Interaction of Hoechst 33258 and Echinomycin with Nucleosomal DNA Fragments Containing Isolated Ligand Binding Sites†

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ABSTRACT: We have examined the interaction of Hoechst 33258 and echinomycin with nucleosomal DNA fragments which contain isolated ligand binding sites. A 145 base pair fragment was prepared on the basis of the sequence of *tyr*T DNA, which contained no CpG or $(A/T)_4$ binding sites for these ligands. Isolated binding sites were introduced into this fragment at discrete locations where the minor groove is known to face toward or away from the protein core when reconstituted onto nucleosome core particles. The interaction of ligands with target sites on these nucleosomal DNA fragments was assessed by DNase I footprinting. We find that Hoechst 33258 can bind to single nucleosomal sites which face both toward and away from the protein core, without affecting the nucleosome structure. Hoechst binding is also observed on nucleosomal fragments which contain two or more drug binding sites, though in these cases the footprints are accompanied by the presence of new cleavage products in positions which suggest that the ligand has caused a proportion of the DNA molecules to adopt a new rotational positioning on the protein surface. Hoechst 33258 does not affect nucleosome reconstitution with any of these fragments. In contrast, the bifunctional intercalating antibiotic echinomycin is not able to bind to single nucleosomal CpG sites. Echinomycin footprints are observed on nucleosomal fragments containing two or more CpG sites, but there are no changes in the cleavage patterns in the remainder of the fragment. Echinomycin abolishes nucleosome reconstitution when included in the reconstitution mixture.

A large number of natural and synthetic compounds have been described which bind reversibly to DNA by both intercalative and nonintercalative mechanisms. Several of these agents are able to recognize particular DNA sequences of between two and four base pairs in length, and many such compounds possess cytotoxic activity and are used in cancer chemotherapy. There have been many in vitro studies on the interaction of ligands with oligonucleotides and polynucleotides, and the molecular details of the interaction of many ligands with their preferred binding sites have been well characterized. However, these studies have almost exclusively studied the interaction of ligands with free DNA, whereas cellular DNA is complexed with various histone and non-histone proteins and is organized into higher order structures which may alter the local DNA conformation or mask potential binding sites.

The first level of organization of cellular DNA is the formation of the nucleosome (*1*) in which 145 base pairs of DNA are wrapped about 1.65 times around the histone octamer in a left-handed superhelix (*2*). Although a wide variety of DNA sequences can be wrapped around the nucleosome core, there is considerable evidence that they adopt well-defined rotational and translational positions on the protein surface (*3*−*8*) and that this may play an important role in gene activation or repression (*9*, *10*). The rotational positioning is largely determined by the anisotropic flexibility of DNA, and sequences that facilitate bending have been implicated in directing nucleosome assembly (*11*, *12*). Although it is not possible to satisfy all of the local sequence preferences within each fragment, GC-rich regions are generally positioned with their wider than average minor grooves facing away from the protein, while the narrow grooves of AT-rich sequences face toward the protein (*5*, *6*). Sequences toward the center of the nucleosome have a greater effect on positioning that those toward the ends (*13*).

The interaction of DNA with the protein surface may present a problem for the binding of sequence-specific ligands. We could imagine that binding sites which face toward the core will not be available for drug binding, while those which face away from the protein surface may adopt an altered conformation which alters the interaction with the ligand. In particular, intercalating agents, which both unwind and lengthen the DNA helix, may have a problem interacting with DNA which is constrained to wrap around the protein surface. In the present study we have therefore examined the interaction of a minor groove binder (Hoechst 33258) and a bifunctional intercalator (echinomycin) with DNA fragments which have been reconstituted into nucleosome core particles.

Hoechst 33258 (Figure 1B) is a member of the large group of AT-selective minor groove binding ligands (*14*, *15*).

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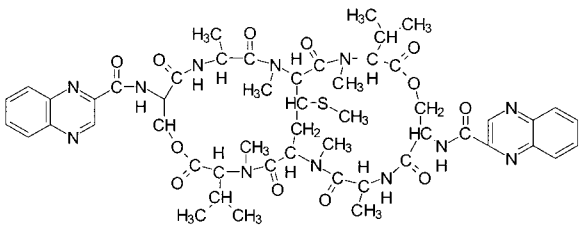
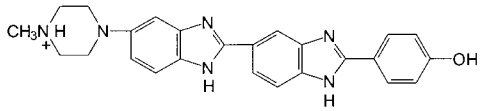


A)



B)

C)

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                                        AAAA-40H AATT-49H AATT-58H                    INWARD
                                  ATTA-35H   AATT-46H                 AATT-73H         OUTWARD
5′-AATTCTGGTCACCTTCAGTCTGTTGTGGATGAAGATCACACAACCAGTTCTTCTTCCTCTTCCTGACACTCTACAGTGGTGTGTCATCT
                                           ACGT-44E                 ACGT-74E         OUTWARD
                    ACGT-20E  ACGT-30E ACGT-39E   ACGT-50E ACGT-59E   ACGT-70E  ACGT-80E  INWARD
          10         20         30         40         50         60         70         80

GATGTGATGTGTCCCACTTCCCAACAAGGGAGTAGGTCAGTAGAGAACATCACCCTGTCCC-3′
         ACGT-100E
90         100        110        120        130        140
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FIGURE 1: Chemical structures of (A) echinomycin and (B) Hoechst 33258. (C) Sequence of the *nobind* DNA fragment. The positions of the various mutations, introducing unique ligand binding sites, are shown above and below the sequence, indicating which ones face toward (inward) or away from (outward) the protein core.

Footprinting studies have shown that it binds to AT-rich sequences (*16*−*18*). Several high-resolution structures have been determined for complexes of Hoechst[1] with short oligonucleotides (*19*−*23*), and these show that the molecule fits snugly within the minor groove, where it replaces a spine of hydration. The AT selectivity is explained by the narrow minor groove adopted by AT-rich sequences which allows close van der Waals contacts with the walls of the groove. In addition, GC base pairs are excluded from the binding sites as the result of steric clash with the 2-amino group of guanine. Although Hoechst 33258 binds to all $(A/T)_4$ sequences, it binds less well to sites containing TpA steps and shows the strongest interaction with AAAA and AATT (*24*).

Echinomycin (Figure 1A) is a bifunctional intercalator (*25*) which is selective for the sequence CpG (*26*, *27*). It binds in the DNA minor groove forming hydrogen bonds between its alanine carbonyls and the 2-amino group of guanine (*28*−*30*). Echinomycin binds especially well to the tetranucleotide ACGT, and binding to this site is stronger when it is surrounded by alternating purines and pyrimidines than by oligopurine tracts (*31*).

There have been a few previous studies on the interaction of sequence-specific drugs with nucleosomal DNA. Early footprinting studies with Hoechst 33258 and echinomycin suggested that these ligands can induce a change in the rotational positioning of nucleosomal DNA fragments (*32*−*35*). However, although the drugs caused clear changes in the DNase I digestion patterns, no simple footprints were observed, suggesting that the effects were occurring at low levels of occupancy. In contrast, mithramycin produced clear footprints with nucleosomal DNA in which the exact location of the binding sites was modified by interaction with the protein (*36*). Actinomycin merely bound to accessible sites at low concentrations and displaced the DNA from the protein surface at higher concentrations (*33*). Each of these studies was performed with natural DNA restriction fragments in which different drug binding sites faced both toward and away from the protein core. Further studies with synthetic DNA fragments containing $(A/T)_4$ sites located every 10 base pairs, facing toward the protein core, also showed that minor groove binding ligands induced a change in the DNA rotational position (*37*). Other recent studies have shown that minor groove binders selectively inhibit the assembly of curved DNA molecules onto nucleosome core particles and destabilize complexes which have previously been assembled (*38*, *39*). Recent studies have shown that pyrrole−imidazole polyamides can bind to nucleosomal sites which face away from the histone octamer and that binding does not disrupt nucleosome structure (*40*). These ligands failed to bind to sites which were fully protected by the histone octamer, and these were rendered accessible by removal of the histone amino-terminal tails (*40*). These studies suggested that much of the DNA in a nucleosome is readily accessible for binding by minor groove ligands.

Related studies have also shown that nucleosome formation affects the formation of intermolecular DNA triplexes (*41*−*43*). Target sites located toward the edge of the nucleosome are available for drug binding, while those positioned 30 base pairs on either side of the dyad are not available for oligonucleotide binding (*41*, *42*). On longer fragments triplex formation has been shown to alter nucleosomal arrangements and acts as a nucleosomal barrier (*43*).

In the present studies we have used DNase I footprinting to examine the interaction of Hoechst 33258 and echinomycin with nucleosomal DNA fragments. For these studies we have introduced drug binding sites at unique positions along the DNA fragment so that they either face toward or away from the protein core.

