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DNA structural and sequence determinants for nucleosome positioning*

Review Article

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Summary

Positioned nucleosomes are thought to be regulators of genome function but the role of DNA sequence and structure in the control of positioning is poorly understood. We examined the intrinsic curvature of DNA sequences that are known to position histone octamers at single translational sites as a first step to investigate this problem and discovered a conserved pattern of intrinsic DNA curvature that was proposed to direct the formation of nucleosomes to unique positions. The pattern consists of two 50-60 base pair regions of curved DNA separated by preferred lengths of non-curved DNA. The conserved pattern was also seen in all 57 satellite sequences present in the GeneBank database and the distances between successive pairs of curved elements in repeated arrays of satellite monomers were similar to the average spacing of nucleosomes in chromatin. To test the significance of the pattern, ten synthetic DNAs were constructed which contain two regions of curved DNA that are separated by non-curved regions of variable length. Translational mapping of *in vitro* reconstituted nucleosomes demonstrated that two of the fragments positioned nucleosomes at a single site while most of the remaining fragments positioned octamers at multiple sites spaced at 10 base intervals. In support of the curvature-based model, the positioning sequences contained non-curved central regions of the same lengths that were seen in natural positioning sequences and displayed an affinity for histone octamers comparable to the strongest known natural positioning sequences.

A detailed study was then carried out to identify the features that were responsible for high affinity and unique translational positioning activity. Nucleosomes assembled onto positioning fragments of different lengths shared a common upstream border suggesting that the positioning signals were located on the upstream half of these nucleosomal DNAs. In this region, the compressed minor grooves of the A-tracts did not assume the typical rotational orientation of facing the histone octamer. This unusual orientation was showed to be required for unique positioning since positioning activity was lost upon the insertion of 4 bp between the upstream tracts and the pseudo-dyad region. A permanganate hypersensitive site was also found in this region 1.5 turns from the pseudo-dyad at a site known to display DNA distortion in the nucleosome. The sequence of the hypersite contained a TA step flanked by an oligo-pyrimidine tract and the rotational orientation of the reactive TA step in the nucleosomal DNAs was such that the minor groove faces the histone octamer. Substitutions were made in the region of the hypersite and the resulting constructs tested for affinity for histone octamers and translational positioning. The results revealed that a single base change in the TA step and a few changes in the adjacent tract were sufficient to dramatically reduce affinity and positioning activity in a manner that appeared to be correlated with the presence of a permanganate hypersite. In addition, the rotational orientation of the sequence was shown to be important for function since altering the orientation of the site in a positioning fragment reduced positioning activity and octamer affinity while altering the orientation of the sequence in a nonpositioning fragment had the opposite effects. The 5S rDNA positioning sequence from *L. variegatus also* contained a permanganate hypersite at 1.5 turns from the pseudo-dyad and other natural positioning sequences were enriched in the sequence motifs that give rise to permanganate hypersensitivity in this location. These results suggest a model in which translational positioning is due to a concerted action between the stabilizing forces associated with the hypersite sequences occupying specific sites within the central three turns of nucleosomal DNA and destabilizing forces which appear when the upstream A- tracts with outward facing minor grooves occupy particular translational positions.

I. Introduction

The first step in the packaging of DNA into a chromosome is the formation of a complex between 146 base pairs (bp) of DNA and an octamer of histone proteins, resulting in a particle known as a nucleosome (Van Holde, 1993). The difference in stability between potential nucleosome positions along bulk genomic DNA is usually small enough that a number of different positions are equally likely to be occupied on a given DNA sequence (Cao *et al.*, 1998; Lowary & Widom, 1998). However, sequences have been identified that direct the deposition of histone octamers to specific locations. The locational preference is usually considered in terms of the translational positioning which refers to the position of the histone octamer along the DNA molecule and the rotational setting, which gives the local orientation of the DNA relative to the direction of curvature on the octamer surface.

Positioned nucleosomes frequently occur in the vicinity of promoters, enhancers and other control sequences in the genome. Consequently, these elements have received considerable attention as potential regulators of genome function. It is now clear that positioned nucleosomes can regulate both transcription and replication by controlling the accessibility of regulatory proteins to DNA (reviewed in Wolffe, 1994; Svaren and Hörz, 1996; Beato and Eisfeld, 1997). For example, some regulatory factors bind their target sequence with high affinity only if this sequence is located along DNA separating two adjoining nucleosomes. Other regulatory factors can bind to nucleosomal DNA, but only if the target sequence is presented in a particular rotational orientation. The translational position of a DNA sequence within a nucleosome can also influence its accessibility for protein factor binding. Thus, a complete understanding of genomic control mechanisms in eukaryotes will require a more thorough description of the determinants of nucleosome positioning than is currently available. An understanding of mechanisms that lead to nucleosome positioning would also likely enhance our knowledge of the physical organization of DNA in the nucleosome and in solution. In addition, a strong positioning sequence could be used to control the site of nucleosome residency *in vivo* which might permit the development of new approaches to regulate gene activity and to further clarify relationships between nucleosome positioning and genome function in the cell.

Some progress has been made in our understanding of sequence features that give rise to a preferred rotational orientation of DNA in the nucleosome. Such DNA has been viewed as an anisotropic rod, which may bend more easily in one plane than another. Sequence features that can give rise to anisotropic bendability include short A/T rich and G/C rich elements, which can bend more readily into the minor and major grooves, respectively. When these sequences are arranged in a 10.0 bp period, the A+T rich sequences are oriented such that their minor grooves face the histone surface while the minor grooves of G+C rich sequences face outward (Drew and Travers, 1985; Satchwell *et al.*, 1986; Shrader and Crothers, 1989). Thus, the proper rotational orientation of such sequences decreases the amount of mechanical work that is required to fold the DNA and results in an increase in nucleosome stability. Intrinsically curved DNA arises from oligo A-tracts arranged in a 10 bp period and such DNAs also generally adopts this preferred rotational orientation in the nucleosome (reviewed in Crothers *et al.*, 1990; Harvey *et al.*, 1995). Intrinsically curved DNA favors nucleosome formation and has been identified within many of the most stable nucleosomes in the genome (Penning *et al.*, 1989; *et al*., 1990, Widlund *et al*., 1997; Fitzgerald and Anderson, 1999a). However, anisotropic bendability or intrinsic curvature *per se* is insufficient to position nucleosomes at single sites (Schrader and Crothers 1990; Fitzgerald and Anderson, 1999a). For example, nucleosomes form preferentially on kinetoplast DNA and on a circular intron segment which are both highly curved, and these sequences assume an optimal rotational setting in the nucleosome. However, nucleosome formation occurs at multiple positions along these circular DNA fragments presumably because of the monotonous bending along these segments (Costanzo *et al.*, 1990, Fitzgerald and Anderson,1999a).

A commonly held view is that multiple distinct sequence elements along the nucleosome contribute to the translational positioning of nucleosomes (Trifonov, 1980; Drew & Travers, 1985; Neubauer *et al.*, 1986; Shrader & Crothers, 1990). Consistent with this proposition are studies which have identified sinusoidal patterns of sequence preference along bulk nucleosomal DNA which are likely to reflect particular sequences occupying favorable and avoiding unfavorable sites. For example, AA and AAA are found most often at sites where their minor grooves face the histone surface while longer A-tracts are frequently observed in distal turns of the nucleosome and along linker regions (Struhl, 1985; Satchwell *et al.*, 1986). Similarly, dinucleotides displaying low stacking energy are known to prefer particular translational positions along nucleosomal DNAs (Satchwell *et al.*, 1986; Satchwell & Travers, 1989; Ioshikhes *et al., 1996*). These sequence patterns are magnified in nucleosome positioning DNAs and thus may play some role in dictating sites of nucleosome residency in the genome (Fitzgerald et. al, 1994).

