Modulation of Progesterone Receptor Binding to Progesterone Response Elements by Positioned Nucleosomes†

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ABSTRACT: In cells, steroid hormone receptors interact with target enhancer elements on nucleosomes to regulate transcription of genes. To elucidate how nucleosomes can potentially regulate the interactions of steroid receptors with steroid response elements, we have examined the effects of nucleosome positioning and histone source on the binding of the progesterone receptor to DNA elements on nucleosomes reconstituted in vitro. We find that the affinity of the receptor for its response element is dependent on the position of the element within the nucleosome, but not on the histone source, active or inactive chromatin. Our results suggest that the strength of DNA–histone interactions within the nucleosome modulates the binding of progesterone receptor to response elements. Thus, nucleosome positioning is likely to influence the function of steroid receptors in vivo.

Regulation of gene transcription is mediated by protein factors which bind to specific DNA sequences often located in the proximity of target genes. The interactions of many of these factors with DNA sequences have been characterized in vitro [for reviews, see Mitchell and Tjian (1989) and Johnson and McKnight (1989)]. In cells, however, DNA does not exist free in solution but rather is associated with basic histone proteins which organize the DNA into chromatin. The basic unit of chromatin is the nucleosome, a particle consisting of an octamer of core histone proteins and at least 145 base pairs of DNA [reviewed by van Holde (1989)]. Since virtually the entire eukaryotic genome is packaged into nucleosome arrays, it seems likely that while nucleosomes may exclude most transcription factors from the DNA, others must have access to a nucleosomal DNA template.

There is some in vivo evidence that suggests that this is the case. In the uninduced state, an array of positioned nucleosomes has been observed over the hormone-responsive mouse mammary tumor virus (MMTV)[1] promoter (Richard-Foy & Hager, 1987). Upon glucocorticoid induction, a specific nucleosome residing over sequences containing glucocorticoid-responsive elements (GREs) is thought to be disrupted or altered, as indicated by the appearance of a nuclease-hypersensitive site over the region. The effects of glucocorticoid are mediated by its cognate receptor, a transcription factor that requires hormone to activate its DNA binding and transactivation functions [for reviews, see Beato (1989) and O'Malley (1990)]. Since the MMTV GREs exist within a nucleosome prior to hormonal induction, the receptor would appear to have to interact with nucleosomal DNA to affect its biological functions. In contrast, the transcription factor NF-1, a constitutive factor required for MMTV induction, could be observed to bind to the MMTV promoter only after nucleosome disruption (Cordingley et al., 1987). As changes in chromatin structure are hallmarks of steroid-induced transcription (Burch & Weintraub, 1983; Kaye et al., 1986; Hecht et al., 1988; Strahle et al., 1989; Pham et al., 1991), it would appear that the modulation of transcription factor access is not a phenomenon unique to the MMTV promoter.

In vitro, the glucocorticoid receptor appears to have some affinity for MMTV GREs on nucleosomes reconstituted with purified histones. The GR has been found to bind to this nucleosomal DNA, apparently with lower affinity compared to free DNA, but does not produce significant changes in nucleosome structure (Perlmann & Wrange, 1988; Pina et al., 1990; Archer et al., 1991). If the path of DNA on the nucleosome must be altered to facilitate its interaction with hormone receptors, one might expect that receptor–nucleosome interactions would be strongly influenced by the relative stability of the nucleosome. This, in turn, is determined by the modifications of the histones and by the sequence composition of the DNA within the nucleosome. It is known, for example, that nucleosomes composed of synthetic repeating sequences of the form $(A/T)_3nn(G/C)_3nn$ are far more stable than a nucleosome reconstituted over a well-characterized natural nucleosome positioning sequence (Schrader & Crothers, 1989). These aspects of chromatin structure may modulate the function of steroid receptors in vivo.