MATERIALS AND METHODS

Chemicals and Enzymes. Oligonucleotides for preparing the various DNA fragments were purchased from Oswel

[1] Abbreviations: Hoechst, Hoechst 33258; EDTA, ethylenediaminetetraacetic acid.

DNA service (Southampton, U.K.). These were stored in water at −20 °C and diluted to working concentrations immediately before use. Plasmid pUC19 was purchased from Pharmacia. DNase I was purchased from Sigma and stored at −20 °C at a concentration of 7200 units/mL. Restriction enzymes and reverse transcriptase were purchased from Promega.

DNA Fragments. The sequence of the 150 base pair DNA fragment *nobind*, containing no binding sites for echinomycin (CpG) or Hoechst 33258 [$(A/T)_4$], was based on the *tyr*T DNA fragment. The 160 base pair *tyr*T has been widely studied, and its interaction with nucleosome core particles has been fully characterized, revealing that the first 145 base pairs are closely associated with the protein (*6*, *32*, *33*, *41*, *42*). The *tyr*T sequence was modified, generating the sequence *nobind* (shown in Figure 1C) so as to remove all of the ligand binding sites while retaining the order of purines and pyrimidines. Oligonucleotides corresponding to this sequence were synthesized, kinased, ligated, and cloned between the *Eco*RI and *Ava*I sites of pUC19. Further fragments, containing isolated ligand binding sites at chosen positions, were prepared from this clone by QickChange site-directed mutagenesis (Stratagene). The modifications were chosen so as to introduce good binding sites for echinomycin (ACGT) or Hoechst (AATT, AAAA, or ATTA) while making as few changes as possible to the DNA sequence. The sequences of the resulting fragments, which are shown in Figure 1C, were confirmed by DNA sequencing using a T7 sequencing kit (Pharmacia). Radiolabeled 150 base pair DNA fragments were prepared by digesting the plasmids with *Eco*RI and *Sma*I and were labeled at the 3′-end of the *Eco*RI site with [α-^{32}P]dATP using reverse transcriptase. Radiolabeled DNA was separated from the remainder of the plasmid on 6% nondenaturing polyacrylamide gels. The bands containing the radiolabeled DNA were excised and eluted into 10 mM Tris-HCl, pH 7.5, containing 0.1 mM EDTA. The DNA was then precipitated with ethanol in the presence of 0.3 M sodium acetate.

Reconstitution of DNA onto Nucleosome Core Particles. H1-stripped chromatin, derived from chicken erythrocytes, was prepared as previously described (*5*, *6*, *44*, *45*) and stored at 4 °C, or −20 °C in 50% (v/v) glycerol. The DNA fragments of interest were reconstituted onto nucleosomes at pH 7.5 by a salt exchange method, as previously described (*3*, *5*, *6*, *44*, *45*). The incorporation of labeled DNA onto nucleosomes was checked by gel retardation on 5% (w/v) polyacrylamide gels; at least 90% of the labeled DNA was included in the retarded species. These reconstituted nucleosomes were stored at 4 °C. For experiments in which nucleosomes were reconstituted in the presence of the ligands, the radiolabeled DNA was first incubated with the ligand before addition of the nucleosomes and high-salt buffer (which also contained the ligand at the same concentration). The ligand concentration was maintained while reducing the ionic strength by including the ligand in the low-salt buffer.

Footprinting of Reconstituted Core Particles. Ten microliters of the reconstituted nucleosomes was mixed with 10 μL of varying concentrations of ligand dissolved in 10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl. These mixtures were allowed to equilibrate for at least 10 min before being digested with either DNase I or a hydroxyl radical generating mixture. DNase I digestion was achieved by adding 4 μL of enzyme (typically 30 units/mL) dissolved in 20 mM NaCl, 2 mM $MgCl_2$, and 2 mM $MnCl_2$. Higher concentrations of DNase I are required for digesting the nucleosome-bound DNA fragments than for free DNA due to the presence of the large amount of unlabeled DNA from the chicken nucleosomes; the enzyme concentration was adjusted so that about 60−70% of the DNA remained uncut. The digestion was stopped after 1 min by the addition of 100 μL of buffered phenol (pH 7.5). Hydroxyl radical cleavage was performed by adding 40 μL of a freshly prepared mixture containing 50 μM ferrous ammonium sulfate, 100 μM EDTA, 2 mM ascorbic acid, and 0.05% hydrogen peroxide. The reaction was stopped after 10 min by adding 100 μL of phenol, and the aqueous phase was made up to 100 μL with water. The samples were then extracted twice with phenol, to remove the protein, followed by two extractions with ether, to remove the remaining phenol. The DNA was finally precipitated with ethanol and redissolved in 8 μL of 80% formamide containing 10 mM EDTA, 10 mM NaOH, and 0.1% (w/v) bromophenol blue.

Footprinting Free DNA. Radiolabeled DNA was dissolved in 10 mM Tris-HCl, pH 7.5, containing 0.1 mM EDTA; 1.5 μL of this DNA solution was mixed with 1.5 μL of ligand, dissolved 10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl. This mixture was allowed to equilibrate for at least 1 h before being digested with either DNase I or a hydroxyl radical generating mixture. DNase I digestion was achieved by adding 2 μL of enzyme (typically 0.01 unit/mL) dissolved in 20 mM NaCl, 2 mM $MgCl_2$, and 2 mM $MnCl_2$. The digestion was stopped after 1 min by adding 5 μL of 80% formamide containing 10 mM EDTA, 10 mM NaOH, and 0.1% (w/v) bromophenol blue. Hydroxyl radical cleavage was performed by adding 6 μL of a freshly prepared mixture containing 50 μM ferrous ammonium sulfate, 100 μM EDTA, 2 mM ascorbic acid, and 0.05% hydrogen peroxide. The reaction was stopped after 10 min by precipitating with ethanol. The DNA was finally redissolved in 8 μL of 80% formamide containing 10 mM EDTA, 10 mM NaOH, and 0.1% (w/v) bromophenol blue.

Denaturing Gel Electrophoresis. Products of DNase I or hydroxyl radical digestion were separated on 6−10% polyacrylamide gels (depending on the location of the target site) containing 8 M urea. DNA samples were boiled for 3 min immediately before being loaded onto the gels. Polyacrylamide gels (40 cm long) were run at 1500 V for 2 h. These were then fixed in 10% acetic acid, transferred to Whatmann 3MM paper, dried under vacuum at 80 °C, and exposed to a phosphorimager screen (Kodak) overnight. Dry gels were exposed to a Kodak Phosphor Storage screen which was scanned using a Molecular Dynamics Storm 860 phosphorimager The products of digestion were assigned by comparison with Maxam−Gilbert marker lanes specific for guanine and adenine.

Nondenaturing Gel Electrophoresis (Band Shifts). A 4 μL aliquot from the reconstitution reaction was mixed with 3 μL of loading dye. This was run on 5% (w/v) nondenaturing polyacrylamide gel (Protogel, National Diagnostics). These gels (20 cm long, 0.3 mm thick) were run at 200 V, 8 W, until the bromophenol blue had run about 15 cm. Gels were fixed, dried, and exposed to phosphorimaging as described above.

