Selective Nucleosome Disruption by Drugs That Bind in the Minor Groove of DNA*

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Previous studies have shown that drugs which bind in the DNA minor groove reduce the curvature of bent DNA. In this article, we examined the effects of these drugs on the nucleosome assembly of DNA molecules that display different degrees of intrinsic curvature. DAPI (4,6-diamidino-2-phenylindole) inhibited the assembly of a histone octamer onto a 192-base pair circular DNA fragment from Caenorhabditis elegans and destabilized a nucleosome that was previously assembled on this segment. The inhibitory effect was highly selective since it was not seen with nonbent molecules, bent molecules with noncircular shapes, or total genomic DNA. This marked template specificity was attributed to the binding of the ligand to multiple oligo A-tracks distributed over the length of the fragment. A likely mechanism for the effect is that the bound ligand prevents the further compression of the DNA into the minor groove which is required for assembly of DNA into nucleosomes. To further characterize the effects of the drug on chromatin formation, a nucleosome was assembled onto a 322-base pair DNA fragment that contained the circular element and a nonbent flanking segment of DNA. The position of the nucleosome along the fragment was then determined using a variety of nuclease probes including exonuclease III, micrococcal nuclease, DNase I, and restriction enzymes. The results of these studies revealed that the nucleosome was preferentially positioned along the circular element in the absence of DAPI but assembled onto the nonbent flanking sequence in the presence of the drug. DAPI also induced the directional movement of the nucleosome from the circular element onto the nonbent flanking sequence when a nucleosome preassembled onto this template was exposed to the drug under physiologically relevant conditions.

The nucleosome is the basic structural unit of chromatin (reviewed in Refs. 1–6). Nucleosomes are often located at discrete sites in regions of chromatin that regulate transcription and replication and this nonrandom positioning is thought to play a key role in controlling the accessibility of DNA sequence elements to protein regulatory factors. Nucleosome positioning in genome control regions is also a dynamic process. Changes in nucleosome arrangement including disruption, unfolding, and short-range lateral mobility have been related to changes in gene activity and replication function in a variety of systems (1–13). Thus, the ability to disrupt or to direct the movement of a specific nucleosome type would provide a method that could be used to further understand the significance of these events and the multifunctional roles of the nucleosome.

Drugs that bind to DNA frequently alter the patterns of transcription and replication. Consequently, there has been a tremendous effort devoted to describing drug-DNA interactions with the aim of understanding the mechanisms that are involved in promoting these changes in the cell. It is often difficult to relate results from in vitro studies that focus on ligand-DNA interactions to pharmacological effects seen in vivo since the DNA in the nucleus is packaged into chromatin. Many studies have shown that chromatin packaging directs sites of drug binding since chromatin accessible regions such as nucleosome linker DNA and the outer facing surface of nucleosomal DNA are preferred targets for drug interactions (see Refs. 14–16 and references therein). Similarly, chromatin accessible regions are preferred cleavage sites for antitumor glycoprotein antibiotics and are hot spots for the formation of photoproducts produced by UV light (17, 18). However, little is known about selective changes in chromatin structure that might occur upon drug binding, although such changes are likely to be important in the mechanism of action of these compounds.

Classical intercalators such as ethidium bromide display little or no sequence specificity (19) which limits their use as selective probes in chromatin structural studies. Intercalation of ring systems into the DNA duplex also leads to gross changes in helical structure which produces marked changes in the structure of all nucleosomes and the nonspecific disruption of higher-order chromatin conformations (20, 21). Drugs that bind in the DNA minor groove display a degree of sequence specificity in that they preferentially bind to short oligo(A) sequences (reviewed in Refs. 22 and 23). The specificity of these drugs for bent DNA may also be enhanced by the cooperative drug binding to multiple closely spaced oligo A-tracks that characterize these sequences (24). These drugs bind to preexisting structures, the narrow minor grooves, which are complementary to the structures of the ligands. Consequently, in contrast to intercalators, these drugs cause little distortion in the DNA duplex and their effects on chromatin structure appear more subtle. Minor groove binders have been reported to interact preferentially with AT-rich linker regions in chromatin, thereby promoting the selective dissociation of histone H1 (25). This effect is presumably responsible for the drug-induced decondensation of higher order structures of AT-rich regions of chromatin, which has been analyzed by both biochemical and microscopic methods (25–27). Some of these drugs have also been reported to promote the rotation of the DNA on the surface of the nucleosome (28–30). However, it has generally been assumed that the nucleosome core is resistant to disruption by these compounds although, to our knowledge, their effects on assembly of the nucleosome have not been assessed. In this article, we present an analysis of the influence of a minor groove binding drug on the nucleosome assembly of DNA mol-

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and subcloned into the rRNA gene from sea urchin (35) was obtained from Dr. Robert Simpson. The dove satellite sequence has also been described (34) while the 5 S rRNA (36). Each of these recombinants contains the same two 40-bp1 bending elements that are separated by variable amounts of nonbent DNA as indicated in Fig. 4. The two recombinants used in this study are designated pSym 20 and pSym 47. Complete sequences of these fragments are presented (36). The high ion strength electrophoresis described in the text was carried out using an 8% polyacrylamide (PA) gel run for 3 days at 4 °C with buffer recirculation at 30 V in Tris-acetate-EDTA/TAE buffer with or without 0.6 mM NaCl.