To examine further how chromatin organization affects the binding of hormone receptors to response elements, we have characterized the interactions of the chicken progesterone receptor (PR) with progesterone response elements on nucleosomes reconstituted in vitro. A potential weakness of most reconstitution studies of this nature is that bulk histones of mammalian or avian origin are often used. As these histones originate primarily from repressed chromatin, they lack the modifications characteristic of transcriptionally active/competent chromatin, such as acetylation [see Van Holde (1989) and Oliva et al. (1990) and references cited therein], which influence nucleosome structure and stability. To examine whether these differences are significant, we have reconstituted nucleosomes using histones from chicken erythrocyte and yeast. Yeast is an appropriate source of active histones since virtually the entire yeast genome is thought to be transcriptionally competent (Lohr & Hereford, 1979), and, furthermore, steroid hormone receptors can function in yeast (Metzger et al., 1988; Mak et al., 1989; Pham et al., 1991a). In this study, we have also examined how changing nucleosome positioning with respect to PREs affects the binding of PR. Our results suggest

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[1] Abbreviations: PR, progesterone receptor; PRE, progesterone-responsive element; GR, glucocorticoid receptor; GRE, glucocorticoid-responsive element; MMTV, mouse mammary tumor virus.

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that the affinity of the receptor for its response element is modulated by its position within the nucleosome but is not dependent on the histone source, active or inactive chromatin.

Experimental Procedures

Preparation of Chicken Erythrocyte Chromatin. Nuclei were prepared from chicken erythrocytes using standard procedures (Bates et al., 1981). Soluble chromatin was obtained by digesting nuclei with micrococcal nuclease (Boehringer Mannheim) at 80 units/mL for 15 min. The nuclei were then lysed by resuspending in 0.2 mM EDTA, pH 7.5, and the insoluble material was pelleted at 8000*g* for 10 min. To remove H1, H5, and non-histone proteins, the soluble chromatin was adjusted to 0.6 M NaCl and chromatographed over a Sephacryl S-300 column (Pharmacia) in buffer containing 10 mM Tris-HCl (pH 7.5)/0.6 M NaCl/0.2 mM EDTA. The flow-through fractions containing the stripped chromatin were collected and stored in 20% glycerol at −70 °C.

Preparation of Yeast Chromatin. Chromatin was prepared from a protease-deficient yeast strain (BJ3505), kindly provided by E. W. Jones (Carnegie-Mellon University). Yeast cells were grown to mid to late-log phase in YEPD medium (Gibco BRL) at 30 °C. The cells were then harvested, washed twice in cold distilled water, and then washed again in homogenization buffer [10 mM NaPipes (pH 6.5)/400 mM NaCl/2 mM spermine/0.25 mM EDTA/0.25 mM EGTA/1 mM $MgCl_2$]. Yeast cells were lysed in homogenization buffer using a cell homogenizer (B. Braun Melsungen AG) between 0 and 4 °C. We preferred this method of lysis over methods that require spheroplast formation because of potential problems with proteases in commercial preparations of lytic enzymes. Spermine was included in the lysis and washing buffers to inhibit the yeast deacetylase; the yeast enzyme is not inhibited by butyrate but is instead inhibited by spermine and spermidine (Vu et al., 1987). After lysis, the nuclear fraction was collected by centrifugation at 2000*g*. The pellet was washed once in homogenization buffer, and once in buffer containing 10 mM NaPipes (pH 6.5)/0.25 M sucrose/1 mM $MgCl_2$/2 mM spermine/0.25 mM EDTA/0.25 mM EGTA/0.5% Triton X-100. Prior to micrococcal nuclease digestion, the pellet was washed in digestion buffer [15 mM Tris-HCl (pH 7.5)/50 mM NaCl/1.4 mM $CaCl_2$/0.2 mM EDTA/2 mM spermine/5 mM β-mercaptoethanol]. Micrococcal nuclease digestion was performed at a nuclease concentration of 100 units/mL for 5 min at 37 °C and stopped by the addition of EDTA. The insoluble material was pelleted, and the supernatant containing soluble chromatin was collected. After the NaCl concentration was adjusted to 0.45 M, the soluble chromatin was chromatographed over a Sephacryl S-300 column in buffer containing 10 mM Tris-HCl (pH 7.5)/0.45 M NaCl/0.2 mM β-mercaptoethanol/0.2 mM EDTA. The fractions containing the stripped chromatin were collected and concentrated to about 400 μg/mL by using dialysis tubing with repeated applications of Sephadex G-200. The concentrated chromatin was stored in 20% glycerol at −70 °C.

Labeling of DNA. The DNA fragments were end-labeled by phosphorylation with polynucleotide kinase using conventional procedures (Sambrook et al., 1989). Labeled DNA fragments were purified as described previously (Bradshaw et al., 1988a).