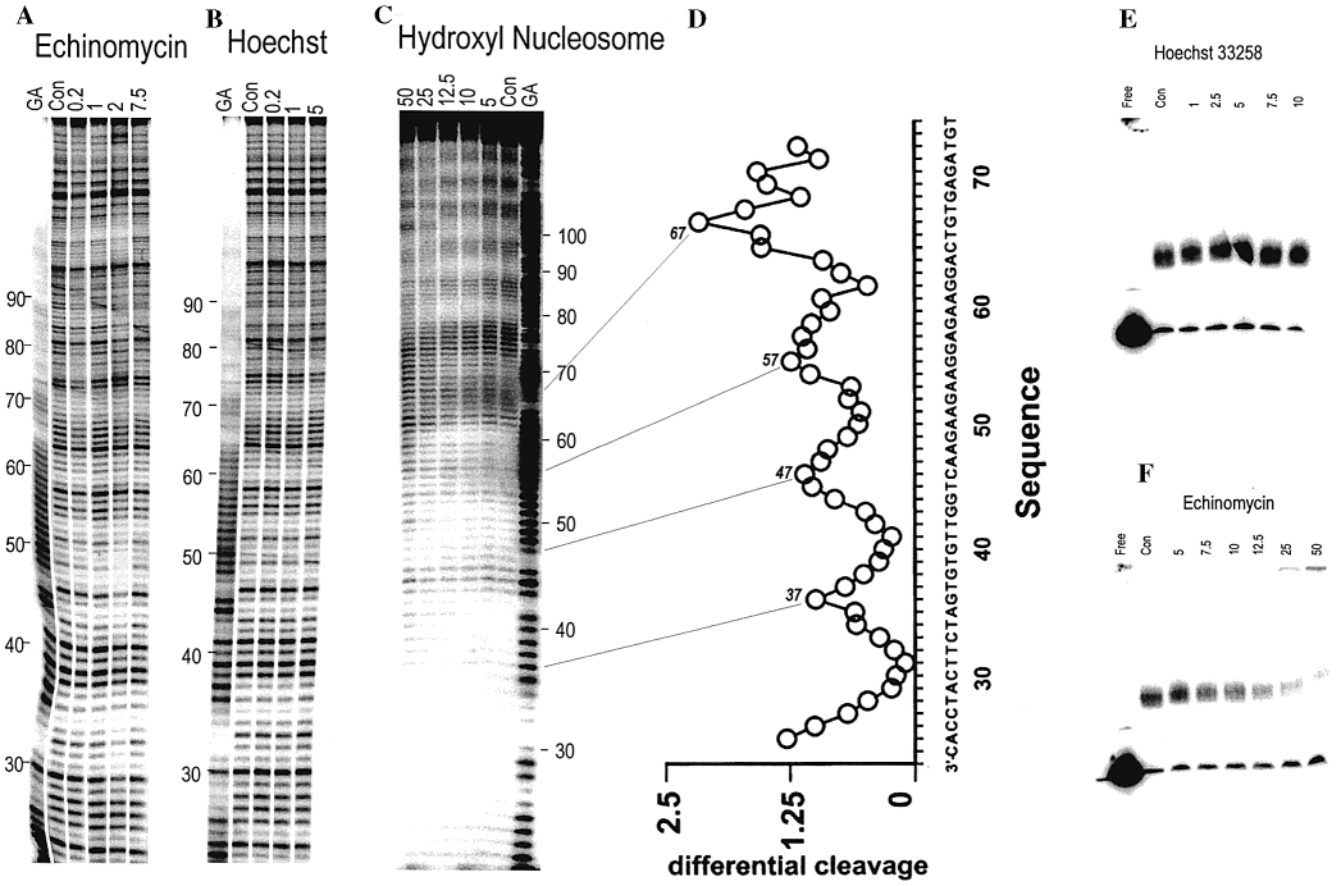

FIGURE 2: (A and B) DNase I cleavage patterns for the interaction of echinomycin and Hoechst 33258 with the *nobind* DNA fragment. (C) Hydroxyl radical digestion of the *nobind* DNA fragment when reconstituted onto nucleosome core particles in the absence and presence of different concentrations of Hoechst 33258. Ligand concentrations (micromolar) are shown at the top of each lane. Con indicates cleavage in the absence of added ligand. Tracks labeled GA are marker lanes specific for purines. (D) Differential cleavage plot showing the difference in hydroxyl radical cleavage of nucleosomal and free *nobind* DNA. Minima correspond to positions where the DNA minor groove faces toward the protein core. (E and F) Band-shift experiments showing the effect of Hoechst 33258 and echinomycin on the reconstitution of *nobind* DNA onto nucleosome core particles.

RESULTS

We first prepared the 150 base pair *nobind* DNA fragment shown in Figure 1C which contains no binding sites for echinomycin (CpG) or Hoechst 33258 [$(A/T)_4$] and examined its interaction with these ligands and with nucleosome core particles. The results are shown in Figure 2. The first two panels show footprinting reactions with free DNA in the presence of echinomycin and Hoechst 33258. It can be seen that, as expected, both ligands do not affect the DNase I cleavage pattern at concentrations which normally produce footprints on free DNA (*26*, *27*, and see below). The third panel shows hydroxyl radical footprinting patterns for this fragment when reconstituted onto nucleosome core particles in the presence and absence of Hoechst 33258. Hydroxyl radical cleavage of the drug-free DNA reveals a sinusoidal cleavage pattern, confirming that the DNA is wrapped around the nucleosomes, revealing the positions in which the minor groove faces toward (low cleavage) or away (maximum cleavage) from the protein surface. This cleavage pattern is used in the remainder of this work to locate drug binding sites in specific orientations with respect to the protein. This pattern is not affected by addition of Hoechst 33258 at concentrations up to 12.5 μM, though some changes are evident at the highest concentrations (25 and 50 μM), which are probably due to nonintercalative binding. The final panel of this figure shows the effects of echinomycin and Hoechst 33258 on the reconstitution of this fragment onto nucleosomes. It can be seen that in each case the mobility of the reconstituted fragment is reduced relative to the free DNA and that about 90% of the total radioactivity is found within this retarded species. This process is not affected by addition of Hoechst 33258 or echinomycin up to 10 μM. These results confirm that the *nobind* DNA fragment, containing no CpG or $(A/T)_4$ sites, adopts a preferred rotational position on the nucleosome surface, and it does not bind echinomycin or Hoechst 33258 when either free or incorporated in a nucleosome.

A potential problem with altering the DNA sequence is that, since the rotational and translational position of a DNA molecule on the nucleosome surface is indirectly determined by its sequence, these changes might alter its position relative to that of the native fragment. This would complicate any comparison of ligand binding at the different locations. The rotational position of these mutated fragments when reconstituted onto nucleosome core particles was therefore checked by examining their DNase I cleavage patterns. In each case, the positions of maximum and minimum cleavage were in very similar locations, suggesting that these base changes have not affected the rotational position adopted by the DNA on the protein surface. The translational position of each

fragment is less well defined, though since they are all 150 base pairs long, it seems reasonable that they should all adopt the same translational position in the absence of added ligands. Slippage of the nucleosome in either direction would expose some of the DNA binding surface of the protein and would be expected to be energetically less favorable. Nonetheless, there is some ambiguity about the position of the dyad which is probably located between 65 and 85. We can therefore be less certain about the rotational setting of sites in this region (e.g., fragments 73H, 74E, and 70E,80E described below).

Outward-Facing Hoechst Sites. Having confirmed that Hoechst does not bind to the parent fragment, we used site-directed mutagenesis to introduce unique binding sites for this ligand at discrete positions along the nucleosomal DNA. In each case the mutations were chosen so as to make as few changes as possible to the DNA sequence, thereby minimizing any alterations to the protein−DNA interaction. Hoechst binding sites were therefore introduced around positions 35 (ATTA), 46 (AATT), and 73 (AATT), regions in which the minor groove faces away from the protein core, and the results of DNase I footprinting experiments with these fragments when free and nucleosome bound are presented in Figure 3. The first two panels show the results with fragment 35H. It can be seen that on free DNA Hoechst 33258, at concentrations of 1 μM and above, produces a single footprint which covers 5−6 bases at the expected target site. The second panel shows that Hoechst 33258 produces a footprint in the same location with nucleosomal DNA. At concentrations of 10 μM and below Hoechst has no effect on cleavage in the remainder of the nucleosomal DNA, and positions of maximal DNase I cleavage can be seen at positions 46, 58, 66−68, 74−78, 89, and 96−99. It therefore appears that at these concentrations Hoechst 33258 can bind to this outward-facing nucleosomal site and that it does so without affecting the integrity of the complex. Other changes in the cleavage pattern are evident at higher ligand concentrations, but since these are greater than that required to generate the footprint, they are unlikely to be due to binding to this site alone and probably reflect a secondary binding mode. The third and fourth panels of this figure show the results of similar experiments with fragment 46H. It can be seen that Hoechst 33258 produces a footprint at the expected binding site with both free and nucleosomal DNA. It should also be noted that the nucleosomal DNase I cleavage pattern in the absence of the ligand is similar to that of fragment 35H. Similar DNase I cleavage patterns are produced with each of the nucleosomal DNA fragments described below, demonstrating that the mutations do not cause any major changes in the interaction with the nucleosome core particles. The fifth and sixth panels of Figure 3 show the results of similar experiments with fragment 35H, 46H containing both of these outward-facing Hoechst binding sites. Footprints are evident at both sites on both free and core-bound DNA. It therefore appears that Hoechst 33258 can bind to outward-facing nucleosome sites, without altering the integrity of the complex.

The last two panels of this figure show the results with fragment 73H, which possesses a single Hoechst binding site (AATT) located close to the nucleosome dyad. Since this site is flanked by A/T residues, in the sequence TAATTT, we can be less certain about the exact location of the bound drug molecule, though previous studies have suggested that AATT is a better binding site than either TAAT or ATTT (*24*). A further complication is that this site is located in the vicinity of the dyad, and we can therefore be less certain about its rotational position. DNase I footprints for Hoechst can again be seen on both free and nucleosomal DNA, confirming that, whatever its rotational position, this site is still accessible for drug binding when bound to nucleosomes.