Radioactive DNA was prepared by polymerase chain reaction using primers (25-mers) that are centered at 19 bp upstream and 33 bp downstream from the EcoRI site and HindIII site in pUC18, respectively (see Fig. 1). DNA was uniformly labeled with [α-32P]dATP or uniquely end-labeled by using one 32P-end-labeled primer in the polymerase chain reaction. DNA fragments were purified by electrophoresis through agarose gels and appropriate bands eluted from gels by the crush-soak method.

Reconstitution and Analysis of Nucleosomes—Erythrocyte nuclei were prepared from chicken blood (Pel-Freeze) as described previously (33) and digested with MNase (200 units/ml) for 20 min at 37 °C. Following centrifugation, nuclei were lysed by suspension in 10 mM Tris-HCl, pH 8.0, containing 5 mM EDTA, centrifuged and the soluble chromatin made 0.6 mM NaCl in order to dissociate histone H2/H5. Mononucleosomes devoid of these histones were then recovered from Bio-Gel A-15M (Bio-Rad) columns run in 20 mM Tris-HCl containing 0.6 mM NaCl and 1 mM EDTA. Mononucleosomes were stored at 4 °C for 4 h and analyzed on 1.3% agarose gels. DNA fragments 135–155 bp in size were then fractionated on 1.3% agarose gels. DNA fragments 135–155 bp in length were recovered from these gels, secondarily cleaved with restriction enzymes, and concentrated by ethanol precipitation prior to electrophoresis. The noncellulosate filter binding assay was carried out as described previously (39) using the NaCl concentrations for sample application and washing indicated in the legend to Fig. 3. For DAPI binding experiments, reconstitution was carried out by gradient dialysis (2 mM NaCl, 10 mM Tris, pH 8.0, to 50 mM NaCl, 10 mM Tris, pH 8.0) at 4 °C for 18 h using purified erythrocyte core histones and the 192-bp EcoRI fragment or wheat genomic DNA at a protein:DNA ratio of 0.9:1.0. Reconstituted nucleosomes and the corresponding naked DNAs were incubated with DAPI (10 μM) at 4, 25, and 37 °C for times that ranged from 1 min to 3 h. Binding at all temperatures as determined by fluorescence enhancement at 485 nm was essentially maximum within 3 min with both nucleosome and naked DNA and binding extents were similar with naked Vs compared nucleosome DNA.

RESULTS

Effects of Minor Groove Binding Drugs on Nucleosome Assembly—The studies described below focus on a DNA segment from an intron of the C. elegans RNA polymerase II gene (33, 40). This linear segment preferentially forms a circle in ligation reactions and displays marked electrophoretic retardation that is comparable to that seen with kintoplan DNA (33). As seen in the bottom of Fig. 1, the 192-bp EcoRI fragment contains 17 AT sites which are arranged in a 10–11 bp periodicity. Each of these regions is a binding site for minor groove-specific drugs.

DNA must bend around the histone octamer to be incorporated into a nucleosome. Thus, it is reasonable to assume that intrinsically bent DNA would facilitate nucleosome formation and several studies seem to support this view (42–45). To determine whether the circular intron segment is preferentially packaged into a nucleosome, nucleosomes were assembled onto four labeled fragments of similar lengths by exchange of histones from chicken erythrocyte mononucleosomes using the salt-dilution method. Reconstituted samples were then analyzed on the PA-glyceral gels shown in Fig. 1. To assess the relative reconstitution efficiencies, experiments were carried out in the presence of increasing amounts of histones in the absence of competitor DNA (45) (lanes 1–5, panel E) and in the presence of maximal amounts of histones and two concentrations of competitor (38) (lanes 6 and 7). The single type of complex formed on each template was judged to be a nucleosome since each comigrated with mononucleosomes from cellular chromatin and each yielded a protected fragment of 140–150 bp following digestion by micrococcal nuclease (data not shown). The circular intron fragment displayed the highest reconstitution efficiency as it required the least amount of histones to form a nucleosome. Reconstitution efficiencies of a synthetic bent segment, a bent DNA satellite, and the sea urchin 5 S rDNA were about 1.3-, 2-, and 5-fold lower than the circular element (Fig. 1E). The competitive reconstitutions (lanes 6 and 7) also demonstrated this trend with the highest competition effect seen with the 5 S rDNA sequence and the lowest with intron DNA. The efficiency of reconstitution of the intron fragment was nearly identical to that reported for the circular segment from the Crithidia fasciculata kinetoplast (45). This efficiency is similar to the TG and GT reference sequences used by Shrader and Crothers (38, 45) (which were found to display higher histone binding affinities than several natural nucleosome positioning sequences).

1 The abbreviations used are: bp, base pair; DAPI, 4,6-diamidino-2-phenylindole; EcoIII, exonuclease III; MNase, micrococcal nuclease; PA, polyacrylamide; PAGE, polyacrylamide gel electrophoresis.