Nucleosome Reconstitution. Nucleosomes were reconstituted by exchange of histones from native chromatin in high salt (Rhodes, 1985; Drew & Travers, 1985). The labeled DNA fragment was incubated with yeast or chicken erythrocyte chromatin, at a chromatin concentration of approximately 200 μg/mL, in 20 mM Tris-HCl (pH 7.5)/1 M NaCl/0.2 mM EDTA/0.2 mM β-mercaptoethanol/0.1 mg/mL bovine serum albumin. After a 2-h incubation at room temperature, the salt concentration was lowered sequentially by dilution to 0.8, 0.6, 0.45, and finally 0.3 M NaCl, with incubations of 30 min at room temperature for each dilution. To separate free DNA and native chromatin from the reconstituted nucleosome, the sample was applied onto a gradient of 5–30% sucrose in 50 mM Tris-HCl (pH 7.5)/1 mM EDTA/0.1 mg/mL bovine serum albumin and centrifuged at 35000 rpm in an SW50.1 rotor at 4 °C for 16 h.

Purification of Chicken Progesterone Receptor. PR type A was purified from chick oviduct essentially as described by Rodriguez et al. (1989). The only significant deviation from this protocol was that PR was eluted from the final DNA–cellulose column in 20 mM Tris-HCl (pH 7.5)/300 mM NaCl/0.2 mM EDTA/10% glycerol/2.5 mM dithiothreitol. By silver staining of SDS gels, the receptor preparations appeared to be between 30 and 50% pure.

Gel Mobility Shift Analysis. PR binding was assayed by incubating PR with the labeled DNA fragment or the reconstituted nucleosomes (sucrose gradient purified) in 90 mM NaCl/10 mM Tris-HCl (Ph 7.5)/2.5 mM $MgCl_2$/2.5 mM dithiothreitol/0.5 mM EDTA/100 μg/mL bovine serum albumin/5% sucrose for 20 min at room temperature. PR complexes were then separated from the unbound probe on a 4% gel (60:1 ratio of monomer to bis) in 0.5× standard Tris–borate/EDTA buffer (Sambrook et al., 1989).

DNase I Footprint Analysis. Reconstituted nucleosomes purified on sucrose gradients were digested at room temperature for 30 s to 1 min with various concentrations of DNase I (as noted in the figure legends; DNase I is from Worthington, approximately 2000 units/mg). The digestion buffer used is 10 mM Tris-HCl (pH 7.5)/50 mM NaCl/7.5 mM $MgCl_2$/2.5 mM DTT/0.5 mM EDTA/100 μg/mL bovine serum albumin/10 ng of poly(dI-dC). The DNA was purified as described (Bradshaw et al., 1988a) and analyzed on a 6% sequencing gel. To analyze PR binding, the reconstituted nucleosome or labeled free DNA was first incubated with PR under the same conditions as described above for 15 min at room temperature and then digested with DNase I.

Exonuclease III Analysis. Reconstituted nucleosomes purified on sucrose gradients were digested with exonuclease III as described (Ramsay, 1986; Linxweiler & Horz, 1984).

Results

In Vitro Nucleosome Assembly on Short DNA Fragments Containing the Progesterone-Responsive Element. To obtain nucleosomes containing the progesterone-responsive element (PRE), we have assembled nucleosomes in vitro on short fragments of DNA carrying PRE sequences. These DNA fragments were excised from the plasma pOVCAT-50PRE2(+) (Bradshaw et al., 1988b), which contains ovalbumin minimal promoter sequences and two copies of the PRE. We have utilized in this study two overlapping fragments in which the PREs are placed at different distances relative to the ends (Figure 1A). These fragments are of sufficient length to accommodate only one nucleosome core particle.