We also examined the effect of Hoechst 33258 on nucleosome reconstitution by including the ligand in the reconstitution mixture. The integrity of the complexes was then examined by performing band-shift experiments using nondenaturing polyacrylamide gels. The results for fragments 35H,46H and 73H are shown in Figure 4A,B. It can be seen that Hoechst 33258 has not affected nucleosome reconstitution. Similar results were also found with fragments 35H and 46H (not shown).

Outward-Facing Echinomycin Sites. Echinomycin binding sites (ACGT) were introduced at positions 44 and 74, regions in which the minor groove is thought to face away from the protein core. The results of DNase I footprinting experiments with these fragments when free and nucleosome bound are presented in Figure 5. In each case the ligand produces clear footprints at the expected locations on free DNA, each of which covers about 6 to 7 bases and is generated at concentrations of 1 μM and above. In contrast to the results with Hoechst 33258, echinomycin does not produce any changes in the cleavage pattern with this nucleosomal DNA. It therefore appears that echinomycin is excluded from these nucleosomal sites, even though their minor grooves face away from the protein surface.

Panels C and D of Figure 4 show the effect of echinomycin on nucleosome reconstitution with these two DNA fragments. It can be seen that echinomycin prevents nucleosome formation with fragment 44E, at concentrations of 10 μM and above. It therefore appears that echinomycin binding at this location is not compatible with nucleosome formation. Surprisingly, the ligand has less effect on fragment 74E, and nucleosome formation is only attenuated at the highest ligand concentrations.

Inward-Facing Hoechst Sites. The results presented above suggest that Hoechst is able to bind to DNA sites for which the minor groove faces away from the protein core and that it does so without affecting nucleosome structure. We were therefore interested in examining the interaction of Hoechst with inward-facing AT sites. Fragment 58H contains a single AATT site in a region in which the minor groove should face toward the protein core; DNase I footprinting experiments with this fragment are shown in Figure 6. The left-hand panel shows the interaction with free DNA in which it can be seen that Hoechst produces a clear footprint at the expected target site at low ligand concentrations. The results of experiments when this fragment is reconstituted onto nucleosome core particles are shown in the second panel. In the absence of ligand DNase I produces a phased cleavage pattern which is very similar to that produced with the other nucleosomal DNA fragments. Cleavage around the intended target site is poor since the minor groove faces toward the protein surface at this position. It is therefore difficult to assess ligand binding to this region. However, cleavage at position 58 does appear to be reduced with increasing

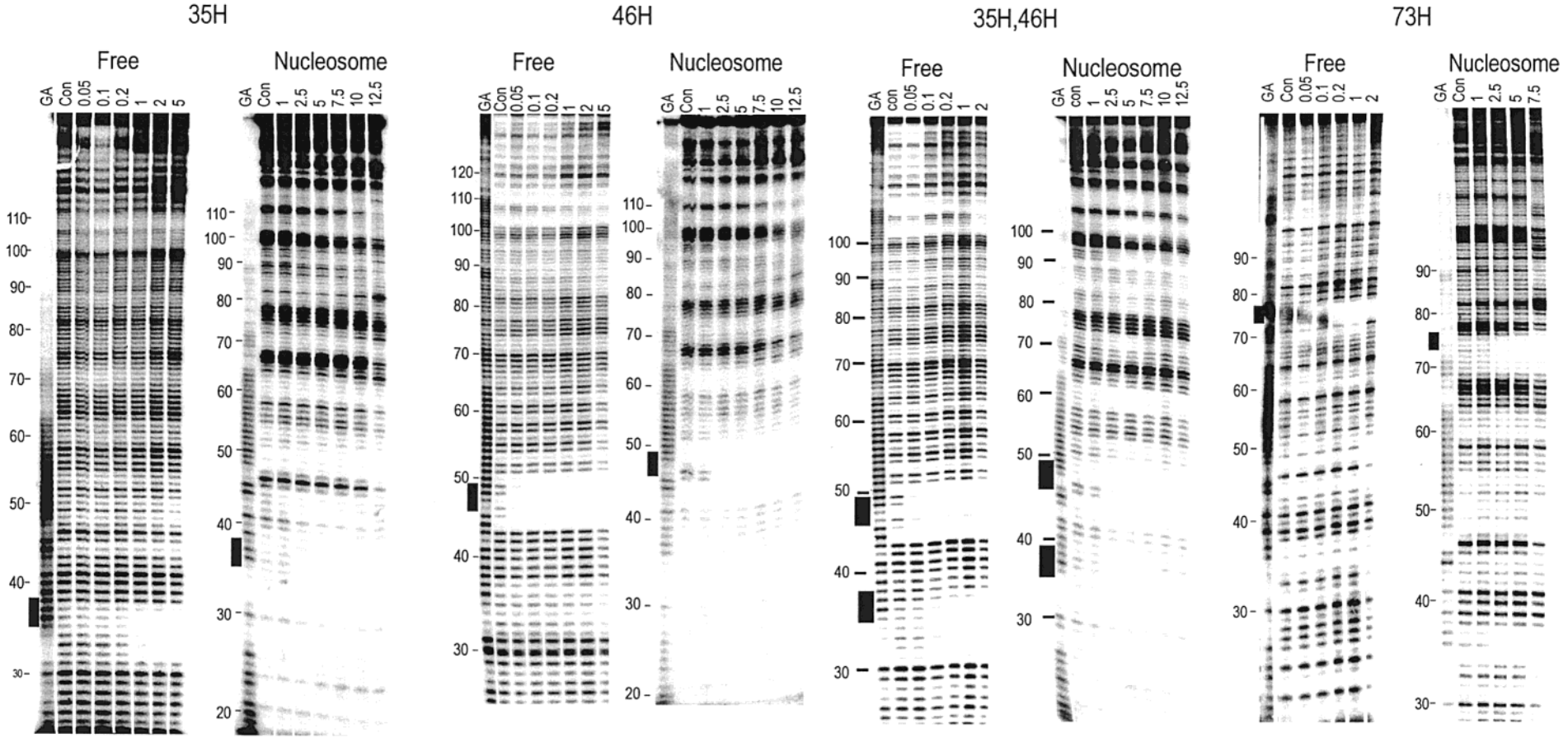

FIGURE 3: DNase I digestion of fragments containing single outward-facing Hoechst sites when free and incorporated into nucleosomes in the absence and presence of various concentrations of Hoechst 33258. Ligand concentrations (micromolar) are shown at the top of each lane. Con indicates cleavage in the absence of added ligand. Tracks labeled GA are marker lanes specific for purines. The locations of the expected target sites are indicated by the filled boxes.