The non-uniform distribution of sequence elements along nucleosomal DNAs is likely to be related to the curvature demands that are imposed on the DNA during nucleosome formation and to the non-uniform shape of the DNA superhelix in the mature nucleosome. The recent high resolution crystallographic study of the nucleosome core particle (Luger *et al*., 1997) confirmed earlier suspicions that nucleosomal DNA does indeed take an irregular path around the histone octamer. Along this path, regions 15 and 45 bps from the pseudo-dyad exhibit marked departures from ideal base stacking. Biochemical studies have also suggested that the DNA in these areas assumes a distinct structure not seen elsewhere along the particle. For example, the region 15 bps from the pseudo-dyad is preferentially attacked by singlet oxygen (Hogan *et al*., 1987) and the enediyne antibiotic calicheamicin (Kuduvalli *et al.*, 1995). This position is also a hot spot for the formation of thymidine dimers (Gale & Smerdon, 1988) and is a preferred

site of action of the HIV integrase where a distorted (or distortable) DNA structure is required for the action of this enzyme (Pruss *et al*., 1994 A&B). A recent analysis of a nucleosome from the *Adh* gene of Drosophila revealed an asymmetric intra-nucleosome structure in the region 1.5 turns from the pseudo-dyad that was suggested to be a result of a unique local DNA conformation (Gao & Benyajati, 1998). In addition, an *in vivo* positioned nucleosome in *C. Glabrata* was shown to display sequence-dependent DNA distortion directly adjacent to a transcription factor binding site in the absence of the factor (Zhu & Thiele, 1996). Since a disruption in base stacking along nucleosomal DNA is likely to be both energetically costly and sequence-dependent (Crick & Klug, 1975; Zhurkin, 1985; Travers, 1991), the localization of sequences that facilitate DNA distortion to distorted sites within the nucleosome might serve as a translational positioning signal (Neubauer *et al.*, 1986; Travers & Klug, 1987; Luger & Richmond, 1998).

It is evident from the above correlation studies that variations in sequence-dependent properties of DNA in the nucleosome such as intrinsic curvature and distortion could be involved in directing nucleosomes to unique sites. However, in spite of the considerable amount that is known about DNA in a nucleosome, the mechanisms responsible for translational positioning remain poorly understood. To this point, all previously published attempts by other laboratories at producing a synthetic sequence that positions nucleosomes at a single site have failed (Schrader and Crothers, 1990; Tanaka *et al*., 1992; Patterton and Simpson, 1995). In this article, we review our studies on identifying the features that are responsible for the translational positioning of nucleosomes.

II. Results and discussion

A. Conserved patterns of curvature in satellite and nucleosome positioning DNA

We described the intrinsic curvature of DNA sequences that are known to position histone octamers at single translational sites. The 11 nucleosome positioning sequences that were analyzed in this study were from satellites, promoters and gene coding regions. The computer program used in the analysis is based on the A-tract bending phenomenon and yields predicted DNA curvature values that closely reflect the results of experimental data on DNA curvature (Eckdahl & Anderson, 1987; Van Wye *et al*., 1991; Wang *et al.*, 1994; Albert *et al.*, 1995). The analysis uncovered a conserved pattern of intrinsic curvature that was proposed to direct the formation of nucleosomes to unique positions (Fitzgerald *et. al,*. 1994). As summarized in **Figure 1**, the pattern consists of two 40-50 base pair regions of curved DNA separated by about 40-50 bp of non-curved DNA and the pseudo dyad was located in the region of low curvature. The conserved pattern was also seen in all 57 satellite sequences present in the GeneBank database and the distances between successive pairs of curved elements in repeated arrays of satellite monomer were similar to the average spacing of nucleosomes in chromatin.

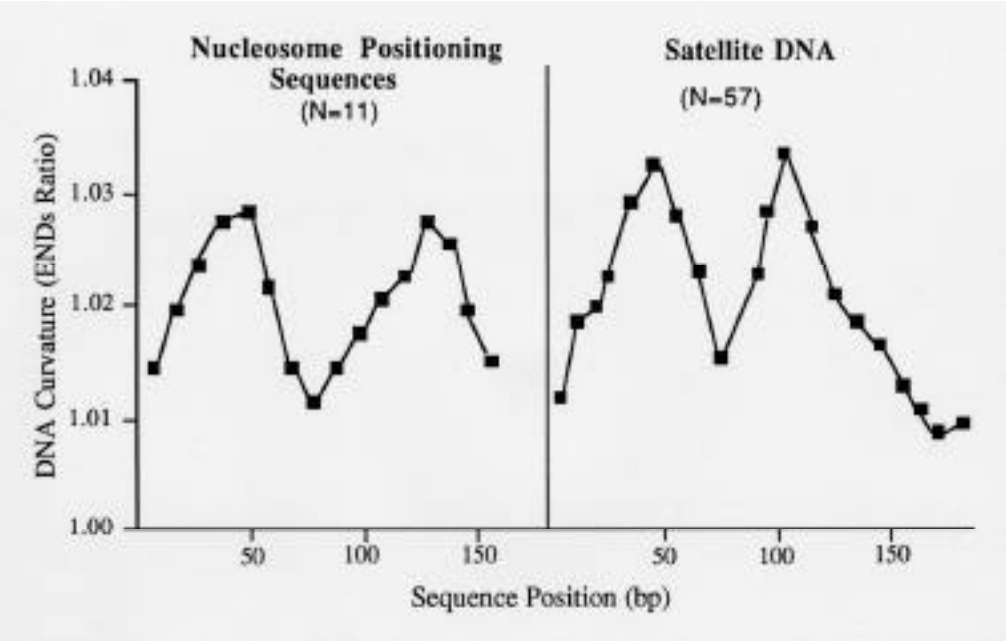

Figure 1. Conserved patterns of DNA curvature in nucleosome positioning and satellite DNAs. The computer program used for this analysis is based on the wedge model for A-tract bending and calculates an index of intrinsic DNA curvature from nucleotide sequence that is called the ENDs ratio (Eckdahl and Anderson, 1997). The ENDs ratio is defined as the ratio of the contour length of a DNA segment to the shortest distance between its ends. The program was used to analyze the curvature along 11 nucleosome positioning sequences and all 57 satellite sequences present in the Gene Bank database and the averages for each sequence set are shown. Each of the sequences displayed the conserved pattern of curvature which consists of two ~ 40-50 bp regions of curvature DNA separated by a ~40-50 bp segment of noncurved DNA. The data is summarized from Fitzgerald *et al.*, 1994.