Nucleosomes are assembled on these fragments by exchange of histone octamers from native chromatin under high salt, using a standard procedure (Rhodes, 1985; Drew & Travers, 1985). In this study, we have used chromatin obtained from two sources, chicken erythrocyte and yeast. These two cell types represent extremes in terms of transcriptional activity. Chicken erythrocyte chromatin is inactive and is a standard

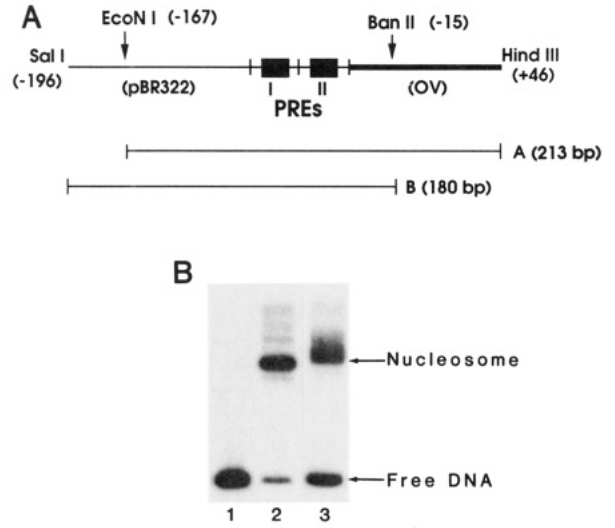

FIGURE 1: Nucleosome reconstitution on short DNA fragments using chicken erythrocyte and yeast histones. (A) DNA fragments used in reconstitution experiments. The two fragments used (labeled A and B) were excised from a plasmid containing two copies of the PRE by using appropriate restriction sites as shown. The fragments contain sequences originating from pBR322, synthetic oligonucleotides containing a PRE, and ovalbumin promoter sequences. (B) Gel mobility shift analysis of the products obtained after nucleosome reconstitution. Fragment A was reconstituted as described under Experimental Procedures and then electrophoresed on a nondenaturing polyacrylamide gel. Shown are the free DNA control (lane 1), reconstitutes containing chicken erythrocyte histones (lane 2), and reconstitutes containing yeast histones (lane 3). This analysis only illustrates in a qualitative sense the complexes formed; it does not reflect differences in efficiency of reconstitution between yeast and chicken erythrocyte histones.

source of histones used in reconstitution studies. In contrast, virtually the entire yeast genome is transcriptionally poised and DNase I sensitive (Lohr & Hereford, 1979), and furthermore, yeast nucleosomes are thought to be less stable than those of higher eukaryotes (Morse et al., 1987; Lee et al., 1982). Chromatin preparations used in reconstitution experiments have been stripped of H1 and other chromosomal proteins, and show no detectable proteolysis (data not shown). As shown in Figure 1B, fragment A subject to exchange reconstitution yields a major species with lower mobility on nodenaturing polyacrylamide gels. Interestingly, the complex containing yeast histones (Figure 1B, lane 3) has slightly lower mobility compared to the complex of chicken erythrocyte histones, consistent with the idea that yeast histones are more loosely organized.

Characterization of Fragment A Reconstituted Nucleosomes. In the initial experiments, we focused on using fragment A in reconstitution studies, as the PREs are centered within this fragment. To examine whether the predominant reconstitution product shown in Figure 1 has characteristics similar to native nucleosomes, the structure of the reconstituted complex was analyzed by nuclease digestion. In this and subsequent analyses, the reconstituted nucleosomes have been purified by sucrose gradient sedimentation. DNase I digestion analysis of the reconstituted nucleosomes (Figure 2A) reveals that one end of the fragment, the upstream portion, is highly DNase I sensitive and is separated by a distinct boundary (at −100) from the rest of the fragment, which is relatively protected. The protected portion is characterized by a periodic pattern of DNase I cleavage separated by approximately 10 base pairs. These results suggest the presence of a positioned nucleosome over the downstream portion of the fragment.

Nucleosomes reconstituted with yeast histones appear to be positioned identically compared to that obtained with chicken erythrocyte histones. Most of the major DNase I cleavage sites are clearly present in both reconstitutes. However, there are definite subtle differences. Cleavage sites at positions −29, −48, and −58 are enhanced in the yeast nucleosome, whereas cleavages at positions −82 and +3 are reduced. Furthermore, position −28 is cleaved by DNase I with yeast nucleosome but not with chicken erythrocyte nucleosomes. Overall, the yeast reconstituted nucleosome consistently shows a less distinct nucleosome ladder compared to the chicken erythrocyte nucleosome.