2 Albert, G. F., Eckdahl, T. T., Fitzgerald, D. J., and Anderson, J. N. (1999) Biochemistry, in press.
The 192-bp intron segment must undergo substantial additional bending when packaged into a nucleosome where 145 bp of DNA are wound about 1.65 times around the histone octamer. Thus, an analysis of inhibiting this bending during reconstitution by minor groove binding drugs may provide a further understanding of the functional significance of sequence-directed curvature and anisotropic bendability in chromatin formation. The effects of the minor groove binding drug DAPI on the assembly of a nucleosome onto the intron segment is shown in Fig. 2A. The drug concentrations used in this experiment were within the range of those previously employed to study the structure of the intron DNA by electrophoretic and ligation techniques and to identify the drug-binding sites by footprinting analysis. The ligand inhibited the formation of a nucleosome from 10 ng of uniformly labeled fragments as described under “Experimental Procedures” with 20, 40, 60, 300, 300, and 300 ng of erythrocyte nucleosomes (lanes 2–7). Lanes 6 and 7 also contained 200 and 400 ng of competitor erythrocyte DNA, respectively. Products were electrophoresed on PA-glycerol gels in order to resolve nucleosomal (N) and free (F) DNA. Templates used were: A, the intron segment (192 bp); B, Syn 47 (205 bp); C, dove satellite (192 bp); and D, 5 S rDNA (195 bp). E, quantitation of DNA assembled into nucleosomes from lanes 1–5 in A-D is shown as a function of chromatin concentration. Symbols are intron (●), Syn 47 (○), satellite (■), and 5 S rDNA (□). The sequence of the circular EcoRI intron fragment and flanking vector DNA is shown in the bottom of the figure. Indicated are AT sites ≥4 nucleotide in length numbered 1–21 with the 192-bp intron segment lying between the EcoRI (R) sites (AT sites 2 and 20). The positions of DNase I cutting sites along the bottom labeled strand of the intron segment are also shown (data from Fig. 8). Indicated are the sites that were unique to naked DNA (■) and those that were found in both naked DNA and reconstituted nucleosome in the absence of DAPI (△).

To study the stage in the assembly reaction that is most sensitive to DAPI, the drug was added to reconstitution mixtures at various steps during salt dilution. After the final dilution to 0.1 M NaCl, samples were resolved electrophoretically in order to determine the fraction of the intron and exon fragments that were packaged into nucleosomes (Fig. 3B). There was no significant effect of drug addition on the assembly
of the nucleosome onto the exon sequence. In contrast, DAPI selectively inhibited assembly onto the intron segment principally during early stages in the reconstitution at NaCl concentrations (\(\geq 0.6 \text{ m}\)) that correspond to those at which the transfer of the histones onto the labeled DNA fragment occurs (37, 38).

An important question concerning the action of the drug is whether the ligand can selectively disrupt a preformed nucleosome containing the intron sequence. As shown in Fig. 3C, the nucleosome gel band broadens in response to drug treatment in contrast to the nucleosome containing the 5 S rDNA sequence. In addition, a small amount of free DNA was produced when the intron-containing nucleosome was exposed to DAPI (Fig. 3, B and C). Thus, the drug may promote a selective modification of the intron nucleosome rather than causing actual histone displacement. The results of the nitrocellulose binding assay in Fig. 3C provide support for this view. In this analysis, nucleosomes containing the intron sequence and 5 S rDNA were incubated with increasing amounts of DAPI and applied to a nitrocellulose filter. Histones were then dissociated from the particles by washing the filter with solutions of increasing NaCl concentrations. The results clearly show that histones are bound less tenaciously to the intron sequence in nucleosomes treated with the drug and the effect is selective in that it was not seen with 5 S rDNA (Fig. 3C) or the exon sequence (data not shown).

The results in Figs. 2 and 3 raised a question as to the possible contribution of DNA curvature to the drug inhibitory effect since the structure of bent DNA may be perturbed in high ionic strength (46). To evaluate this possibility, the electrophoretic mobilities of the intron fragment were compared in 8% PA gels run at 4 °C in the absence and presence of 0.6 m NaCl. Although glycerol in PA gels reduces the anomalous migration of bent DNA (8), it did not completely normalize the mobility of the intron segment in the absence of the drugs. Consequently, free intron DNA migrates slightly faster in the presence of the drugs. Similar analyses were carried out with the indicated templates (bottom panel) in the presence of no drug (lane 1) and 5 \(\mu\text{m}\) DAPI, distamycin and Hoechst (lanes 2–4, respectively).

The results demonstrated that the ligand can selectively disrupt a preformed nucleosome on the intron segment as indicated by the anomalous migration of bent DNA (8), it did not completely normalize the mobility of the intron segment in the absence of the drugs. Consequently, free intron DNA migrates slightly faster in the presence of the drugs. Similar analyses were carried out with the indicated templates (bottom panel) in the presence of no drug (lane 1) and 5 \(\mu\text{m}\) DAPI, distamycin and Hoechst (lanes 2–4, respectively).

**FIG. 2.** Selective inhibition of nucleosome assembly by DAPI. Labeled DNA fragments A–E were incubated in 1 m NaCl with 0, 1, 5, and 10 \(\mu\text{m}\) DAPI (lanes 1–4, respectively) prior to addition of 300 ng of erythrocyte nucleosomes. The DNA fragments are intron (A), 5 S rDNA (B), Syn47 (C), exon (D), satellite (E), and genomic DNA (F). Fragments ranged in size from 180 to 222 bp. Although glycerol in PA gels reduces the anomalous migration of bent DNA (8), it did not completely normalize the mobility of the intron segment in the absence of the drugs. Consequently, free intron DNA migrates slightly faster in the presence of the drugs. Similar analyses were carried out with the indicated templates (bottom panel) in the presence of no drug (lane 1) and 5 \(\mu\text{m}\) DAPI, distamycin and Hoechst (lanes 2–4, respectively).