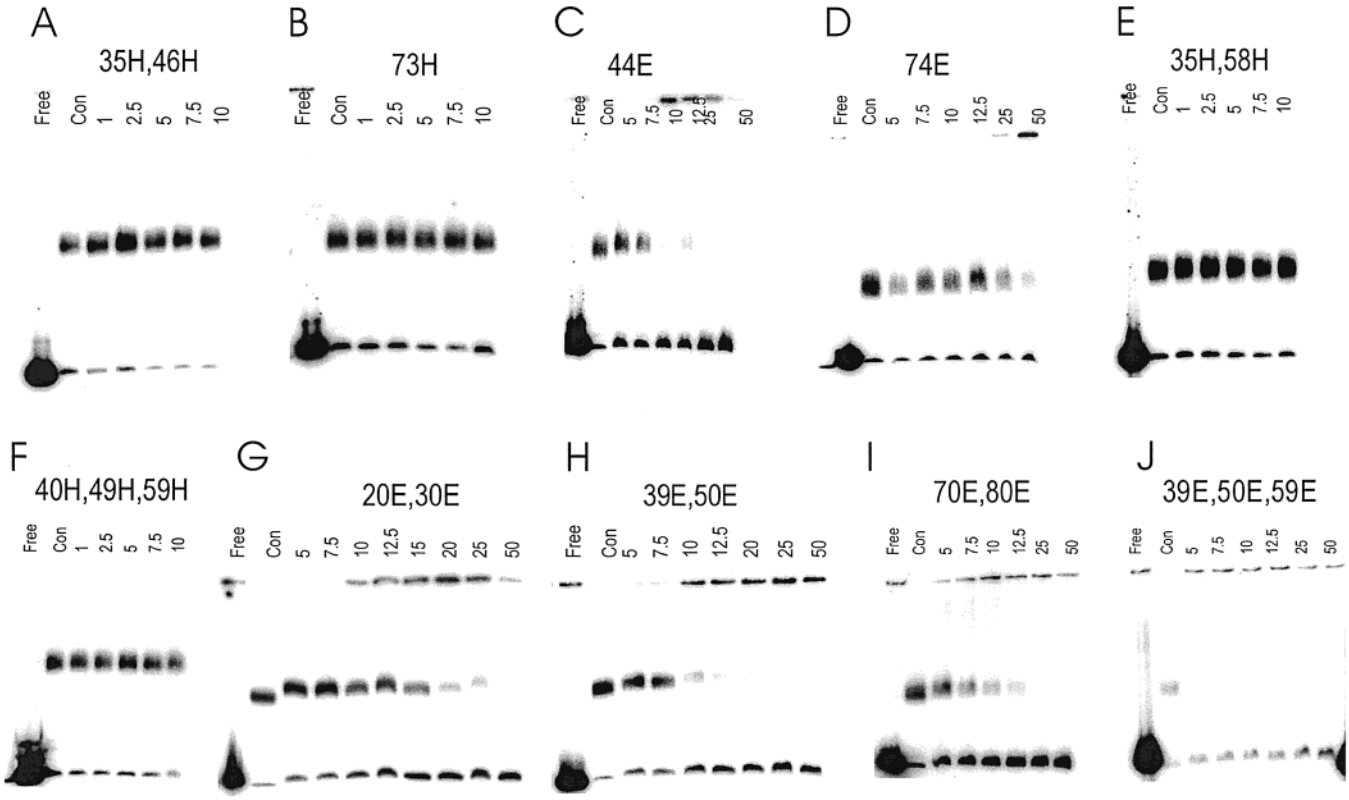

FIGURE 4: Band shifts showing the reconstitution of different fragments onto nucleosomes in the presence of Hoechst 33258 (A, B, E, F) and echinomycin (C, D, G, H, I, J,). Ligand concentrations (micromolar) are shown at the top of each lane. Con indicates reconstitution in the absence of added ligand. Tracks labeled free show the mobility of DNA which has not been complexed with nucleosome core particles. In each panel the upper band corresponds to the nucleosomal DNA, while the lower species is free DNA.

concentrations of Hoechst 33258. No drug-induced changes in the cleavage pattern are evident for the remainder of this sequence.

Fragment 35H,58H was designed to contain one inward-facing Hoechst site at position 58 and one outward-facing site at position 35. The results with this fragment are shown in the third and fourth panels of Figure 6. As expected, the ligand produces two clear footprints on free DNA. DNase I cleavage is also attenuated at both sites with nucleosomal DNA, suggesting that the ligand can access both inward- and outward-facing sites. Figure 4E shows the effect of Hoechst on nucleosome reconstitution with this fragment. It can be seen that the ligand has no effect on the reconstitution at concentrations as high as 10 μM. Similar results were also found with fragment 58H (not shown).

Inward-Facing Echinomycin Sites. Fragments 39E,50E and 100E were designed to contain single echinomycin binding sites (ACGT) in regions where the minor groove faces toward the protein surface. DNase I footprinting experiments with these fragments are shown in Figure 7. It can be seen that in each case the ligand produces a clear footprint at the expected target sites on free DNA. In contrast, no changes are produced on any of the nucleosomal DNA fragments. It therefore appears that echinomycin cannot access inward-facing nucleosome-bound target sites. Reconstitution experiments (not shown) also demonstrate that echinomycin prevents the wrapping of these fragments around nucleosomes. With 39E there is a steady fall in histone reconstitution down to less that 2% with 50 μM echinomycin. With 50E nucleosome formation falls to 28% with 5 μM echinomycin and is abolished with 50 μM ligand. Similarly, nucleosome formation with 100E is abolished with 50 μM ligand.

Multiple Inward-Facing Hoechst Sites. Fragment 49H,58H contains two good Hoechst binding sites (AATT), which are expected to be positioned with their minor grooves facing the protein surface. There are five base pairs between the end of one site and the beginning of the second, and over this region the minor groove turns away from the protein core toward solution. DNase I footprinting results for the interaction of Hoechst 33258 with this fragment are presented in Figure 8. As expected the ligand produces a clear footprint with free DNA, though the two sites coalesce into a single region of protection between positions 46 and 61, separated by a single (weak) cleavage product at position 53. The second panel of Figure 8 shows DNase I cleavage of this fragment when bound to nucleosome core particles. As expected, the target sites lie in regions where the DNA is cut poorly since the minor groove is facing toward the protein core. On addition of Hoechst the intensity of bands at positions 49 and 58 is attenuated, suggesting that the ligand is able to interact with these nucleosomal sites. These attenuations are accompanied by other changes in the cleavage pattern throughout the fragment. DNase I cleavage is attenuated at positions 25, 36−39, 49−52, 57−60, 78, 87−89, 97−99, and 108 bp which correspond to cleavage maxima in the ligand free control, while other regions of enhanced cleavage (indicated with arrows in the second panel of Figure 8) are observed at positions 28, 43−44, 53, 63, 74, 84, 92−94, and 102−105. These changes in digestion cannot be directly attributed to drug binding and presumably reflect changes in the interaction of DNA with the protein

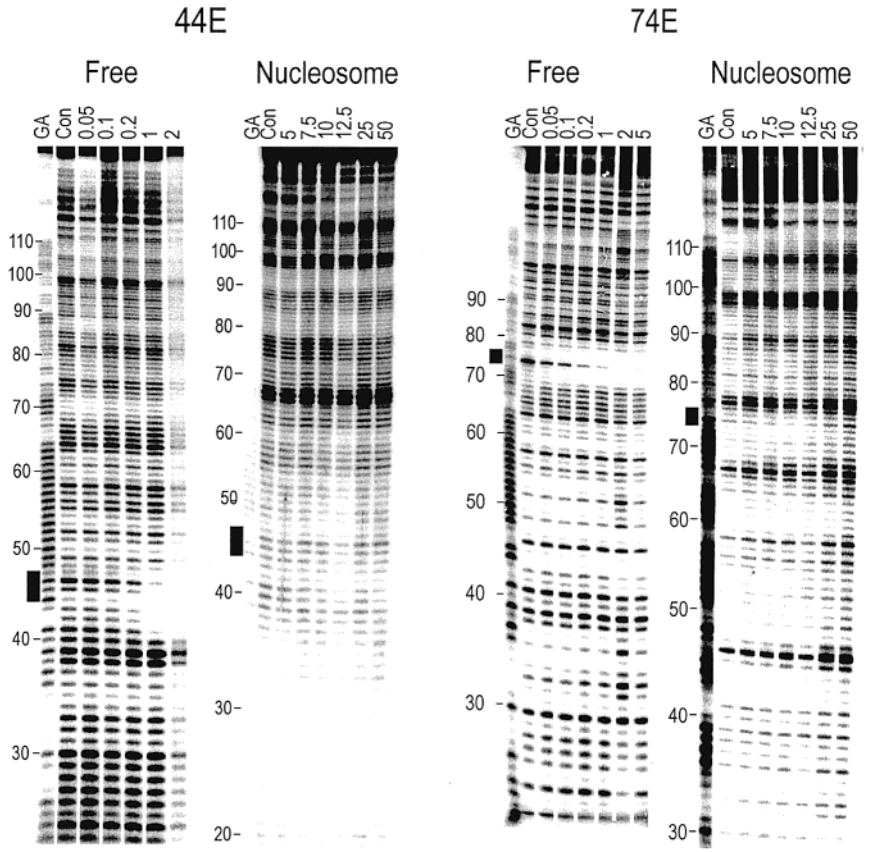

FIGURE 5: DNase I digestion of fragments containing single outward-facing echinomycin sites when free and incorporated into nucleosomes in the absence and presence of various concentrations of echinomycin. Ligand concentrations (micromolar) are shown at the top of each lane. Con indicates cleavage in the absence of added ligand. Tracks labeled GA are marker lanes specific for purines. The locations of the expected target sites are indicated by the filled boxes.

surface. The enhancements lie almost halfway between the original nucleosome peaks, suggesting that the DNA has rotated on the nucleosome surface.

Fragment 40H,49H,58H contains the same binding sites as 49H, 58H with an additional target site (AAAA) positioned so that its minor groove also faces toward the protein core. DNase I footprinting results for the interaction of Hoechst 33258 with this DNA fragment are shown in the third and fourth panels of Figure 8. As expected, the ligand produces footprints at all of the sites on free DNA, though the interaction with AAAA appears weaker than with AATT. With nucleosomal DNA similar changes in the cleavage pattern are seen to those observed with 49H,58H. Bands are attenuated in the vicinity of the intended target sites; these are most evident from the attenuated cleavage of bands at positions 41, 46, and 58. These are accompanied by other sites of reduced cleavage at positions 35, 67, 76, 86, 96, and 107. In addition, regions of enhancement are evident at positions 63, 72−73, 82, 92−94, and 103, which are indicated by the arrows in this figure. These results again suggest that Hoechst 33258 has altered the rotational position of the DNA on the protein surface. We also examined the effect of Hoechst on nucleosome reconstitution with this DNA fragment, and the results are presented in Figure 4F. It can be seen that Hoechst does not affect nucleosome formation. It therefore appears that nucleosome formation can tolerate the presence of up to three ligand binding sites at positions which normally face toward the protein surface.