To characterize more precisely the boundaries of the core particle on the DNA fragment, we performed exonuclease III analysis. The exonuclease digests 3′ to 5′ from the ends of the DNA, but is strongly inhibited by bound proteins. By analyzing the positions of the exonuclease stops, the boundaries of the core particle could be determined. Figure 2B shows an analysis of the upstream boundary of the nucleosome core. The 5′ end of the bottom strand was labeled and reconstituted as before, and the reconstituted nucleosomes were digested with exonuclease III for different time intervals. The first exonuclease stop occurs at position −99 for both yeast and chicken erythrocyte nucleosomes, suggesting that this is the core particle upstream boundary. Further digestion gives rise to a second stop, more stable than the first, and a third stop could be seen at the longest digestion times. We believe that the second and third stops represent exonuclease digestion through the nucleosomal DNA, pausing at 10 bp intervals where the particular DNA strand comes into closest contact with the histone octamer. Nucleosome positioning heterogeneity is unlikely because the later stops appear upon continued exonuclease digestion, with concomitant disappearance of the first stop.

By performing similar analysis with the same fragment labeled on the top strand, it is possible to map the downstream boundary of the reconstituted nucleosome. As shown in Figure 2C, this boundary occurs near or at the downstream end of the fragment for the chicken erythrocyte reconstituted nucleosome, as indicated by the strong exonuclease stop. Similar results were obtained with the yeast reconstituted nucleosome (data not shown). Thus, the nucleosome is positioned over the downstream portion of the fragment, occupying approximately 145 bp of DNA.

Important to any consideration of interactions between transcription factors and DNA sequences on nucleosomes is the orientation of the DNA binding site with respect to the histone octamer. Because of the external path of the DNA around the histone octamer, protein contact sites that face outward in the nucleosome will be much more accessible to transcription factors compared to those that face inward toward the center of the nucleosome. To determine the orientation of the PRE in the reconstituted nucleosomes, we have analyzed the positions of the DNase I cleavage sites within the nucleosome. In the nucleosome, DNase I is known to cleave DNA where the DNA minor groove faces away from the histone octamer. Figure 3A shows a high-resolution analysis of the DNase I cleavage sites in the reconstituted nucleosome. Shown are the data for nucleosome reconstituted with chicken erythrocyte histones; the major cleavage sites are positioned identically in the nucleosome reconstituted with yeast histones. Analyses of the top and bottom strands reveal periodic cleavage sites that are staggered by approximately 3 or 4 base pairs.

A schematic of the translational and rotational positioning of the nucleosome on the fragment is shown in Figure 3B. The upper part of the panel shows the translational positioning as