**FIG. 3.** A, DAPI-sensitive stages during nucleosome assembly. The indicated templates were incubated in the presence (+) and absence (−) of 5 \(\mu\text{m}\) DAPI for 20 min. Erythrocyte nucleosomes were then added and the NaCl concentrations reduced to the indicated levels by addition of 10 mM Tris-HCl, pH 8.0. Samples were applied to a nitrocellulose sheet and washed 5 times with the same NaCl levels prior to autoradiography. B, reconstitution reactions containing intron and exon segments were adjusted to 10 \(\mu\text{m}\) DAPI during dilution from 1 to 0.1 m NaCl. After the final dilution, reaction mixtures were applied to a PA-glycerol gel. The NaCl concentrations were, from 1 to 5, 1.0, 0.8, 0.6, 0.4, and 0.1 m. C, nucleosomes assembled onto the intron segment and 5 S rDNA were incubated at 37 °C for 30 min in 50 mM NaCl with the indicated concentrations of DAPI (in \(\mu\text{m}\)). Samples were then electrophoresed on the gel shown in the figure and applied to the nitrocellulose sheet which was washed with solutions containing the indicated NaCl concentrations.

The results of the nitrocellulose binding assay in Fig. 3C provide support for this view. In this analysis, nucleosomes containing the intron sequence and 5 S rDNA were incubated with increasing amounts of DAPI and applied to a nitrocellulose filter. Histones were then dissociated from the particles by washing the filter with solutions of increasing NaCl concentrations. The results clearly show that histones are bound less tenaciously to the intron sequence in nucleosomes treated with the drug and the effect is selective in that it was not seen with 5 S rDNA (Fig. 3C) or the exon sequence (data not shown).

The two approaches shown in Fig. 4 were taken to define the template requirements for inhibiting nucleosome formation by DAPI. Each of the 9 fragments shown in Fig. 4A contained the same two segments of synthetic bent DNA. The sequences that comprise these segments are designated as black bars and are of the form \((\text{A}_n \text{N}_m \text{G})_n\), where \(n = G\) or C. The fragments differ in the lengths of nonbent vector DNA (white spaces) which is located between and outside of the binding elements as indicated in the figure. In the absence of DAPI, each fragment assembled into a nucleosome. A nucleosome also formed on fragments 2–9 in the presence of the drug. However, the drug completely inhibited nucleosome formation on fragment 1 which lacks most of the nonbent vector sequences. The inability of a nucleosome to form on this 133-bp fragment was not due to the short fragment length since the formation of a nucleosome on a 134-bp segment containing exclusively nonbent vector DNA was not inhibited by the drug (data not shown). These results imply that the disruption of nucleosome formation by DAPI is favored by multiple oligo A-tracts (DAPI-binding sites) distributed over most of the length of the DNA.

The studies of the nucleosomes assembled onto fragments...
containing the circular intron segment and variable amounts of flanking vector DNA shown in Fig. 4B are consistent with this view. DAPI inhibited nucleosome formation on the fragment containing the circular segment (black bars) and on the nonbent DNA (white spaces) on both sides (Fig. 4B, lines 1–3). In contrast, the drug inhibitory effect was lost when the nonbent DNA on the 3′ side of the circular element was extended to 100 bp (Fig. 4B, line 4). Similarly, in the presence of DAPI, a nucleosome was not formed on a 161-bp segment of the circular element or on templates containing this segment flanked on the 3′ side by 24 and 39 bp of nonbent vector sequences (Fig. 4B, lines 5–7). However, a nucleosome was formed on this segment flanked by 63 bp of vector DNA as shown in Fig. 4B, line 8. This abrupt transition is illustrated by the gels in the bottom of the figure which show the effects of DAPI on the assembly of a nucleosome onto fragments 7 and 8. Digestion of nucleosomes assembled onto fragment 8 in the presence and absence of DAPI did not show any sites of cleavage (data not shown) revealed the characteristic patterns of DNA cleavages expected of nucleosome DNA (1). Thus, DAPI may selectively direct the assembly of a nucleosome onto the nonbent end of fragment 8 by excluding the assembly onto the circular element. The four approaches described below were carried out to test this proposal.

Nucleosome Positioning Induced by DAPI—As a first approach to study the effects of minor groove binding drugs on nucleosome positioning, the 214-bp HaeIII fragment from Fig. 4B, line 8, was reconstituted with histones in the presence and absence of DAPI. The reconstituted chromatin and naked DNA were then digested with five restriction enzymes in order to determine the relative accessibility of the 6 restriction sites depicted in the map at the top of Fig. 5. In naked DNA, all sites were cleaved to completion (95%) in the presence and absence of the drug (data not shown), suggesting that the reductions in the percent cleavages seen in chromatin (Fig. 5) were not due to drug inhibitory effects on the restriction enzymes but rather to histone interactions. This observation is relevant for the AT-rich site of EcoRI since the ligand-bound site in naked DNA is somewhat resistant to cleavage by the enzyme (41, 47). In the absence of DAPI, the chromatin sites within the circular element were resistant to digestion while the sites in the flanking nonbent portion of the fragment display enhanced sensitivities. These results imply that a nucleosome preferentially formed on the drug inhibitory effect was lost when the nonbent end of the fragment. An example of the analysis is depicted in the map at the top of Fig. 5. In naked DNA, all sites Were cleaved to completion (95%) in the presence and absence of the drug (data not shown), suggesting that the reductions in the percent cleavages seen in chromatin (Fig. 5) were not due to drug inhibitory effects on the restriction enzymes but rather to histone interactions. This observation is relevant for the AT-rich site of EcoRI since the ligand-bound site in naked DNA is somewhat resistant to cleavage by the enzyme (41, 47). In the absence of DAPI, the chromatin sites within the circular element were resistant to digestion while the sites in the flanking nonbent portion of the fragment display enhanced sensitivities. These results imply that a nucleosome preferentially formed on the drug inhibitory effect was lost when the nonbent end of the fragment. An example of the analysis is...
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FIG. 6. Alteration in nucleosome positioning induced by DAPI as determined by MNase digestion. Nucleosomes reconstituted onto uniformly labeled complete template (panel A) or the HaeIII template (panel B) in the absence (-) and presence (+) of 10 μM DAPI were digested with MNase. Templates are shown in Fig. 4B, line 4, and B, line 8. After extraction, protected fragments of 135-155 bp in length were isolated from agarose gels and digested with the indicated restriction enzymes. N₀ and N₁ refer to DNA that was not digested with restriction enzymes before and after agarose gel electrophoresis, respectively. M₃, pUC19 digested with MspI as size markers. Panels C and D show restriction maps of the two template fragments used in the analysis indicating the positions of intron DNA (thick bar), vector DNA (thin bar) and nucleosomes which have one border at the 3’ end of the fragments (wide thick bar). Abbreviations are as described in the legends to Figs. 4 and 5. The positions of observed restriction sites when the core particle DNA was obtained from reconstitutions carried out in the presence of DAPI are indicated by the boxed letters. These positions were deduced from the lengths of the major fragments in panels A and B.