Multiple Inward-Facing Echinomycin Sites. Since echinomycin is unable to interact with single CpG binding sites which face either toward or away from the protein surface, we examined how the ligand might affect DNA fragments which possess multiple inward-facing binding sites. Construct 20E,30E contains two CpG sites located so that their minor grooves should face toward the protein surface. Fragment 39E,50E contains the binding sites present in fragments 39E and 50E. Fragment 70E,80E was designed to study the interaction at inward-facing minor grooves in the vicinity of the nucleosomal dyad. The final fragment (39E,50E,59E) contains the same sites as found in 39E,50E with an additional site at position 59. DNase I footprinting gels showing the interaction of echinomycin with these fragment are presented in Figure 9. In each case it can be seen that echinomycin produces clear footprints at its expected binding sites on free DNA. The interaction of echinomycin with nucleosomal 20E,30E is presented in Figure 9. As expected, each target lies in a region of poor DNase I cleavage, where the minor groove faces the protein core. It can be seen that echinomycin produces DNase I footprints at each of these target sites, even though they are associated with the nucleosome core, facing toward the protein. This is therefore the first example of clear echinomycin footprints on nucleosomal-bound DNA. The positions of the cleavage maxima in the remainder are not changed by the addition of echinomycin, though there appears to be a general increase in cleavage at most positions.

In fragment 39E,50E the two inward-facing echinomycin sites are placed closer to the center of the nucleosome. DNase I cleavage patterns for the interaction of echinomycin with this nucleosomal DNA are shown in the fourth panel of

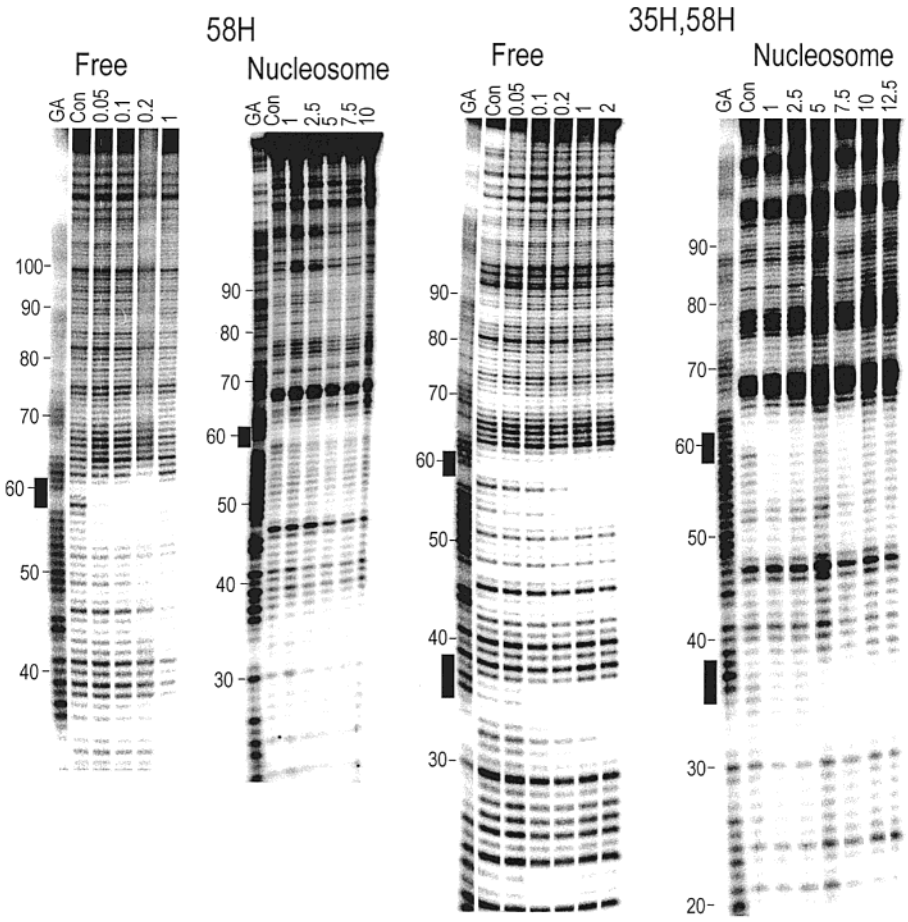

FIGURE 6: DNase I digestion of fragments containing Hoechst 33258 binding sites which face toward the protein core (58H) when free and incorporated into nucleosomes in the absence and presence of various concentrations of Hoechst 33258. Ligand concentrations (micromolar) are shown at the top of each lane. Con indicates cleavage in the absence of added ligand. Tracks labeled GA are marker lanes specific for purines. The locations of the expected target sites are indicated by the filled boxes.

Figure 9. The digestion pattern of the drug-free control is similar to that of the other fragments, confirming that this DNA fragment adopts the same rotational position and revealing the poor cleavage at each target site, as expected since the minor grooves of these sites face toward the protein core. Direct binding to the target sites is therefore not easily detected. However, footprints are produced at both sites, as evidenced by the reduced cleavage at positions 39 and 50. The lower footprint is accompanied by enhanced cleavage at position 32/33, similar to that seen with free DNA.

Fragment 70E,80E possesses two echinomycin sites which are positioned so as to be in the vicinity of the nucleosome dyad. As a result of their proximity to the dyad we can be less certain about their rotational setting, though they probably face toward the protein core. DNase I cleavage patterns for the interaction of echinomycin with this nucleosomal DNA are shown in the sixth panel of Figure 9. In this case the digestion pattern of the drug-free control is not the same as that of the other nucleosomal fragments, suggesting that these sequence changes have altered the way in which the DNA interacts with the nucleosome. This cleavage pattern is more even than that seen with other nucleosomal fragments and more closely resembles that of free DNA. Addition of echinomycin to this nucleosomal DNA produces footprints at the two ligand binding sites, suggesting that echinomycin can still bind to these sites. However, this result should be interpreted with caution in view of the altered cleavage pattern and will be considered further in the Discussion.

The final panel of Figure 9 shows DNase I digestion of nucleosomal 39E,50E,59E in the presence and absence of echinomycin. The digestion pattern for the drug-free DNA is similar to that seen with the other fragments, confirming that the target sites face toward the protein surface. In the presence of echinomycin reduced cleavage is seen around each of the target sites, and enhanced cleavage is evident at positions 33/34 as seen with fragment 39E,50E. The cleavage pattern above position 70 is not affected, demonstrating that echinomycin has not caused any long-range changes in the interaction of DNA with the protein core. At first sight these results suggest that echinomycin is able to bind to these inward-facing sites, without affecting the remainder of the nucleosome. However, the cleavage pattern between the potential binding sites resembles that of free DNA plus echinomycin, except that the band at position 55 in free DNA is not present in the nucleosomal DNA. It is therefore possible that this portion of the DNA fragment is no longer associated with the protein; this will be considered further in the Discussion.

The effects of echinomycin on nucleosome formation with these fragments is shown in Figure 4G−J. Nucleosome reconstitution with 20E,30E is only inhibited at relatively high ligand concentrations (20 μM and above), while 39E,50E and 70E,80E are inhibited at 10 μM ligand. In