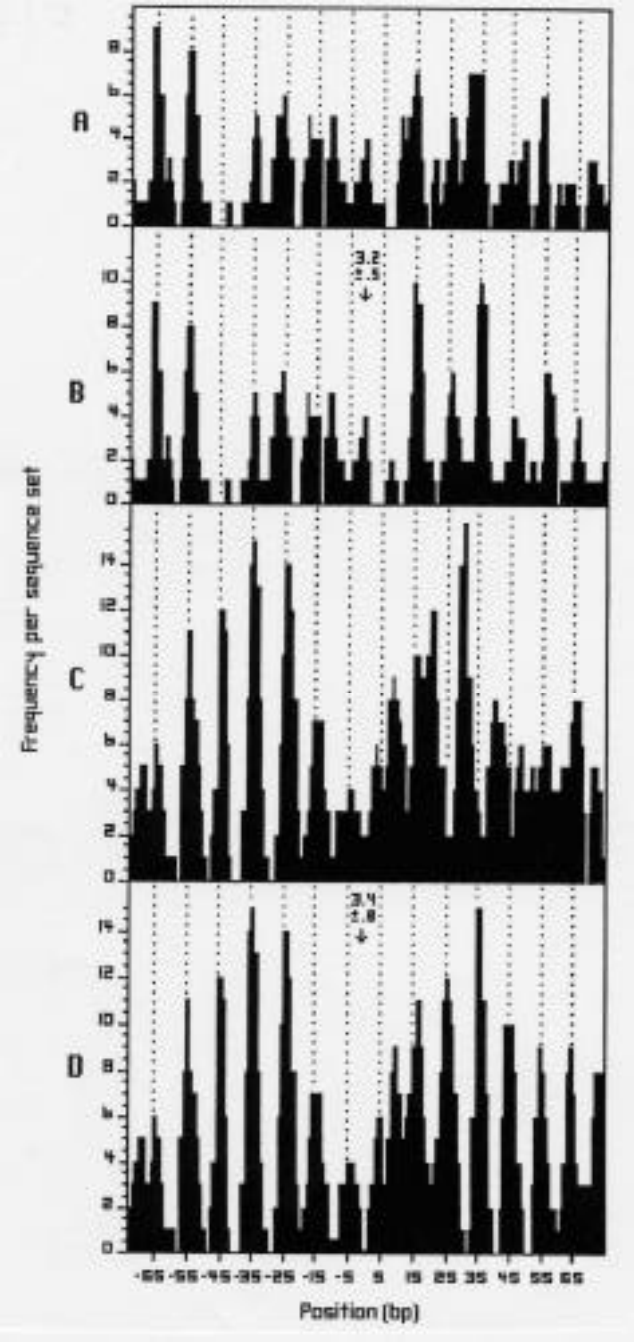

Figure 2. Rotational sequence patterns in nucleosome positioning and satellite DNA. Sequences of the forms $(A/T)_2$ $(N)_4$ (G/C) $(N)_3$ $(A/T)_2$ and $(A/T)_2$ $(N)_3$ (G/C) $(N)_4$ $(A/T)_2$ were identified in the nucleosome positioning (A and B) and satellite (C and D) DNA. The distribution of these motifs along the 5' 73 bp halves of the segments were used to align the 11 sequences which comprised each set. The occurrences of these elements was then summed over a window of 3 bp and plotted as a function of sequence position. In panels B and D, G's were inserted at position 73 until the motifs on the 3' side came into phase with those on the 5' side. The mean lengths $\pm$ SEM of the insertions for the 11 sequences are given above the arrows. The data is from Fitzgerald *et al.*, 1994.

An analysis of the nucleotide sequences responsible for the curvature patterns revealed the expected enrichment of oligo A-tracts in a 10.0 bp period on both sides of the A-tract deficient central regions. The studies also demonstrated that the sequence phase of the oligo A-tracts on the left side of the central region was offset from that on the right side by about 3 bp as seen in **Figure 2**. That is, an insertion of an average of about 3 bp into the central regions was required to bring the two sides of the nucleosomal and satellite DNA into a single phase of 10 bp.

B. Unique translational positioning of nucleosomes on synthetic DNA

To test the significance of the conserved curvature pattern, ten synthetic DNAs were constructed which contained two regions of curved DNA of the form $(A_5 \cdot T_5)(G/C)_5)_4$ that were separated by non-curved regions of variable length. The fragments were named according to the phase relationship between upstream A-tracts 1-4 and downstream A-tracts 5-8 as shown in **Figure 3**. For example, fragment 67 contains 67 bp from the center of tract 4 to the center of tract 5. The two major groups of sequences were the +1 series (fragments 41, 51, and 61) and the -3 series (fragments 47, 57, 67, and 77). The -3 series matches the sequence phase we previously reported as the average for natural nucleosome positioning sequences (**Figure 2**).

Nucleosomes were assembled onto labeled DNA fragments by exchange of histones from chicken erythrocyte mononucleosomes using the salt-dilution method. In order to assess the relative reconstitution efficiencies, samples were reconstituted in the presence of varying amounts of competitor DNA and then analyzed on polyacrylamide-glycerol gels. A summary of the results of these experiments is provided in the top panel of **Figure 4**. Nucleosomes that formed on all of the synthetic fragments were significantly more stable than those that formed on bulk DNA and on the 5S rDNA nucleosome positioning sequence from *L. variegatus* . In addition, a general trend seen in the figure is that the -3 fragments form nucleosomes of greater stability than the +1 fragments. Translational mapping of *in vitro* reconstituted nucleosomes demonstrated that the two longest fragments in the -3 series (#67 and 77) also positioned nucleosomes at a single site as summarized in the bottom panel of **Figure 4**. This major position, as well as other minor positions, was also seen with the shorter members of the -3 series (fragments 57,47). In contrast, all fragments in the +1 series positioned octamers at multiple sites that were spaced at 10 base intervals. In support of the curvature-based model, the positioning sequences contained non-curved central regions of similar length and phase that were seen in natural positioning sequences and these fragments displayed an affinity for histone octamers comparable to the strongest known natural positioning sequences. In addition, both the upstream and downstream curved segments were shown to be required for unique positioning as revealed by translational mapping studies of nucleosomes assembled onto derivative 67 fragments that lacked these segments (Fitzgerald &Anderson, 1999).

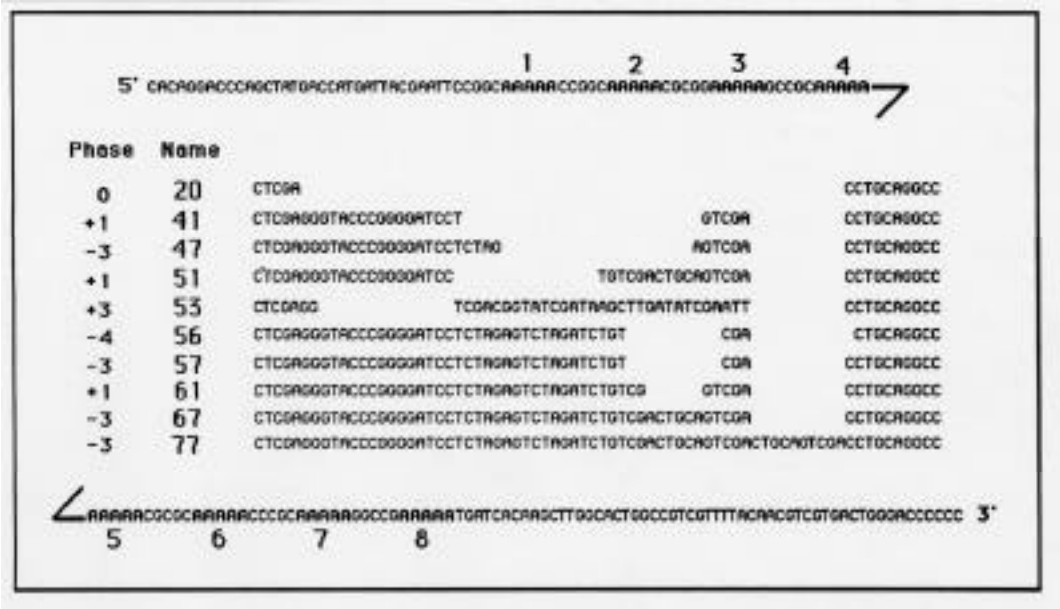

Figure 3. The synthetic sequences. Synthetic DNA fragments were constructed which contained two regions of curved DNA separated by non-curved regions of variable length. To generate the fragments, two 45 bp synthetic oligonucleotides of the form $(A_5 \cdot T_5)(G/C)_5)_4$ were inserted into the polylinker of pUC 18 to yield a recombinant parent plasmid. Insertions and deletions were then made in the region separating the two synthetic bending elements. The fragments were named according to the phase relationship between upstream A-tracts 1-4 and downstream A-tracts 5-8. For example, fragment 67 contains 67 bp from the center of tract 4 to the center of tract 5. The two major groups of sequences were the +1 series (fragments 41, 51, and 61) and the -3 series (fragments 47, 57, 67, and 77). The plasmids were used as templates in the PCR to produce end-labeled or uniformly labeled fragments that ranged in length from 178 bp to 235 bp. These fragments were then used for nucleosome reconstitution.