given by the gel in the figure which shows the effects of DAPI on the differential chromatin sensitivities of the two MspI sites in the fragment. As indicated by the map below the gel, the MspI site in the bent sequences is selectively protected in the absence of DAPI as seen by the preferential appearance of the 46- and 178-bp bands. The selective protection of the MspI site in the nonbent sequences in the presence of the drug is seen by the preferential formation of the 101- and 123-bp fragments.

In order to further study the effects of DAPI on nucleosome positioning, the two templates shown in Fig. 6, C and D, were uniformly labeled and assembled into nucleosomes in the presence and absence of the drug. These fragments correspond to fragments 4 and 8 from Fig. 4B and the sequence of fragment 4 is shown in Fig. 1. The reconstituted chromatin was then digested with MNase, deproteinized, and the ~135-155-bp protected fragments isolated by elution from agarose gels. Approximately 80% of the DNA in the digest was of this size class (see Fig. 6B). The DNA was further digested with the restriction enzymes in order to map MNase cleavage sites and thus the nucleosome positions relative to the positions of the restriction sites. The rational and limitations associated with this general strategy have been discussed in detail (48, 49). Denaturing gels were used for fragment analysis in Fig. 6, A and B, since preliminary studies demonstrated that the marked bending of some fragments precluded accurate determination of fragment length under native conditions (data not shown). Thus, a single double-stranded fragment frequently appeared as two closely spaced gel bands with similar intensities because of compositional strand variation. The method also detects single stranded nicks introduced by MNase and consequently the gel background was higher than that seen by native gel analysis.

The dark bars along the maps in Fig. 6, C and D, represent the positions of a precisely phased nucleosome with one border at the 3’ end of the fragments. The positions of the restriction sites in the boxes were deduced by determining the lengths of the major band sets from the reconstitutions that were carried out in the presence of DAPI. None of these bands were seen in naked DNA digested by MNase and restriction enzymes which implies that their lengths reflect authentic nucleosome positions (data not shown). The agreement between the expected and observed results shown in Fig. 6, C and D, suggests that the nucleosome is positioned primarily as in maps with one border in the vicinity of the 3’ end of the fragments. This arrangement would maximize the contacts of the histone octamer with the nonbent sequences on both templates.

Interpretation of the positioning data from the reconstitutions carried out in the absence of DAPI was more difficult. If a nucleosome is positioned exclusively on the bent portion of the fragments, no further cuts should be made by BamHI and PstI since their cleavage sites would lie outside the region containing the nucleosome. Most of the DNA from the nucleosomes reconstituted in the absence of the drug was indeed resistant to cleavage by these enzymes. However, additional bands were also formed (Fig. 6, A and B). Some of these bands were present in MNase digests of naked DNA and the two ~110-nucleotide bands in the BamHI digest were members of this group. However, approximately 40% of the bands were unique to nucleosomal DNA which indicates that the nucleosome is positioned at multiple sites with some of the nucleosome borders extending into the nonbent sequences along the fragment. The presence of multiple bands in digests by enzymes which cut within the bent sequences (Bst and MspI) and immediately flanking these sequences (EcoRI) is consistent with this view.

In order to study the effects of DAPI on the boundaries of the histone octamer, nucleosomes reconstituted onto the top and bottom labeled strands of the 322-bp template were digested with ExoIII (Fig. 7). This fragment corresponds to number 4 in Fig. 4B and the sequence is presented in Fig. 1. These digests were carried out in parallel with digests of naked DNA which had been taken through all steps in the reconstitutions in the absence of histones. In the presence of DAPI, prominent ExoIII pause sites were noted in the right terminal portions of the sequence at bp positions 310, 315, and 320. These chromatin sites persisted throughout the time course of digestion but were not seen in naked DNA. The strength and stability of these sites suggest that they arise from the presence of a fixed nucleosome boundary at this position which is in agreement with the results in Fig. 6B. This nucleosome should extend to the approximate center of the 322-bp fragment. Consequently, ExoIII was expected to remove about half of the DNA and pause at around position 150 when the labeled bottom strand template was used in the analysis. As noted from the digests of this chromatin template, the pattern of fragments was indeed indistinguishable from that generated by naked DNA for about 150 nucleotides. The absence of a discrete chromatin pause site.
FIG. 7. Effects of DAPI on nucleosome boundaries as determined by ExoIII digestion. The 322-bp intron containing fragment from Fig. 4B, line 4, with 5’-end labeled top (left) or bottom (right) strands, was reconstituted into nucleosomes using 1 µg of template/ml and 10 µg/ml erythrocyte nucleosomes in the absence (−) or in the presence (+) of 10 µM DAPI. The reconstituted nucleosomes (Nuc) and correspondingly treated naked DNA (DNA) were digested at room temperature with 180 units/ml ExoIII for 3, 10, and 30 min (−3). DNA samples were extracted and electrophoresed on an 8% denaturing PA gel which was calibrated with MspI digests of pUC18 and A/G sequencing reactions (not shown). The numbers correspond to the sequence positions given in Fig. 1. The labeled extremities (+), directions of ExoIII digestion (±), and all AT sites ≥4 bp in length (bold face) are also indicated in Fig. 1.