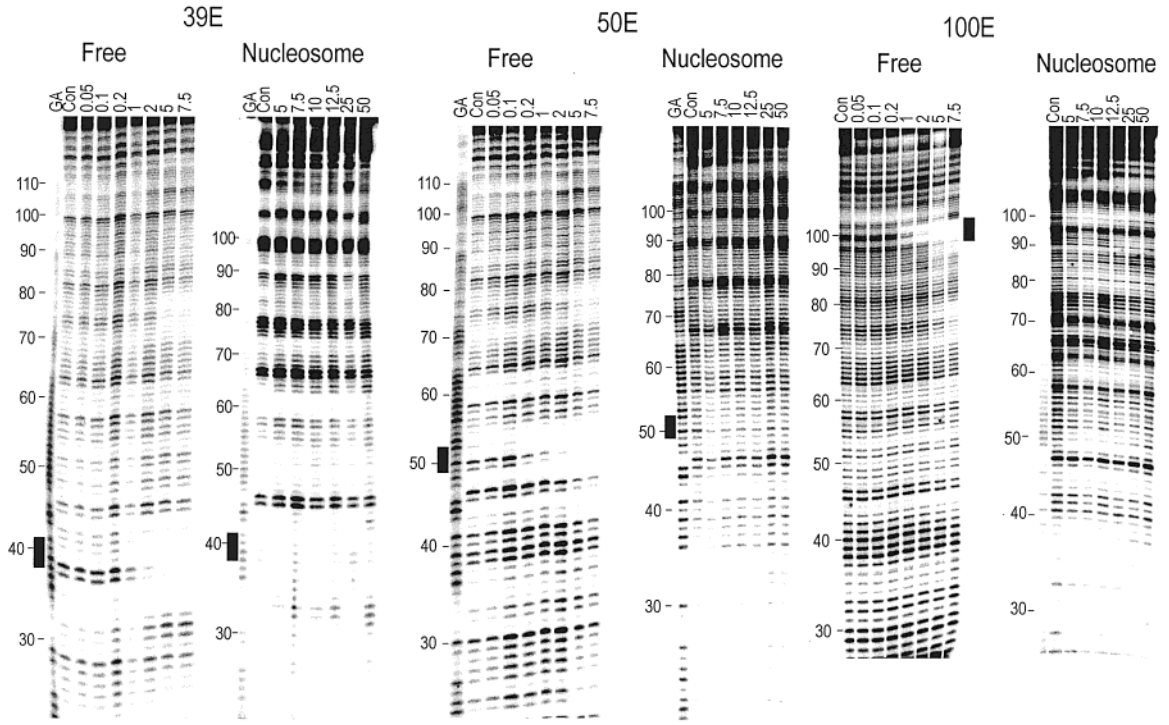

FIGURE 7: DNase I digestion of fragments containing single inward-facing echinomycin sites when free and incorporated into nucleosomes in the absence and presence of various concentrations of echinomycin. Ligand concentrations (micromolar) are shown at the top of each lane. Con indicates cleavage in the absence of added ligand. Tracks labeled GA are marker lanes specific for purines. The locations of the expected target sites are indicated by the filled boxes.

contrast, nucleosome formation with 39E,50E,59E is prevented even with 5 μM echinomycin.

DISCUSSION

This paper has systematically examined the binding of echinomycin and Hoechst 33258 to defined positions on nucleosomal DNA fragments. In general, we find that the minor groove binding ligand does not prevent nucleosome formation and that it can bind to single AT sites which face both toward and away from the protein without disrupting the nucleosome structure. The interaction of Hoechst 33258 with multiple inward-facing AT sites causes a proportion of the DNA molecules to adopt a different structure, consistent with a change in the rotational positioning of the DNA. In contrast, echinomycin prevents nucleosome formation and cannot bind to both inward- and outward-facing nucleosomal CpG sites. Addition of echinomycin to nucleosomal fragments which contain multiple CpG sites causes the DNA to dissociate from the protein.

A proper interpretation of the results presented in this paper requires that we know the rotational positioning of each of the DNA fragments. This was first determined for the *nobind* fragment by determining the regions of maximum and minimum cleavage by DNase I and hydroxyl radicals. This was confirmed by examination of the control lanes for the other nucleosomal fragments which revealed that they each produced similar DNase I digestion patterns with maxima and minima in the same positions. This is not surprising since these fragments only differ by a few bases (out of 150) and most of the changes are not at the nucleosome dyad, which is thought to play a major role in determining nucleosome assembly. We can therefore estimate the rotational position of each of the sites with reasonable confidence. The only exception is for fragment 70E,80E which possesses two inward-facing echinomycin sites close to the nucleosome dyad. In this fragment the sequences CTCT and GTGT in *nobind* are each replaced by ACGT. Although this retains the GC/AT content, the DNase I cleavage pattern of this nucleosomal fragment is different from the other fragments used in this work. This fragment is clearly bound to the nucleosomes as demonstrated by the band shifts and by the observation that the cleavage patterns of free and nucleosomal DNA are different. It is possible that this fragment does not adopt a unique rotational position.

Hoechst 33258. These results demonstrate that Hoechst 33258 can bind to both inward- and outward-facing AT sites and produce DNase I footprints. This is in contrast to previous studies with natural DNA fragments for which no Hoechst-induced footprints were observed, even though the ligand altered the digestion patterns (*32*−*35*). These results were interpreted by suggesting that the ligand-induced changes occurred at low levels of occupancy and that they might be induced by the binding of only one or two ligand molecules. Our results demonstrate that single Hoechst molecules can be accommodated within the nucleosomal DNA. The results for outward-facing sites may not be surprising since these sites should be readily available, and Hoechst is known to bind without causing any major changes in local DNA structure. The results also demonstrate that Hoechst can bind to inward-facing sites. This is more surprising since one would imagine that they should be occluded by interaction with the protein. However, this ligand fits snugly within the DNA minor groove, and there may be sufficient space for it to bind without affecting the interaction with the protein. These results are consistent with those of a recent study which showed that minor groove binding

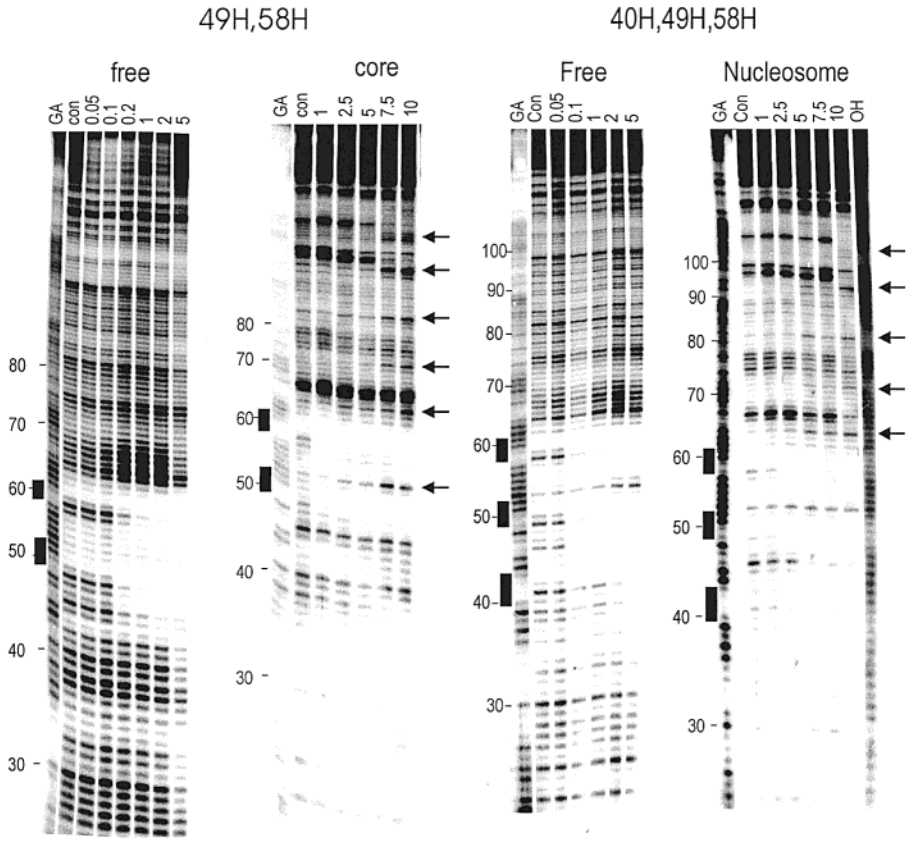

FIGURE 8: DNase I digestion of fragments containing multiple inward-facing binding sites for Hoechst 33258, when free and incorporated into nucleosomes, in the absence and presence of various concentrations of Hoechst 33258. Ligand concentrations (micromolar) are shown at the top of each lane. Con indicates cleavage in the absence of added ligand. Tracks labeled GA are marker lanes specific for purines. The locations of the expected target sites are indicated by the filled boxes. The arrows indicate the positions of enhanced cleavage which are produced in the presence of the ligand.

hairpin polyamides can successfully bind to both inward- and outward-facing sites (*40*). This still leaves the question of how Hoechst gains access to these inward-facing sites. We can suggest a number of mechanisms. First, it might slide along the minor groove, from regions which face outward until it locates its preferred binding site on the inward-facing surface. Alternatively, the nucleosome structure may be dynamic rather than static, and the ligand may bind to regions which transiently dissociate from the protein surface (*46*, *47*). In this case the ligand will bind to any site when it becomes available before the DNA reassociates with the protein surface, so long as ligand binding is rapid compared with the rate of reassociation. If the ligand is buried within the minor groove and does not alter the local structure, then this rebinding will be in the same orientation as previously. This process will be more likely for sites located toward the ends of the nucleosomal DNA than for those in the center.

In contrast to the results with single sites we find that Hoechst causes the appearance of new bands in the digestion of nucleosomal fragments which contain multiple inward-facing sites. We suggest that these arise from rotation of a proportion of the DNA molecules on the protein surface, as previously noted in experiments with natural (*32*−*35*) and synthetic (*37*) DNA fragments. In the original experiments with *tyr*T DNA it was suggested that rotation was caused by binding of the ligand to outward-facing sites, causing the superhelix to rotate through 180° on the surface of the histone core (*32*). The driving force for this conformational change was thought to be an increase in nonbonded interactions between the walls of the minor groove and the bound ligand, which would now be located on the histone-facing side of the superhelix and hence on the inner surface of the coil. In contrast, the results presented in this paper, together with those with synthetic DNA fragments (*37*), show that the changes are caused by binding of the ligand to inward-facing sites, which subsequently turn to face out.