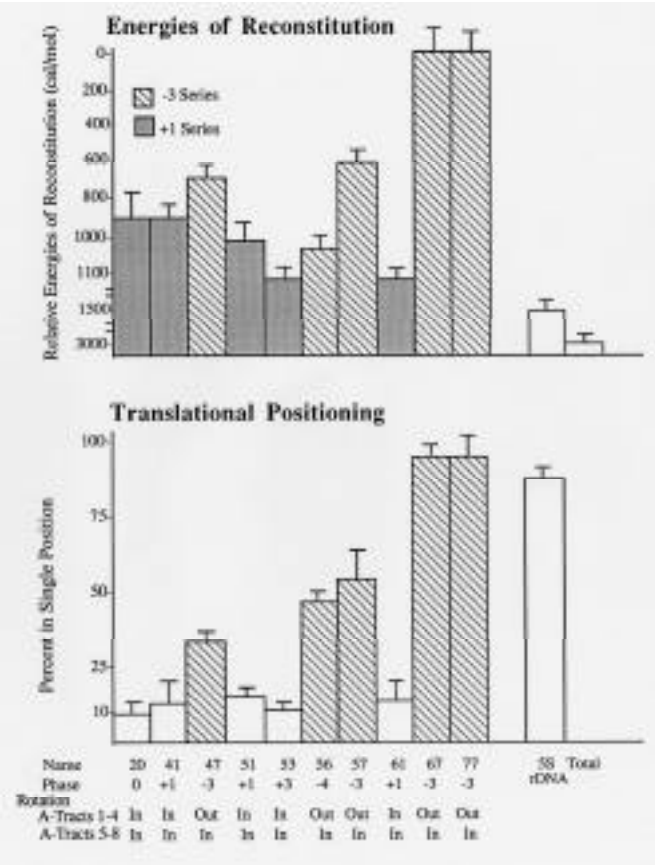

Figure 4. Characteristics of nucleosomes assembled onto the synthetic segments. Nucleosomes were reconstituted onto the fragments by the standard exchange-salt dilution method (1.0M NaCl to 0.1 M NaCl) using H1/H5 deficient nucleosomes from chicken erythrocytes as a source of core histones (Drew and Travers, 1985). Energies of reconstitution relative to fragment 67 were determined by competitive reconstitution analysis (Shrader and Crothers, 1990). Translational positioning was assessed by exonuclease III (Exo III) and micrococcal nuclease (MNase) digestion studies. Hydroxyl radical cleavage analysis and DNase 1 digestion studies were used to determine the rotational orientation of sequences along the nucleosomal DNAs. The hydroxyl radical cleavage data in conjunction with the results of the Exo III and MNase experiments was also used in assigning the position of the pseudo-dyad. Note that the upstream curved element containing A-tracts 1-4 assumed a variable rotational orientation between constructs that depended on the phase: the narrow minor grooves of the tracts pointed inward (IN) in the +1 series but outward (OUT) in the positioning -3 series. Data summarized from Fitzgerald and Anderson, 1998.

All of the synthetic fragments assumed standard pitches when packaged into nucleosomes of about 10.0-10.3 bp/turn in distal areas and 10.5-10.7bp/turn in the central three turns (Hayes, *et al*., 1990, 1991). Consequently, the minor grooves of A-tracts in the +1 series should display the same rotational orientation on both sides of the nucleosomal dyad while the opposite should be true for the -3 series fragments. Hydroxy radical cleavage analysis and DNase I digestion studies confirmed these expectations as summarized in the bottom of **Figure 4**. These results showed that the synthetic curved element on the downstream side of the nucleosomal dyad in all fragments existed in the same rotational orientation with the narrow minor grooves of A-tracts 5-8 facing inward toward the histone surface. We therefore suggested that these tracts set the rotational orientation of all fragments. In contrast, the curved element on the other side of the nucleosome displayed variable rotational orientations which appears to be related to translational positioning. The narrow minor grooves of the A-tracts 1-4 faced inward in nucleosomes assembled onto +1 series fragments but outward in the -3 series nucleosomes.

C. The positioning signals

1. Location

A detailed study was then carried out to identify the features that were responsible for high nucleosome stability and unique translational positioning activity of the fragments in the -3 series. The unique translational position in fragments 67 and 77 and the major position in 57 share a common upstream border as shown schematically in **Figure 5**. We therefore proposed that the translational positioning signals were located on the upstream half of these nucleosomal DNAs. One unusual feature that characterized this upstream region was that the compressed minor grooves of the A-tracts did not assume the typical rotational orientation of facing the histone octamer as described above. An additional unusual feature in this region was detected using the oxidative reagent potassium permanganate. Previous studies have shown that permanganate oxidizes mononucleotides with a relative rate of T>>C>A=G (Hayatsu & Ukita, 1967). Double stranded DNA displays up to 20-fold lower reactivity than single stranded DNA (Hänsler & Rokita, 1993) with the highest reactivities also being observed at thymines. Since the 5-6 ∏ bond of pyrimidines is expected to be involved in base-stacking with the adjacent base in B-DNA, hyperreactivity of this reagent is considered to be indicative of a melted or distorted DNA structure (reviewed in Nielsen, 1990; John & Workman, 1998). Although permanganate has been used to detect DNA distortion around transcription start sites, enhancers, and other DNA binding sites (Borowiec *et al*., 1987; Hsieh *et. al*., 1993; Summers *et al*., 1997; Wilkins & Lis, 1999), it had not been used previously to study DNA distortion in the nucleosome. As shown in **Figure 6**, a single permanganate hypersensitive site was found in the upstream DNA region of nucleosomes assembled onto fragments 67 and 77. This site was not hypersensitive in the naked DNA samples which shows that the nucleosome packaging of the positioning fragments was required for formation of the hypersensitive site. There was no KMnO4 hypersensitivity on the top strands of these fragments or on either strand of fragment 61 (see below). Of crucial importance is that the prominent reactive site is located 1.5 turns away from the dyad in the single position of 67 and 77 at a site known to display DNA distortion in the nucleosome. The 5S rDNA positioning sequence from *L. variegatus* also contained a permanganate hypersite at 1.5 turns from the pseudo-dyad as seen in **Figure 6** and other natural positioning sequences were enriched in the sequence motifs that give rise to permanganate hypersensitivity in this location as will be discussed below .

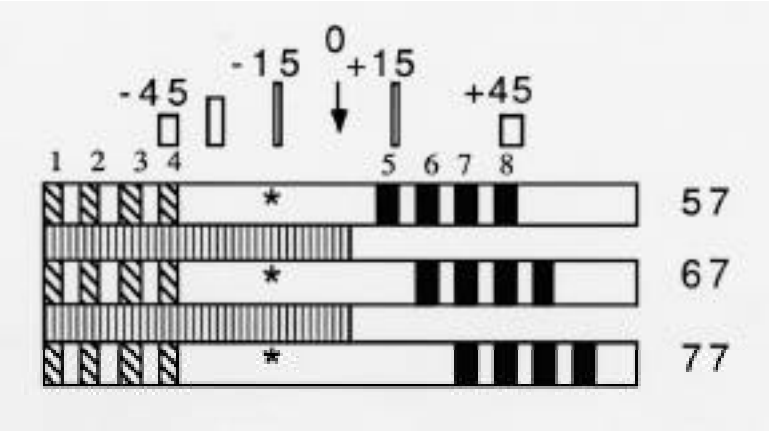

Figure 5. Upstream location of the positioning signals. The unique translational position in fragments 77, 67 and the major position in 57 are depicted by the rectangles. The A-tracts are indicated by dark squares (tracts 5-8) if their minor grooves face the protein surface and by hatched squares (tracts 1-4) if they do not. Areas of common sequence are shown by horizontal lines between the rectangles. The mapped pseudo-dyad is indicated by an arrow and presumptive sites of DNA distortion at ±15 bp and ±45 bp from the pseudo-dyad are indicated above the fragments. Note that the nucleosomes assembled onto each of the fragments share a common upstream border suggesting that the positioning signals are located on the upstream half of these nucleosome DNAs. The permanganate hypersite is indicated by the stars.