FIG. 8. Effects of DAPI on DNase I digestion patterns. Procedures and abbreviations are as described in the legend to Fig. 7 except DNase I was used to digest nucleosomes (0.5, 1, and 4 units/ml; −3) and naked DNA (0.05, 0.2, and 0.5 units/ml; −3) for 10 min at room temperature. Vertical bars indicate protected regions in nucleosomal DNA in the presence of DAPI relative to the naked drug-treated DNA sample. Positions of DNase I cutting sites in DNA and in the nucleosome in the absence of DAPI are indicated in Fig. 1.

at this site presumably resulted from strong inhibitory effects of DAPI on the progress of ExoIII digestion and by the inability of the enzyme to progress much beyond the center of the fragment.

ExoIII digestion of the nucleosome assembled in the absence of the drug revealed multiple pause sites which differed in DNA position by multiples of the helical repeat of 10–11 bp. These sites were not seen along the ~60 nucleotides in the nonbent right terminus where the weak pauses in the chromatin sample were in the same positions as those seen in naked DNA. The strongest sites were distributed over the first 60 nucleotides of the fragment and the sites at base pair positions 12 and 22 lie within nonbent vector DNA. The formation of these two sites was apparently dependent on the flanking circular element since they were not seen on an assembled template containing this vector DNA flanked by the exon sequence (data not shown). We presume that the pause sites are authentic nucleosome boundaries since ExoIII does not generally penetrate the core particle by more than 10–20 bp under the conditions used in the analysis (Refs. 49 and 50, also see below). The presumptive boundaries seem to be related to the periodic sequence nature of the circular element since the positions of the sites within the element roughly coincide to the positions of the AT sites. This can be seen by the similarities in gel positions of the chromatin pause sites and the DAPI-induced pause sites which occur at the AT regions in naked DNA. However, the nucleosome ladders appear somewhat more regular than the AT sites which implies that the multiple nucleosome frames are related to repetitive structural elements along the circular segment.

The circular element contains AT sites in a 10–11-base period that are separated by GC-rich and mixed sequence DNA (see Fig. 1). DNA with this sequence pattern is expected to adopt a specific rotational setting when bent in solution or in the nucleosome with the AT narrow minor grooves facing inward toward the direction of curvature (37, 51–53). DNase I is commonly used to study this periodic modulation in groove width since the enzyme preferentially cleaves the wider minor grooves on the outside of curved molecules but avoids the narrow minor grooves that face inward. Fig. 8 shows the effects of DAPI on DNase I footprints of naked and nucleosomal DNA. In the absence of DAPI, naked DNA displayed a complex pattern which is most readily seen on the bottom-labeled strand template. Many of the predominant bands in the circular element occurred in a 10–11-base period but several additional bands did not follow this regular pattern. All sites map to regions located between the A/T-tracts as indicated in Fig. 1 and were within one nucleotide of a G or C. Those sites that did not follow the 10-base pattern are often found within the longer GC regions. DAPI eliminated these non-phased sites producing a striking ladder with a 10–11-base period that extended throughout the length of the circular element (Fig. 8). Upon packaging the DNA into a nucleosome in the absence of DAPI, the 10–11-base pattern was also seen which suggests that the rotational orientation of the drug-DNA complex in solution is the same as the orientation of the DNA in the nucleosome in the absence of drug (Fig. 8). The regular chromatin ladder spans an area that encompasses most of the fragment including the 30-base nonbent segment at the 5′ end of the fragment. However, the pattern was not readily apparent in the 60-base nonbent region at the 3′ end of the fragment which displayed a cleavage pattern that was similar to that seen in naked DNA. These results are consistent with studies in Fig. 6 and provide
fig. 9. Effects of DAPI on assembled nucleosomes. Nucleosomes reconstituted onto the HaeIII-EcoRI template from Fig. 4B, line 5 (A), or 5 S rDNA (B) were incubated for 1 h at the indicated temperatures in 150 mM NaCl in the presence (+) or absence (−) of 10 μM DAPI prior to gel analysis. In C, the above analysis was carried in the presence of DAPI at 37 °C for the indicated times using nucleosomes reconstituted onto the HaeIII-EcoRI template ( ), HaeIII-PstI template ( ), HaeIII-HindIII template ( ), and HaeIII-HaeIII template ( ). The HindIII site is midway between the downstream HaeIII and PstI sites in Fig. 4B. The length of the curved intron DNA in the four fragments was 161 bp and the lengths of the non-intron DNA were 0, 39, 51, and 61 bp, respectively.

Additional support for the existence of multiple nucleosome phases distributed in an asymmetric manner along the fragment as indicated by the ExoIII experiments in Fig. 7. In several footprinting experiments in the presence of DAPI, a protected region of ~140–160 nucleotides was seen from approximately 320 to 160 on the top strand and from ~180 to >260 on the bottom. The sequence position of this nucleosome is also in agreement with results in Figs. 5–8. This protected region did not display a distinct 10-base period ladder which presumably reflects a heterogeneous group of rotational settings.

