming a positioned nucleosome, it seemed likely that the spatial relationship of elements to either side of this site might be important in determining positioning. However, it remained a possibility that the sequence at this position could in itself be important. In order to explore this possibility we constructed the fragment s1. This fragment consists of the parent sequence with the replacement of the 6 bp (AAATAG) between the AhaIII and AluI sites with a SmaI linker (CCCGGG). As shown in Fig. 6, this alteration in the sequence had virtually no effect on the cutting pattern of the core particle as compared to that of d0. The only difference is in the cutting site at position 65, a site that is within the substituted region. It should be pointed out that the two sites at positions 68 and 69, which also lie within the substitution, are cut as "normal." Variations in the cutting patterns of the naked DNA are also seen in this region and probably reflect a recognition by DNase I of the local variation in the sequence or structure of the DNA.

The cutting patterns of fragments d0 and a1, a fragment which contains a 7-bp insert at the AluI site, are shown in Fig. 7. This mutant is perhaps the best example we have observed of a random arrangement of histones and DNA, as reflected by the near identity of the patterns for naked and complexed DNA. Minor differences in the two patterns do exist, but these are qualitatively of the type one would expect based on a statistical distribution of the core particle position over the length of the DNA present.
DISCUSSION

We classify the mutant DNA sequences described here into two groups, those which form positioned core particles and those which do not, interacting randomly, or perhaps in multiple positions, with histones. The first class includes the propositus 5 S RNA sequence, deletions of 7 and 17 bp at the AluI site, a substitution of 6 bp to the left of the AZuI site and a variety of mutants at and to the right of the HpaII site at position 120. The second group includes deletions of 1, 5, 6, 10, and 15 bp at the AluI site and a 7-bp insertion at that site. The variable effect of deletions at the 70-bp position suggests that positioning signals exist both to the right and to the left of this site; observations of deletions which allow and disallow positioning suggest that the signals must be in the proper relationship to one another. The substitution mutant, s1, demonstrates that the actual sequences at the AluI site are not of import, rather it is the spacing between sequences which span or flank this restriction site that is involved in nucleosome positioning. The fact that major alterations in the DNA sequence at and to the right of the 120-bp position are without effect on positioning suggests that discriminatory signals such as those to the left of the AluI site are likely to be absent in this region.

The ability of h2 and h3 to form positioned nucleosomes is also informative in that it demonstrates that the pyrimidine isotach C(T)2CT(C)T2TT between positions 208 and 235 can be incorporated into nucleosomes without any detectable effect on the positioning pattern of the particle. This is of particular significance in light of the demonstration by Simpson and Kunzler (15) that poly(dG)-poly(dC) did not form nucleosomes. Thus, while it is clear that a nucleosome will not form with long homopolymeric DNA, it would seem that shorter stretches of a single base, T8 or C10, do not in themselves constitute a sufficiently disruptive element to preclude formation of a nucleosome. This result is consistent with the findings of Kunkel and Martinon (16) who demonstrated that a 20-bp stretch of poly(dA)-poly(dT) was efficiently incorporated into nucleosomes whereas an 80-bp stretch of poly(dA)-poly(dT) was preferentially excluded from nucleosomes.

A prerequisite to making an accurate determination of the nature of the positioning signal that allows precise interactions of d0, d7, and d17 with core histones is determination of the exact position of the nucleosome on these DNA fragments. Unfortunately, while DNase I is very useful in defining a positioned core particle, producing a clear footprint of cut and noncut regions, it does not yield a clear definition of the ends of the particle. Attempts to define the ends of these particles by digestion with exonuclease III were not totally successful. In part this was because exonuclease III digestion of naked DNA yielded a pattern that contained definite pauses. Additionally, the enzyme did not produce a single major stop site at the end of the core particle but rather multiple pauses at about 10-bp intervals within the core particle (data not shown). However, based on the data from these analyses and the DNase I mapping experiments, our best estimation of the positioning of the core particle would put one edge of the particle about 20 bp from the left-hand end of the DNA (as shown in Fig. 1 A) for the fragments d0 and d17 and 13 bases from the same end for d7. In each case, the core particle extends for 140 to 145 bases along the DNA.

We have examined the sequences of these three DNA segments for elements of homology that might exist for areas of the different positioned DNA sequences that occupy the same positions within the core particle. We have made such comparisons using both our best estimates of the core particle position and variations of this position over a ±3-bp range. We have compared both the primary sequences themselves and the predicted secondary structures of twist and roll angle variations using the Calladine rules of structure as presented by Dickerson (17). To date we have been unable to identify any sequence or structural region that could be assigned unambiguously as a positioning signal. It seems likely from the data that the specificity of the interactions between the DNA and histones is most likely not based exclusively on the recognition of a specific DNA sequence by the histones.

We suggest that the major determinant of nucleosome core particle positioning on the 5 S gene sequence resides in the central region of the DNA, between positions 70 and 120. This conclusion is strengthened by the nucleosome position on a derivative of the 5 S sequence assembled in vivo in a yeast plasmid (9). The central region of the core particle DNA probably interacts with histone H3 (18, 19), a highly conserved protein. Of interest in considering our results in the context of the recent X-ray crystal structure of the core particle (18) is the observation that the DNA in the core particle is not completely smoothly bent around the histone octomer. Rather, bends occur in the nucleic acid at about 15 and 40 bp on either side of the center of the core particle segment. It is possible that the central region of the 5 S segment contains sequences which are particularly amenable to such a structural deformation (18). In this interpretation, the discriminatory sequences to the left of the AluI site might be (i) a region which bent easily and allowed nucleosome positioning when in the proper spatial relationship to (i.e. distance from) the central signals or (ii) a sequence which resisted bending and interfered with DNA wrapping if it was located at the site where such a structural deformation is required in forming a core particle.

One of the criticisms that has been leveled against the use of DNase I as a probe of DNA-protein interactions is the danger that the results may be influenced by the digestion specificity of the enzyme itself. Many studies have shown that the enzyme does exhibit a great deal of specificity in the manner in which it cuts DNA, although the exact nature of the specificity is still somewhat in doubt. While some concept of the cutting specificity of the enzyme can be obtained by digesting naked DNA, there has been little or no defense to the argument that the specificity of the enzyme may be different for free and complexed DNA. This is perhaps more true in light of the recent studies of Drew (20, 21) that suggests that the specificity of DNase I is largely structure rather than sequence specific in its cutting of naked DNA. We feel that these current experiments shed some light on this subject. The DNA segments examined in this study are identical over the vast majority of their length and appear to form nucleosomes equally well. The digestion patterns of the different particles are remarkably different; these differences, however, do not manifest themselves in the digestion patterns of the fragments cut as free DNA. This is perhaps most clearly demonstrated by comparison of the digestion patterns for the 7-bp insertion fragment, a1, and the parental 5 S fragment shown in Fig. 7. With the exception of the inserted region, cutting patterns for the two naked DNA samples are essentially identical. In contrast, the cutting patterns for the complexes of the fragments with histones differ strikingly. Although both fragments form nucleosomes, random positioning on the a1 fragment leads to a digestion pattern for the complex which closely resembles that of naked DNA; unique positioning of the complex for the 5 S propositus fragment leads to a distinctive core particle cutting pattern. Thus the cutting pattern of the DNA in the nucleosomes is apparently not...
merely due to complexing of the DNA with histones giving rise to a distinct cutting pattern on the basis of an altered specificity of the enzyme, but is dependent on the nature of specific interactions, or positioning of the DNA within the core particle.

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