How might the actual process of rotation occur? The information gained from the crystal structure of the nucleosome suggests that the DNA superhelix is unlikely to rotate across the surface of the histone core. Such a process would involve the simultaneous breaking and re-forming of many salt linkages, and it is unlikely that the binding of Hoechst would provide sufficient energy for this mechanism. It seems more likely that the DNA is transiently displaced from the surface of the protein and redocks in a new rotational position. It is not clear whether this occurs by wholesale displacement of the DNA or by local displacement and redocking, which may be subsequently propagated around the structure. Target sites further away from the nucleosome dyad will be exposed more often than those close to the center (*46*). Therefore, we might imagine that AAAA across positions 39−42 bp (in construct 40H,49H,58H) will be more readily occupied than the AATT sites at positions 49−52 and 58−61 bp. This model suggests that binding to this AAAA will increase the binding to AATT (49−52 bp) which in turn will increase the binding to AATT (58−52 bp). These successive interactions will be cooperative: the binding of

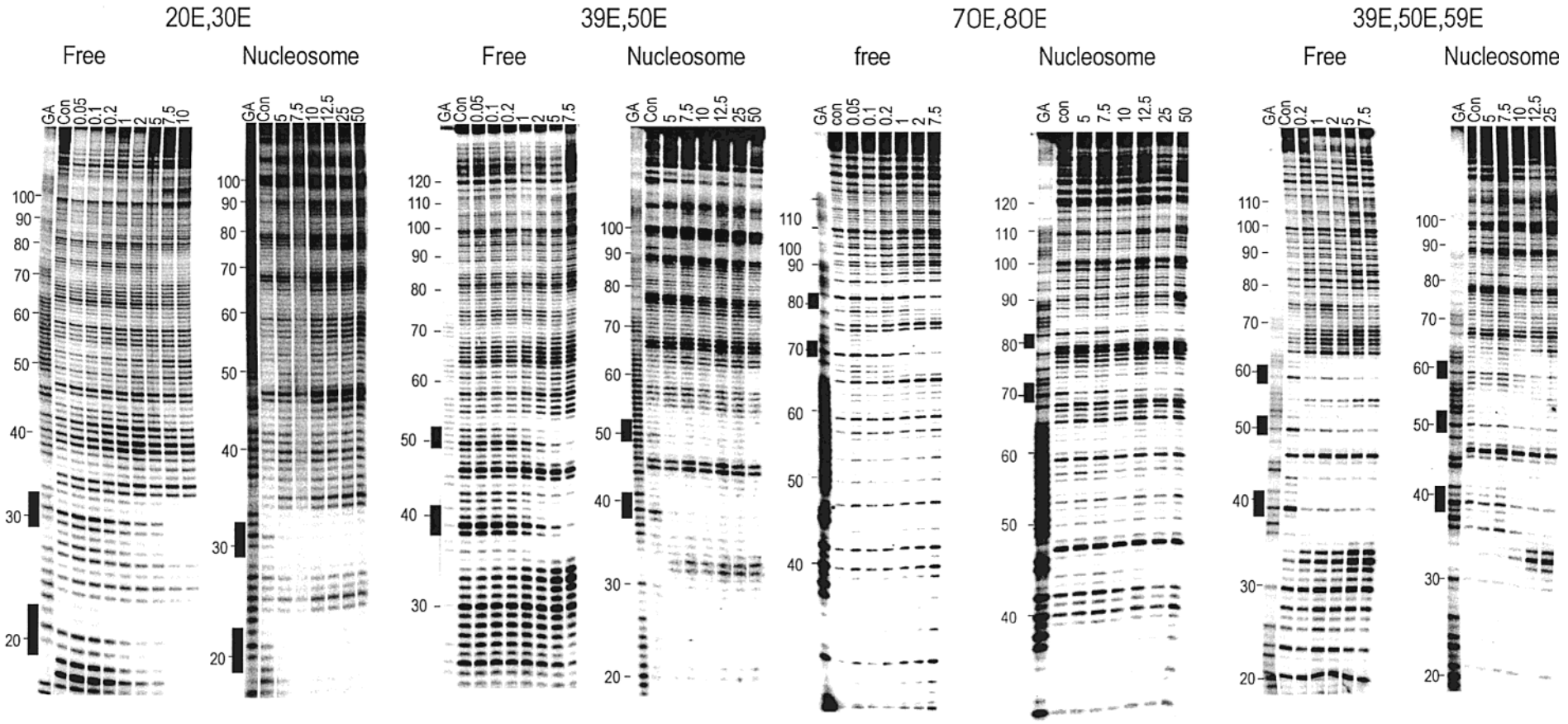

FIGURE 9: DNase I digestion of fragments containing multiple inward-facing echinomycin sites when free and incorporated into nucleosomes in the absence and presence of various concentrations of echinomycin. Ligand concentrations (micromolar) are shown at the top of each lane. Con indicates cleavage in the absence of added ligand. Tracks labeled GA are marker lanes specific for purines. The locations of the expected target sites are indicated by the filled boxes.

one Hoechst molecule will increase the binding of a second molecule and so on. Since Hoechst 33258 causes this fragment to rotate on the nucleosome surface, there must be some unfavorable interaction between the bound ligands and the surface of the histone octamer. This is unlikely to be a steric effect, as discussed above, but might arise from repulsion between the positively charged drug and amino acid side chains.

Echinomycin. In contrast to the results with Hoechst, echinomycin does not produce DNase I footprints at isolated nucleosomal ACGT sites, showing no binding to both inward- and outward-facing sites. The absence of any clear footprints is similar to that shown in previous experiments with the *tyr*T DNA fragment, for which changes in the cleavage pattern were not accompanied by footprints. The difference between echinomycin and Hoechst 33258 can be explained by their different modes of binding. Whereas Hoechst causes few changes to the DNA structure, echinomycin both unwinds and extends the DNA helix. The helix extension would be expected to alter the local DNA phasing by the equivalent of two base pairs. This clearly prevents the drug from binding to preformed nucleosomes. In addition, efficient intercalation must require good stacking between the chromophore and the base pairs. This will not be possible for curved nucleosomal DNA since the peptide backbone of the ligand restricts the movement of the chromophores.

Although echinomycin does not produce footprints on nucleosomal fragments which contain single binding sites, clear footprints are seen with the fragments containing two or more binding sites. However, it should be noted that these footprints are not accompanied by changes in the remainder of the fragments. It therefore appears that, in contrast to previous work with natural DNA (*35*) and the results with multiple Hoechst sites, echinomycin does not cause any changes in the DNA rotational position. The appearance of nucleosomal footprints on these fragments containing multiple echinomycin binding sites is harder to explain. One possibility is that the ligand is able to bind to sites which are located toward the edge of the nucleosomal DNA, as they are transiently exposed. These drug-bound sites would then be unable to rebind to the protein surface and so will enhance the binding to other sites which are located closer to the dyad. The drug-bound DNA will therefore be dangling in free solution with the remainder of the fragment wrapped around the protein in its usual fashion. This explanation may be reasonable for fragment 20E,30E. However, it does not adequately explain the results for fragments 39E,50E and 39E,50E,59E. Looking at the patterns for 39E,50E, it can be seen that there is good DNase I cleavage around positions 24−30 in the drug-bound free DNA. We would expect a similar cleavage pattern in the drug-treated nucleosome DNA if echinomycin binding to sites 39 and 50 had promoted dissociation of this end of the fragment from the protein. However, this is still a region of poor cleavage in the drug-treated nucleosomal DNA, suggesting that it is still bound to the protein with the minor groove in this region facing the protein surface. A similar effect is seen toward the bottom of the gel with fragment 39E,50E,59E, in which the patterns of free and nucleosomal DNA are very different in the presence of echinomycin. It therefore appears that these fragments are still incorporated into nucleosomes, even though they contain bound drug molecules.

Echinomycin also prevents nucleosome formation when added during the reconstitution process. Echinomycin−DNA complexes are known to dissociate slowly (*48*) ($t_{1/2}$ up to 30 min), and the drug will remain bound to the DNA during the reconstitution process. It seems likely that the rigid ligand−DNA complex is unable to wrap around the protein. The inability of echinomycin to target nucleosomal DNA may mean that it will have little effect on inactive chromatin, which is highly condensed and inaccessible to intercalation. In contrast, it may have a greater effect on active chromatin, in which the DNA may transiently dissociate from the protein. In these regions echinomycin will prevent nucleosome reassembly.

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