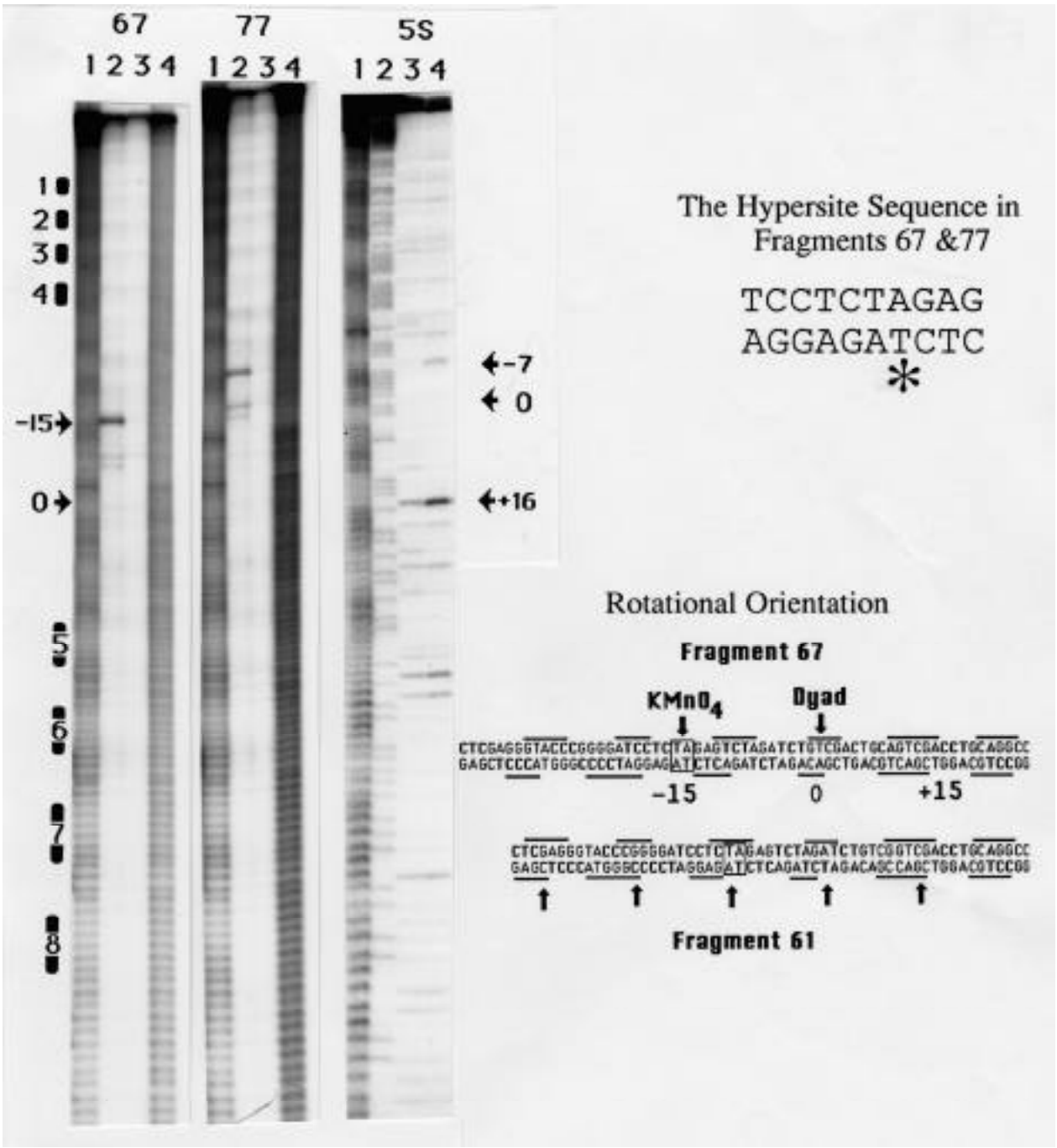

Figure 6. The permanganate hypersite and its rotational orientation. **Left:** DNA distortion in the nucleosome detected by permanganate hypersensitivity-For fragments 67 and 77, lanes 1 and 2 are mononucleosomes treated with hydroxyl radical and permanganate, respectively, and lanes 3 and 4 are naked DNAs digested with permanganate and hydroxyl radical, respectively. For the 5S rDNA sequence from *L. variegatus,* lanes 1 and 4 are mononucleosomes treated with hydroxyl radical and permanganate, respectively, while lane 3 is naked DNA digested with permanganate. Lane 2 is a G/A sequencing lane. The bp positions of the permanganate hypersites at -15 (fragment 67) and +16 (5S rDNA) relative to the pseudo-dyad at 0 are indicated by the arrows. The hypersite in fragment 77 is also located at -15. Note that all three hypersites are located 1.5 turns away from the dyad which is a site of DNA distortion in the nucleosome. **Right:** Rotational orientation of the hypersite- The hyperreactive T residue at -15 bp relative to dyad along the bottom strand of 67 is indicated by the star at the top. Portions of the sequences of positioning fragment 67 and nonpositioning fragment 61 are shown at the bottom. The hyperreactive T residue is contained within a TA step which is flanked on the 5' side by Y_3 (Y=pyrimidine) and on the 3' side by R_5 (R = purine). The possible positions of this TA step in fragment 61 are indicated by the upward facing arrows. Hydroxyl radical cutting sites are represented by lines above and below the bases that were maximally cleaved. Note that minor groove of the hyperreactive TA in 67 faces inward toward the histone surface while the minor groove of the TA in 61 faces away and can be no closer than half a helical turn from the major distortion site at -15 bp from the dyad (from Fitzgerald and Anderson, 1999b).

2. Sequence characteristics and rotational orientation of the permanganate hypersite

The sequence of the region containing the permanganate hypersite in the nucleosomal DNA from fragment 67 and the corresponding sequence from fragment 61 are shown in **Figure 6**. The hyperreactive T residue is contained within a TA step which is flanked on the 5' side by Y_3 (Y=pyrimidine) and on the 3' side by R_5 (R = purine). The TA step has been shown to be a major site of protein-induced kinking (Dickerson, 1998). In contrast, purine homopolymers resist protein-induced bending due to their mechanical rigidity (Hagerman & Hagerman, 1996), and are also known to form unusual structures under a variety of conditions (Wells, 1988; Ohyama *et al.*, 1998). An initial question we considered is why fragment 61 did not display hyperreactivity at the same site as was observed with 67 and 77 since each of these fragments share a common sequence spanning >15 bp on either side of the hyperreactive thymine. A comparison of the rotational orientation of fragments 67 and 61 as deduced by hydroxyl radical mapping studies suggested that rotational orientation might be involved in generating the permanganate hypersite (**Figure 6**). The hydroxy radical cleavage data demonstrated that minor grooves of the hyperreactive TA steps displayed by fragments 67, 77, and 5S rDNA faced inward toward the histone surface as is expected for sequences positioned at the -15 position in the nucleosome. In contrast, the minor groove of the permanganate-insensitive TA step in fragment 61 faced away from the histone surface. One possibility is that potentially distortable sequences in the nucleosome positioning fragments 67, 77, and 5S rDNA occupy a rotational orientation that facilitates distortion in response to strong bending forces while the same sequences in fragment 61 do not occupy a rotational orientation that allows such a conformation. In the following section we describe 11 additional constructs that contain mutations in the hypersite region of the synthetic constructs which were designed to determine sequence and rotational orientation requirements for hypersite production. These constructs were subsequently analyzed to determine the effects of the mutations in the hypersite region on nucleosome stability and translational positioning.