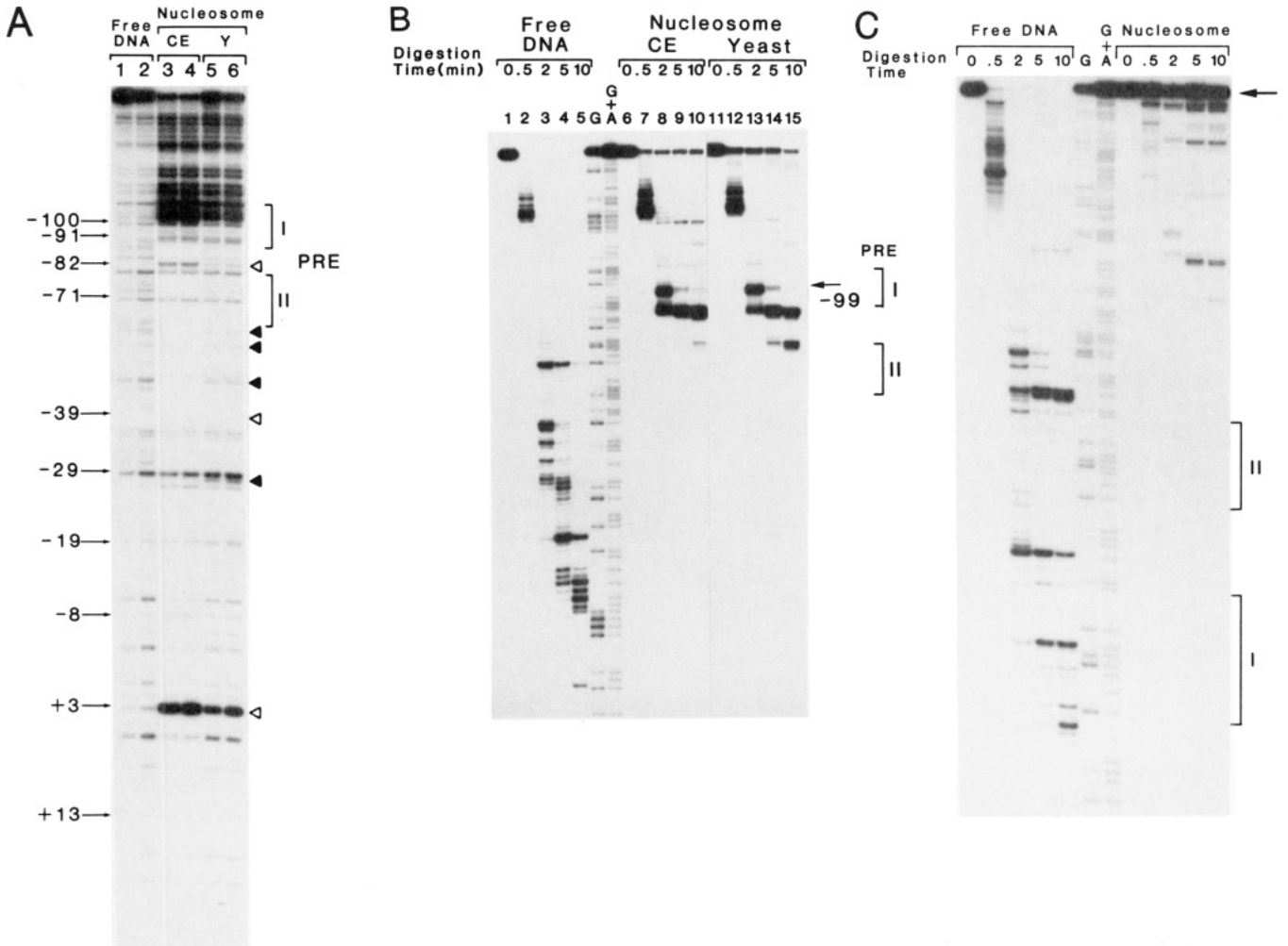

FIGURE 2: Nuclease digestion analyses of nucleosomes reconstituted on fragment A. (A) DNase I digestion analysis. The bottom strand of the DNA was labeled, reconstituted with chicken erythrocyte or yeast histones, and then subjected to digestion with DNase I. The samples were digested with 2 μg/mL (lanes 1, 2,) or 3 μg/mL (lanes 3, 6) for 25 s (lane 1), 45 s (lane 2), 1.5 min (lanes 3, 5), or 2.5 min (lanes 4, 6). The arrows on the left show the positions of the more distinct periodic cleavage sites within the nucleosome core. Open arrowheads on the right denote sites that are digested less readily in the yeast nucleosome compared to the chicken erythrocyte nucleosome. Filled arrowheads denote sites that are enhanced in the yeast nucleosome. The positions of the PREs are marked by brackets, and labeled I and II as shown in Figure 1A. (B) Exonuclease III digestion analysis of the upstream boundary of the nucleosome. Reconstituted nucleosomes labeled on the bottom strand (lanes 6–10 for chicken erythrocyte; lanes 11–15 for yeast) or control free DNA was digested with exonuclease III for the different time intervals as shown. The DNA was then purified and electrophoresed on a sequencing gel. The first visible stop site (at −99) is indicated by the arrow. The locations of the PREs are marked by brackets. (C) Exonuclease III digestion analysis of the downstream boundary of the nucleosome. Fragment A labeled on the top strand was reconstituted with chicken erythrocyte histones and then digested with exonuclease III for different time intervals as shown. The arrow denotes the prominent exonuclease III stop near or at the downstream end of the fragment. The brackets denote the locations of the PREs.

deduced from exonuclease III and DNase I analyses. The lower part of the panel illustrates the orientation of the PREs with respect to the histone octamer. The DNase I minor groove cleavage sites within the reconstituted nucleosome (determined from the data shown in Figure 3A and shown as arrowheads on the sequence) define the accessible DNA surface (shown facing the reader). This surface is presumably oriented outward, away from the histone octamer. The purine contacts in the major groove, known to be important for PR binding (Tsai et al., 1988; Chalepakis et al., 1988), appear to be positioned on the same DNA surface, the same surface accessible to DNase I. These data suggest that the PR contact sites within the major groove are facing outward, and could potentially accommodate PR binding within the major groove.