Effects of DAPI on Assembled Nucleosomes—Fig. 3C shows that DAPI selectively destabilized nucleosomes that had been previously assembled onto the intron segment but that histones remain bound to the DNA. In order to further characterize this unstable particle, interon-containing nucleosomes were incubated in 150 mM NaCl in the presence and absence of DAPI for 1 h at the temperatures indicated in Fig. 9A. The results reveal that DAPI induced a temperature-dependent nucleosome loss which was not seen with nucleosomes assembled onto 5 S rDNA (Fig. 9B), the exon sequence, or total genomic DNA (data not shown). Nucleosome loss was a gradual process that occurred over a period of about 2 h at 37 °C in 150 mM NaCl (Fig. 9C) or 1 h at 44 °C (data not shown). This slow rate was unaffected by the addition of a 100-fold excess of free DNA but was dependent on ionic strength since the nucleosome loss proceeded at a 5–10-fold slower rate in 50 mM NaCl and a 2–3-fold faster rate in 200 mM NaCl (data not shown). The gradual loss was apparently not due to a lag in DAPI binding since maximal or near maximal binding to nucleosomes containing either the intron segment or total genomic DNA occurred within 1 min at 4, 25, and 37 °C as revealed by fluorescence enhancement analysis (data not shown). Taken together, these results imply that the effect of DAPI on the mature nucleosome is a two-step process involving a rapid binding-induced destabilization followed by a more gradual temperature-dependent loss of the histone octamer from the DNA. Since both processes can occur under physiologically relevant conditions (37 °C, 150 mM NaCl), the selective disruption of assembled nucleosomes could be important in the action of the minor groove binding drugs in the cell.

As shown in Fig. 9C, the template requirements for the DAPI-induced loss of nucleosomes were similar to those seen when the drug was added prior to reconstitution (Fig. 4B, lines 5–8). In both cases, there was an abrupt increase in nucleosome loss as the length of the noncurved DNA that flanked the intron segment was decreased from 61 to 51 bp. Thus, DAPI might promote the movement of the nucleosome from the curved intron segment onto the noncurved segment of the fragment provided that the later segment is sufficiently long to enable the nucleosome to establish stable contacts on drug-free DNA sequences. To test this proposal, a nucleosome assembled onto the 322-bp fragment was incubated with and without DAPI in 200 mM NaCl for 1 h at room temperature. Following incubation, the chromatin and protein-free DNA were digested with ExoIII in order to assess the effects of the drug on the boundaries of the nucleosome. The results of these studies were similar to those seen in Fig. 7 (data not shown) which suggests that the drug favors the movement of the nucleosome onto the nonbent DNA that flanks the circular element. Drug-induced changes in positioning were also studied in nucleosomes that were reconstituted onto the 214-bp HaeIII-HaeIII fragment from Fig. 4B, line 8, and Fig. 9C. These nucleosomes were incubated in the presence and absence of drug for 1 h at 4, 25, and 37 °C in 150 mM NaCl. The results of this analysis revealed that the extent of DAPI-induced translocation of the nucleosome onto the flanking noncurved DNA was temperature dependent (data not shown) and roughly paralleled the rate of drug-induced loss of nucleosomes from the intron DNA that lacked noncurved flanking sequences (Fig. 9A).

Discussion

The positioning of nucleosomes along the DNA fiber can be controlled by structural properties of the DNA or be directed by proteins that bind to specific DNA sites (1–13). The results of this study show that minor groove binding drugs may serve as important tools to study these processes since DAPI can direct the positioning of a nucleosome in the vicinity of highly bent sequences. In the absence of these drugs, a nucleosome is preferentially formed on a circular intron segment at positions that are multiples of the DNA helical repeat of 10–11 bp (Figs. 1 and 7). These multiple translational positions share a common rotational orientation and are likely to result from a combination of the 10–11 bp sequence period (37, 51–53) and the correspondingly spaced periodicity of DNA-docking sites that are formed by specific histone motifs on the surface of the nucleosome (54, 55). Some of the nucleosome boundaries extend beyond the circular element onto flanking nonbent DNA.
sequences in the intron fragment that are
The minor groove binding drugs interact with each of the AT
length2 and these sequences are found at 10–11-bp intervals
AT minor groove persists during nucleosome assembly
ers (22, 56) which implies that the narrow configuration of the
AT minor groove persists during nucleosome assembly in vitro.
The minor groove binding drugs interact with each of the AT
sequences in the intron fragment that are ≥4 nucleotides in length2 and these sequences are found at 10–11-bp intervals along the entire 192-bp segment (Fig. 1). This interaction prevents a nucleosome from forming on the bent sequence and the DNA remains in a histone-free state during the assembly reaction (Figs. 2 and 3). Consequently, the ligand can direct the assembly of a nucleosome onto nonbent DNA that immediately flanks the segment by an exclusion mechanism as revealed by digestion studies with restriction enzymes, MNase, ExoIII, and DNase I (Figs. 5–8).