3. New constructs with mutations in the hypersite region

Oligonucleotide-directed mutagenesis was used to modify fragments 61 and 67. For these studies, the positioning fragment 67 was renamed 1 and the nonpositioning fragment 61 was renamed 11. Permanganate reactions performed on mononucleosomes reconstituted onto the parent and derivative fragments revealed that several factors were required for permanganate hypersite induction as shown in **Figure 7**. Permanganate hypersensitivity along nucleosomal DNAs was only observed at particular TA steps. Among the 9 derivatives of fragment 1, substitution of the reactive TA step eliminated permanganate hypersensitivity in every case (fragments 2, 3, 4, 5, and 6) in spite of the fact that several new T residues and TA steps were introduced into these fragments. The second factor involved in generating permanganate reactivity is the identity of sequences immediately flanking the reactive TA step. Fragment 8 contains the intact hypersensitive TA step of the parent but carries two substitutions in the sequences flanking the hypersite. The lack of hyperreactivity in this fragment suggests that flanking sequences are involved in permanganate sensitivity. Similarly, TA steps flanked by homopolymeric Y or R tracts have been shown to be hypersensitive to permanganate in other systems where protein-induced kinking is thought to occur (Borowiec *et al.*, 1987; *et al.*, 1996). In addition, the two prototype kinked DNAs analyzed by Dickerson (1998) occur at YR steps flanked by Y stretches of 6 and 7 bp. The third factor involved in determining permanganate reactivity is the local rotational orientation of the hypersite in agreement with our initial interpretation described above. This was seen with the hypersite displayed by nucleosomes reconstituted onto fragment 13. This fragment was produced by a combination 6 bp insertion and 6 bp deletion flanking the 5' sequence in fragment 11 (61) which altered the local rotational orientation of this sequence rendering it hypersensitive to permanganate.

We reasoned that if the deformation detected by $KMnO_4$ was important for transitional positioning and high nucleosome stability, mutations that alter permanganate reactivity should also alter these properties. A comparison of reconstitution efficiencies of fragment 1 and its derivative fragments (fragments 2-10) revealed that substitutions that eliminated permanganate reactivity in the parent positioning fragment resulted in 2-10 fold decreases in affinity for histone octamers and a partial to total loss in positioning activity while substitutions in the parent non-positioning sequence that rendered the fragment sensitive to permanganate resulted in nearly a 20-fold increase in affinity as seen in **Figure 8**. However, induction of a second hypersite at position +15 (fragment 9) had little effect on affinity or positioning activity. The results also demonstrated that alteration of the rotational orientation of A-tracts 1-4 (fragment 10) did not result in a significant change in octamer affinity when compared to the parent fragment but did cause a large reduction in positioning activity. With this fragment, the nucleosomes assembled at four positions with permanganate hypersite sequences occupying positions at + and/or -15 bps and to a lesser extent ±5 bps from the pseudo-dyad. These results show that both the permanganate hypersite and the unusual rotational orientation of the A-tract region 1-4 are required for the unique translational positioning activity displayed by the synthetic fragments.

D. A model for the translational positioning of nucleosomes

A general model for the positioning of nucleosomes on the synthetic fragments is shown in **Figure 9**. According to the model, A-tracts 5-8 with their narrow minor grooves facing the histone surface set the rotational orientation of the sequence (Fitzgerald and Anderson, 1998, 1999b). This rotational orientation was seen in this upstream region of all fragments

studied in **Figures 1** and **2** as well as in a fragment that contained A-tracts 5-8 but not A-tracts 1-4. All of the synthetic fragments assumed standard pitches when packaged into nucleosomes of 10.0-10.3 bp/turn in distal areas and 10.5-10.7bp/turn in the central three turns (Hayes, *et al.*, 1990&1991). Consequently, the narrow minor grooves of A-tracts 1-4 in the +1 series face the histone surface while the narrow minor groove of the TA step at position -15 faces away. The opposite is true in the -3 series fragments which places the translational signals (A-tracts 1-4 and the hypersite) into a rotational orientation that favors high stability and unique positioning of the nucleosome.