Binding of PR to Yeast and Chicken Erythrocyte Nucleosomes Reconstituted on Fragment A. To characterize the binding of chicken progesterone receptor to the nucleosome assembled on fragment A, we utilized gel mobility shift and DNase I footprint analyses. Nucleosomes were reconstituted as described in the preceding sections and separated from residual free DNA by sucrose gradient sedimentation. Mobility shift assays suggest that there is some binding to reconstituted nucleosomes (Figure 4A). Binding of PR to free DNA results in two complexes, II and IV. Complex II forms at lower concentrations of receptor and has been shown in previous work to result from the binding of a receptor dimer to one of two PREs on the fragment (Tsai et al., 1989). Complex IV results from occupation of both elements. The binding of PR to the PREs appears to be cooperative, consistent with previous results (Tsai et al., 1989). When PR is added to reconstituted nucleosomes, a distinct complex (II′) was formed having a mobility slightly slower than complex II. This complex was observed for both yeast and chicken erythrocyte reconstituted nucleosomes. The mobility of this complex is consistent with the binding of one receptor dimer to the nucleosome. However, a complex reflecting the full occupation of both elements (IV′) is formed extremely inefficiently. This suggests that in the reconstituted nucleosome, one element has much higher affinity for PR compared to the other. Note that PR appears to bind in the same manner to