The displacement of the histone octamer from DNA by regulatory proteins may represent a key step in the control of eukaryotic gene expression (Refs. 6–13 and references cited therein). The binding of a protein to a single site on nucleosomal DNA is usually not sufficient to promote displacement since it is energetically favorable for the adjacent DNA in the nucleosome to remain bound to histones (57–59). Protein-induced disruption may occur by ATP-dependent processes or by an energy-independent mechanism when multiple factor binding sites are clustered on the nucleosomal DNA (57–62). The results in Fig. 4 and 9 also suggest the importance of multiple AT-binding sites spanning over half of the length of the nucleosome as the critical determinant for nucleosome disruption and inhibition of assembly by DAPI. We attribute the marked template specificity of the drug shown in Figs. 2, 3, and 9 to this sequence feature. The drug inhibitory effects seem to occur in an all-or-none fashion as was indicated by the abrupt increase in nucleosome loss upon decreasing the length of nonbent sequences flanking the curved DNA from 61 to 51 bp (Figs. 4 and 9). In addition, intermediates were not seen in these experiments. These characteristics seem consistent with the model advanced by Marky and Manning (63, 64) which focuses on the all-or-none elastic response of the DNA to explain structural transitions in the core particle. In addition, the length of drug-free DNA required for the formation of a stable nucleosome deduced from the present studies (about 60 bp, Figs. 4 and 9) is similar to the 70–80 bp predicted by the elastic model of the nucleosome (64). These results are also consistent with our studies on synthetic nucleosome positioning DNAs which have shown that only a fraction of the nucleosome DNA-histone contacts are necessary for high nucleosome stability (36).8 In contrast, a sequential DNA unpeeling model (58, 59) where histone-DNA contacts are broken one by one does not seem to fit our data since this process should not follow an all-or-none dissociation pathway and should give rise to significant concentrations of intermediate products (63, 64). An all-or-none octamer dissociation mechanism has also been advanced from salt- and temperature-induced nucleosome disruption studies and points to the inherent instability of the nucleosome arising from the elastic tendency of the DNA to unfold when stable histone-DNA contacts drop to a critical level (1, 63, 64, 66–69).

DNA-histone interactions in the nucleosome are sufficiently strong so that the DNA is held to the protein surface against its tendency to straighten (1, 63, 64). However, if the histone-DNA interactions are weak or if the stiffness of the DNA against bending is enhanced the complex will dissociate. Thus, minor groove binding drugs could inhibit the nucleosome assembly of A-tract-rich molecules and promote their disruption by directly inhibiting histone binding or by increasing the stiffness of DNA. It is unlikely that the inhibition of histone binding is solely responsible for the inhibitory actions of DAPI since the ligand lies deep within the minor groove and consequently should not restrict the accessibility of DNA phosphates to histones (23). The failure of the cationic DAPI to alter the electrophoretic mobility of the circular intron DNA in agarose gels is also consistent with this view (data not shown). Similarly, drug-induced inhibition of histone-DNA contacts within the minor groove is probably not the major contributor to the effect since minor groove binding drugs apparently do not displace basic polypeptides from high affinity AT sites in DNA (22, 70).

Drug-induced inhibition of DNA flexibility provides the most plausible mechanism for the effects as is evidenced by previous studies which have shown that minor groove binders render A-tract molecules rigid and resistant to bending forces (4, 22, 24, 26).

The correlation between the effectiveness of DAPI in inhibiting nucleosome assembly of the intron segment (Fig. 2) and inhibiting the cyclization of this segment in ligation reactions2 is also consistent with this proposal. In addition, a comparison of the relative effectiveness of three different minor groove binding drugs in nucleosome assembly, ligation, and electrophoretic assays has revealed that their potencies in inhibiting intrinsic DNA curvature and anisotropy bendability are mirrored by their inhibitory activities on nucleosome assembly.2

The effects of DAPI on the mature nucleosome containing the circular element can be viewed as a process involving rapid drug binding and nucleosome destabilization followed by octamer dissociation or by octamer migration onto adjacent drug-free DNA. In the first stage of this process, the drug binds to multiple AT sites and these sites face the histone octamer in the nucleosome as revealed by the DNase I studies in Fig. 8 and hydroxyl radical footprinting experiments.2 This binding site orientation apparently does not restrict ligand binding since high affinity DAPI-binding sites are preserved when DNA is packaged into nucleosomes (see Ref. 71, “Results,” data not shown). The site exposure model described by Widom and coworkers (58, 59, 72) provides a plausible mechanism by which the drug could gain access to the AT sites since short stretches of DNA are proposed to be transiently released from the octamer surface thereby exposing these DNA segments as naked DNA. The ability of the minor groove binding drugs to induce the rotation of the DNA on the surface of a nucleosome by 180° (28–30) could also serve to propagate site exposure although there is no evidence to support this view. The bound ligand then promotes the rapid destabilization of the nucleosome which is disrupted liberating free DNA in a temperature-dependent process (Figs. 3C and 9). It is unlikely that disruption or destabilization is due solely to the preferential dissociation of the H2A/H2B dimers since major intermediates were not detected in any of the experiments shown in Fig. 9. In addition, DAPI apparently inhibits the DNA interaction of all histone components of the octamer since the H3/H4 tetramer and the

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3 D. J. Fitzgerald and J. N. Anderson, submitted for publication.
H2A/H2B dimers are stably bound to DNA in 0.5 M NaCl in the presence of DAPI is derived from other octamers in the octamer observed to occupy the nonbent end of the fragment of noncurved DNA, the histone octamer is rendered mobile in some destabilization induced by DAPI may be analogous to the this process most likely by enhancing the stiffness of the DNA.

Our results suggest that DAPI reduces the salt requirement for to free DNA without the formation of stable intermediates (69).

Previous studies have shown that histone-DNA dissociation in abruptly been proposed for the folate-sensitive fragile sites (65). In

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Selective Nucleosome Disruption

In these fragile sites, long stretches of repeated CCG triplets inhibit nucleosome formation because of intrinsic structural properties of the DNA.
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