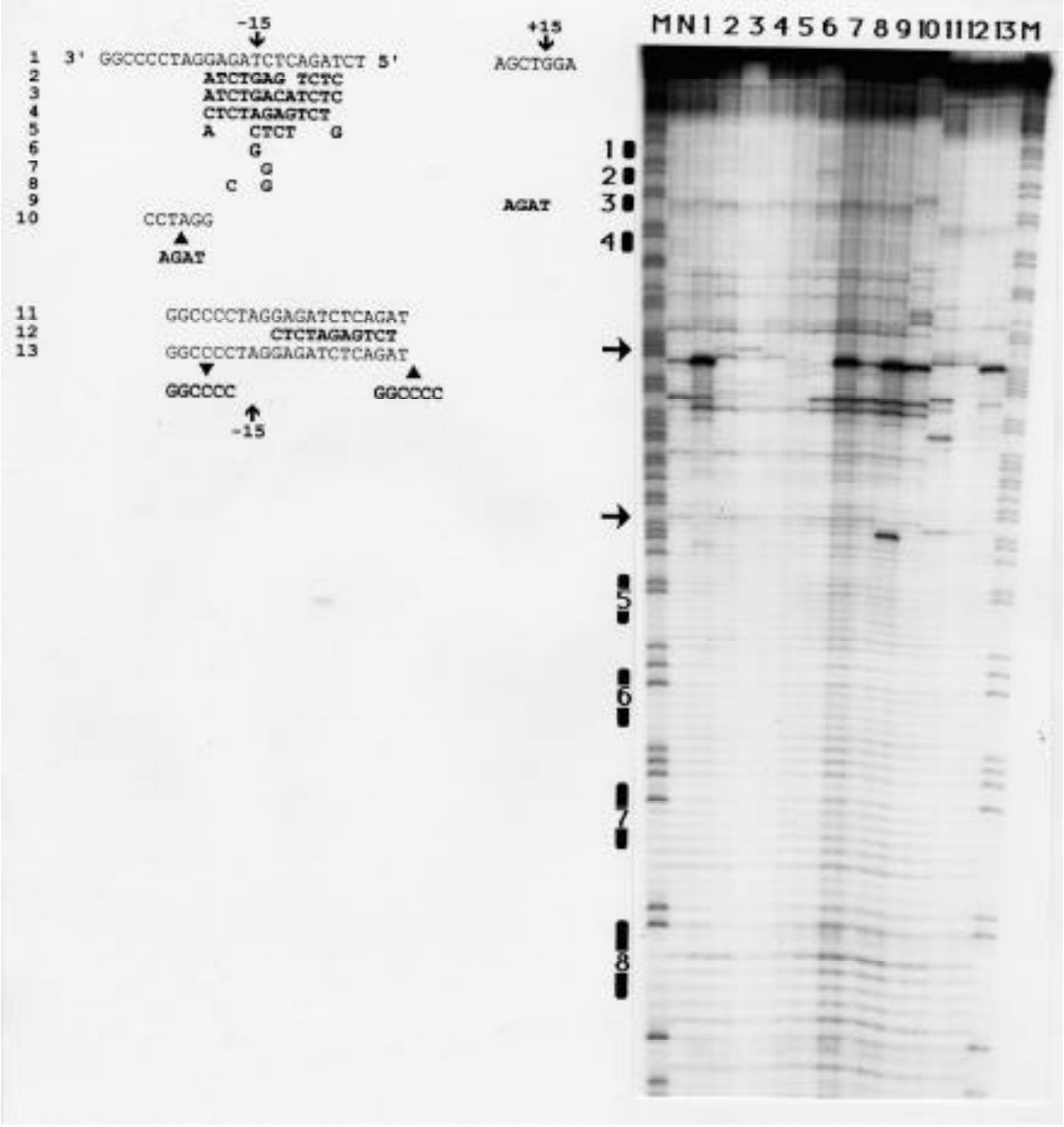

Figure 7. Mutations and permanganate hypersensitivity. Oligonucleotide-directed mutagenesis was used to modify fragments 67 and 61 in order to determine the importance of the unusual features to nucleosome stability and translational positioning. **Left:** Sequences are designated by the number to the left. The positioning fragment 67 was renamed 1 and fragments 2-10 are derivatives of this fragment. The nonpositioning fragment 61 was renamed fragment 11 and fragments 12 and 13 are derivatives of this sequence. Shown is the bottom strand (3' to 5') with the position indicated above the sequences. Bold-faced type indicates a substitution while triangles refer to insertions (points upward) or deletions (points downward). **Right:** Permanganate probing of reconstituted fragments 1-13. Bottom-labeled reconstituted fragments 1-13 (lanes 1-13) were modified with permanganate and the cleaved products run on a sequencing gel. The fragments were co-electrophoresed with permanganate-treated naked fragment 1 (N), the products of a fragment 1 G/A sequencing reaction (leftmost M), and a fragment 11 G/A sequencing reaction (rightmost M). A-tracts 1-8 and the permanganate hypersites of fragment 9 are indicated by the arrows (from Fitzgerald and Anderson, 1999b).

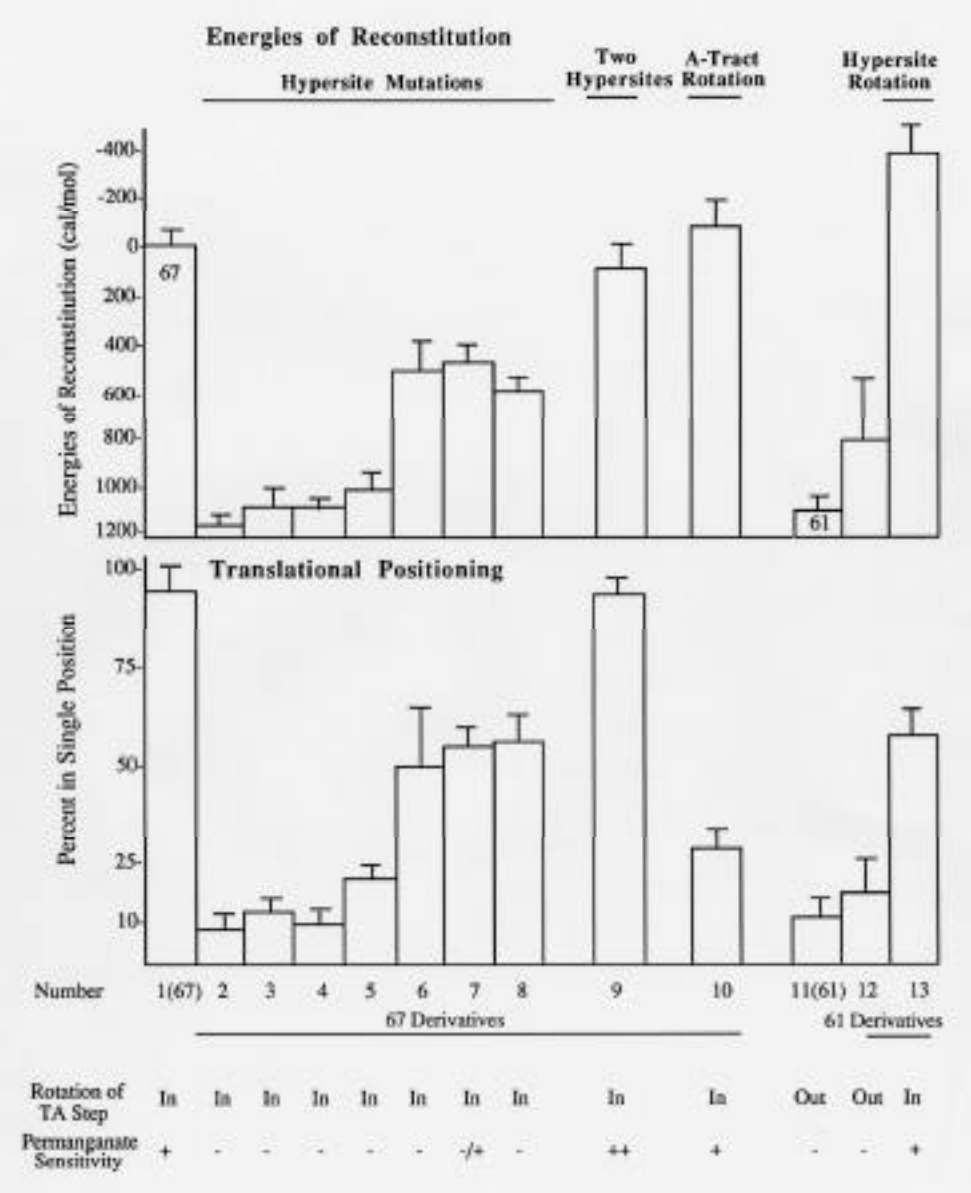

Figure 8. Properties of Nucleosomes Assembled onto Mutagenized Fragments. The mutation type is indicated along the top of the figure and the sequence number from Figure 7 is given along the bottom. Nucleosome stability, translational positioning, permanganate reactivity (+ or -), and rotational orientation of the TA in the hypersite sequence (inward or outward facing minor grooves) were determined as described in Figures 4 and 6. Results are summarized from Fitzgerald & Anderson, 1999b.

The mode of action of both positioning determinants is most likely related to the irregular path of the DNA around the histone octamer. Along this path, regions 15 and 45 bps from the pseudo-dyad exhibit marked departures from ideal base stacking Luger et al., 1997). Biochemical studies have also suggested that the DNA in these areas assumes a distinct structure not seen elsewhere along the particle as described in the "Introduction". Although correlation studies have implicated this irregular DNA path in nucleosome positioning, the studies described in this report provide the first direct evidence for this view.

The marked stability conferred by a functional permanganate hypersite can be attributed to either variations in histone-DNA contacts in the hypersite region, or to differences in histone-DNA contacts formed elsewhere along the sequence due to a change in the mechanical properties of the DNAs at the hypersite. We favor the second alternative because the 1 Kcal/mol drop in affinity resulting from substitutions in this 12 bp region is incompatible with studies that show substitutions generally result in changes of affinity on the order of only 100 cal/helical turn (Shrader & Crothers, 1989). Studies of the effect of sequence changes on other types of protein-DNA complexes suggest a mechanism for the propagation of histone-DNA contact changes beyond the immediate vicinity of the hypersite.

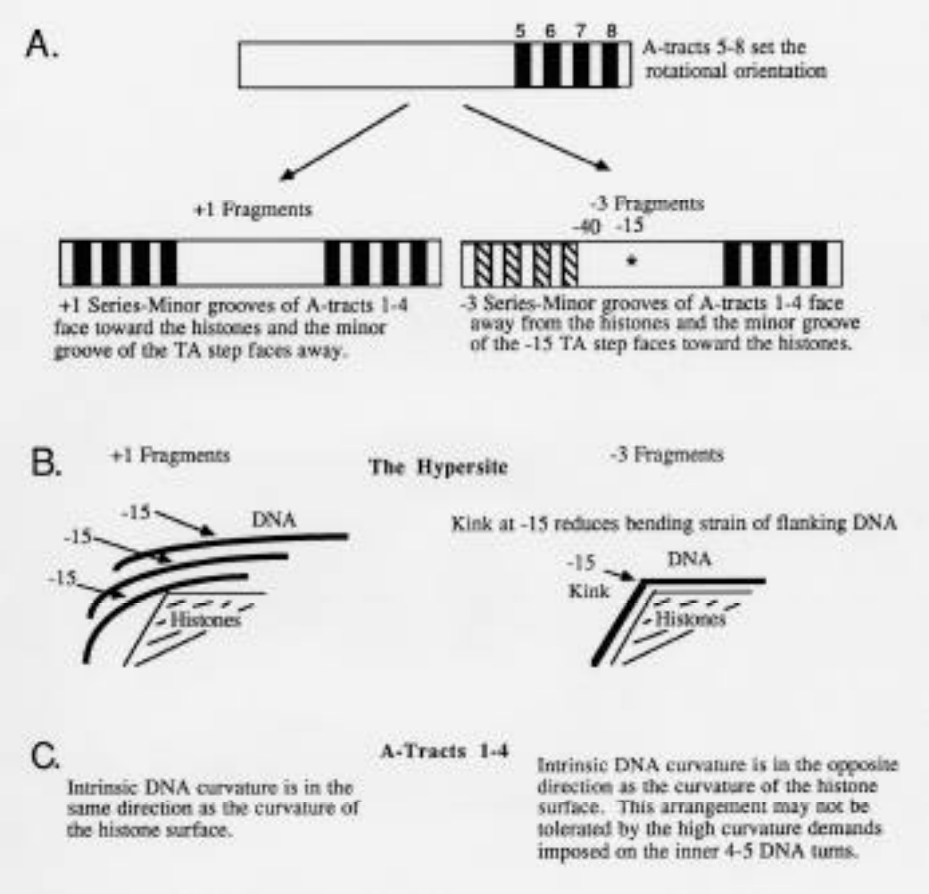

Figure 9. A model for nucleosome positioning. The role of the sequence phase in dictating the rotational orientation of the positioning determinants (the hypersite and A-tracts 1-4) is shown in (**A**). The proposed modes of action of the determinants are shown in (**B**) and (**C**). Further details are provided in the text.

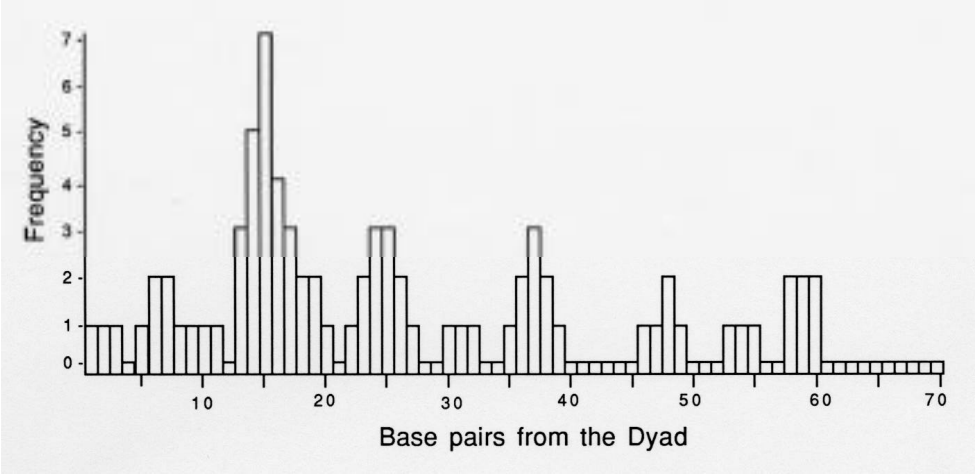

Figure 10. $Y_{>2}$ TA/$TAR_{>2}$ in natural positioning DNA. The frequency of the $Y_{>2}$TA or $TAR_{>2}$ sequence motifs in eight natural positioning sequence counted at the T residue were summed over a window of three bp and plotted as a function of distance from the dyad. Sequences were from : *Drosophila Adh* Promoter (Jackson & Benyajati, 1993); African green monkey a-Satellite (Neubauer *et al.*, 1986); *L. variegatus* 5S rDNA (Flaus *et.al.*,1996; Fitzgerald and Anderson, 1999b); simian virus 40 major-late transcription site (Powers & Bina); *Xenopus* vitellogenin B1 promoter (Schild *et al.*, 1993); mouse mammary tumor virus promoter (Piña *et al.*, 1990); *Xenopus* thyroid hormone receptor ßA (Wong *et al.*, 1997); *S. cerevisiae* pet56-his3-gioded1 ren (Losa *et al.*, 1990).

The CAP protein induces large kinks at two Y-R steps in its binding site (see Table 1 Dickerson, 1998), and substitutions in this vicinity cause up to 2.5 Kcal drops in the relative affinity of the protein for its binding site and an accompanying decrease in the magnitude of the deflection of the helical axis induced by CAP binding (Gartenberg & Crothers, 1988). Other studies of protein induced DNA bending also suggest that substitutions at the bend site can have a significant effect on the extent of post-binding DNA bending (Bareket-Samish *et al.*, 1997; Hotrod & Perona, 1998). The nucleosome hypersite region may therefore act as a hinge to accommodate protein-induced bending in a manner that affects the stability of flanking contact sites as indicated in the figure.

Short oligo A/T tracts are most often oriented in the nucleosome such that their narrow minor grooves face the histone surface. A-tracts 1-4 in the positioning fragments display a different rotational orientation and this unusual orientation is required for positioning. In the absence of these outward facing A-tracts as in fragments 10 and 13, nucleosomes are found at four positions with permanganate hypersite sequences occupying positions at + and/or -15 bps and to a lesser extent ±5 bps. We are left with the question regarding the mechanism by which A-tracts 1-4 fix the permanganate hypersite sequence to -15 bps from the pseudo-dyad in fragment 1 while the same element can be found at ±15 and to a lesser extent ±5 bp from the pseudo-dyad in fragments 10 and 13. It is conceivable that A-tracts with outward facing minor grooves are restricted to the relatively flat surface of the histone octamer present in the distal three turns of nucleosomal DNA because they cannot tolerate the high curvature demands that are found inside 40-50 bps from the pseudo-dyad. Unique translational positioning on the synthetic fragments therefore can be viewed as a concerted action between both the stabilizing forces associated with the hypersite sequences occupying specific sites within the central three turns of nucleosomal DNA and destabilizing forces which appear when A-tracts 1-4 with outward facing minor grooves occupy particular translational positions.

E. Relationship to natural sequences

The synthetic sequences that position nucleosomes at single sites were modeled after natural nucleosome positioning sequences in terms of two regions of curvature and the length of the central region. Our analysis of the nucleosomes that assemble onto the synthetic sequences has revealed that the similarities extend beyond the original model. For example, the 5S rDNA nucleosome positioning sequence from *X. borealis* and a tobacco satellite positioning sequence share with constructs 67 and 77 an unusual rotational orientation of A-tracts on one side of the nucleosomal DNA (Hayes *et al., 1990*; Kralovics *et al., 1995*). The 5S rDNA positioning sequence from *L. variegatus* also contained a permanganate hypersite at 1.5 turns from the pseudo-dyad as seen in **Figure 6**. To determine if other positioning sequences were enriched in the sequence motifs that give rise to permanganate hypersensitivity in this location, we analyzed other known natural positioning sequences for the frequency of TA dinucleotides flanked by pyrimidine or purine homopolymers. *In vivo* mapped nucleosomal DNAs were not included, nor were positioning sequences that were mapped by low resolution techniques (see Trifonov, 1993). We found 8 sequences that met the above criteria. The distribution of these sequence motifs throughout the nucleosomal DNAs is illustrated in **Figure 10**. Seven of the eight sequences had at least one of the Y_3TA or TAR_3 motifs within 5 bp of + or - 15 bp from the mapped pseudo-dyad. The remaining sequence had a Y_2TA motif at position -15. It is evident that the TA sequence motifs cluster in the ±15 region of the positioning sequences. The likelihood that the observed number of Y_3TA and TAR_3 sequence motifs occurred by chance in the 10bp region flanking ±15 from the pseudo-dyad is vanishingly small ($P<0.005$ correcting for base composition). Studies are in progress to evaluate the functional significance of these sequence features for the positioning of nucleosomes on natural DNA.

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