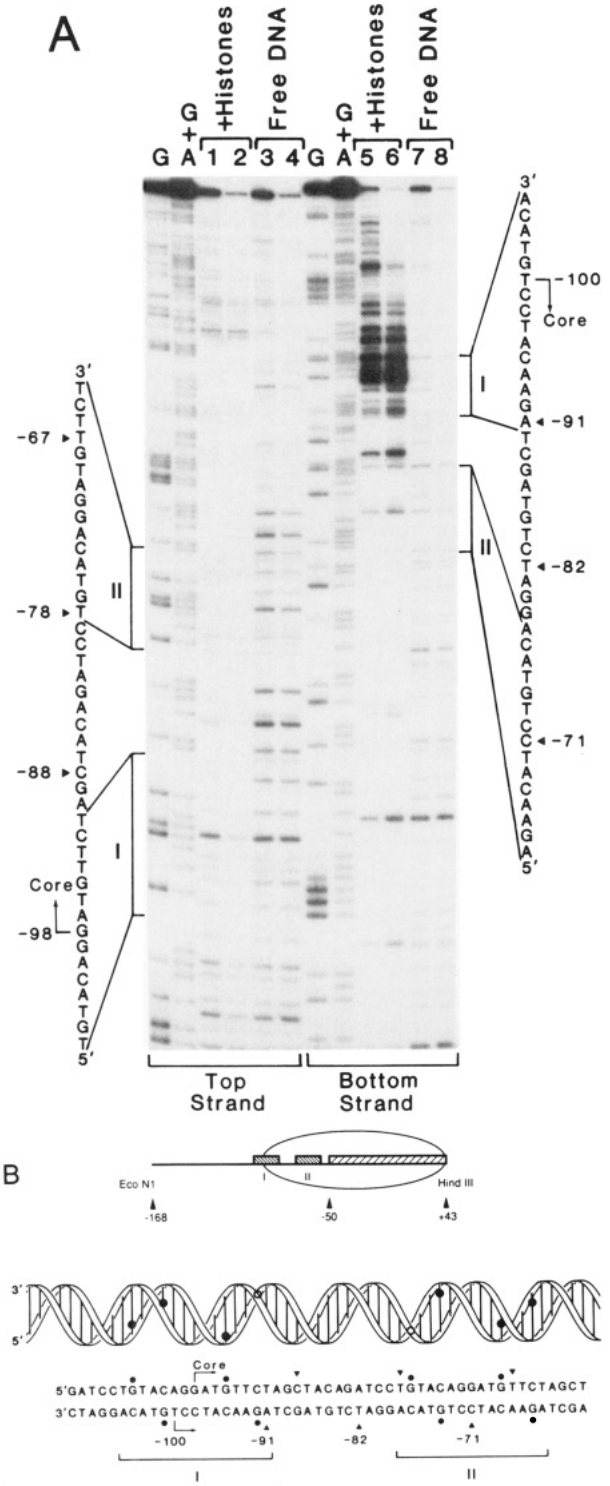

FIGURE 3: Position and orientation of the PREs within the nucleosome reconstituted on fragment A. (A) Higher resolution DNase I analysis of nucleosomes reconstituted with chicken erythrocyte histones. The nucleotides within the PREs most sensitive to DNase I are marked by arrowheads. The locations of PREs I and II are marked by brackets. The digestion conditions used are as follows: 0.75 μg/mL DNase I (lanes 1, 7), 1.5 μg/mL (lanes 2, 8), 1.1 μg/mL (lanes 3, 5), 2.3 μg/mL (lanes 4, 6) for 25 s (lanes 1, 2, 7, 8) or 2.5 min (lanes 3, 4–6). (B) Schematic diagram showing the positions and orientation of the nucleosome core. The upper part of this panel depicts the translational positioning. The orientation of the PREs on the nucleosome is shown in the lower part of the panel. This drawing depicts the positions of the purine contact sites important for receptor binding (shown as circles) on the DNA double helix relative to the DNase I minor groove cleavage sites in reconstituted nucleosomes (shown as arrowheads on the sequence). The DNase I cutting sites mark the DNA surface accessible to the nuclease; this accessible surface is shown facing the reader.

chicken erythrocyte and to yeast nucleosomes, and with similar affinities.

To demonstrate that it is the receptor, and not a minor contaminant, that is binding to the nucleosome, we added an antibody to the binding reaction to attempt to supershift the putative receptor complex (Figure 4B). As expected, the complex II′ can be further shifted by a specific antibody (PR22), but not by a control antibody (PR6). This proves that PR is present in the complex.

DNase I footprint analysis gives further evidence of specific PR binding to reconstituted nucleosomes (Figure 4C). In the presence of PR, footprints are visible both on the free DNA as well as on the reconstituted nucleosomes. PR footprints both PREs I and II on free DNA. On reconstituted nucleosomes, the footprints are weaker, requiring more PR, and seem to be confined to PRE I. The footprint pattern does not appear to be significantly different between the yeast and chicken erythrocyte reconstitutes (compare lanes 3–6 and 7–10), suggesting that the use of yeast histones does not facilitate PR binding. Clearly, the receptor has much higher affinity for PRE I compared to PRE II in reconstituted nucleosomes, although the PR binding surface of both PREs appears to be facing outward. The much higher affinity of PR for PRE I may be a consequence of its location on the edge of the nucleosome. Note that no change in nucleosome structure accompanies PR binding, as indicated by the absence of altered DNase I sensitivity over the rest of the nucleosomal DNA.

Altered Nucleosome Positioning on Fragment B. The results decribed above suggest that the binding of PR to PRE sequences is greatly decreased by nucleosome formation, except when the sequence is on the edge of the nucleosome. This seems to contradict other data that suggest that the receptor has relatively high affinity for nucleosomal DNA (Pina et al., 1990). Possible explanations for this discrepancy include different positioning of PREs within the nucleosome, and different stabilities of nucleosomes formed over different DNA sequences. To evaluate these possibilities, additional experiments were performed using a different DNA fragment (fragment B as shown in Figure 1A), which overlaps with fragment A. Fragment B is mostly contained within fragment A but lacks the downstream portion of fragment A over which most of the nucleosome occupies. Since the nucleosome reconstituted on fragment A occupies a single translational and rotational position, this position must result in a considerably more stable nucleosome compared to alternative positions on the DNA fragment. By removing a large portion of the nucleosomal DNA of fragment A, we hope to shift the nucleosome to a different position, one that should be less stable.

To characterize the nucleosome reconstituted on fragment B, we again performed DNase I and exonuclease III analyses. Since reconstituted yeast and chicken erythrocyte nucleosomes appeared to be highly similar, in the following experiments only chicken erythrocyte histones were used for reconstitution. As shown in Figure 5A, DNase I analysis indicates that the nucleosome is positioned, at least rotationally, and occupies much of the DNA upstream of the PREs. The 10 bp DNase I repeat pattern characteristic of nucleosomal DNA is visible, although this is not pronounced. The lack of a strong 10 bp cleavage ladder is a likely result of the sequence-specific cleavage properties of DNase I. Some of the more distinct DNase I cleavage sites on nucleosomal DNA are indicated by arrows in Figure 5A. It should be noted that there is considerable protection of the DNA by histones since a 4-fold greater level of digestion was utilized for the nucleosomal samples compared to free DNA. However, it is difficult to

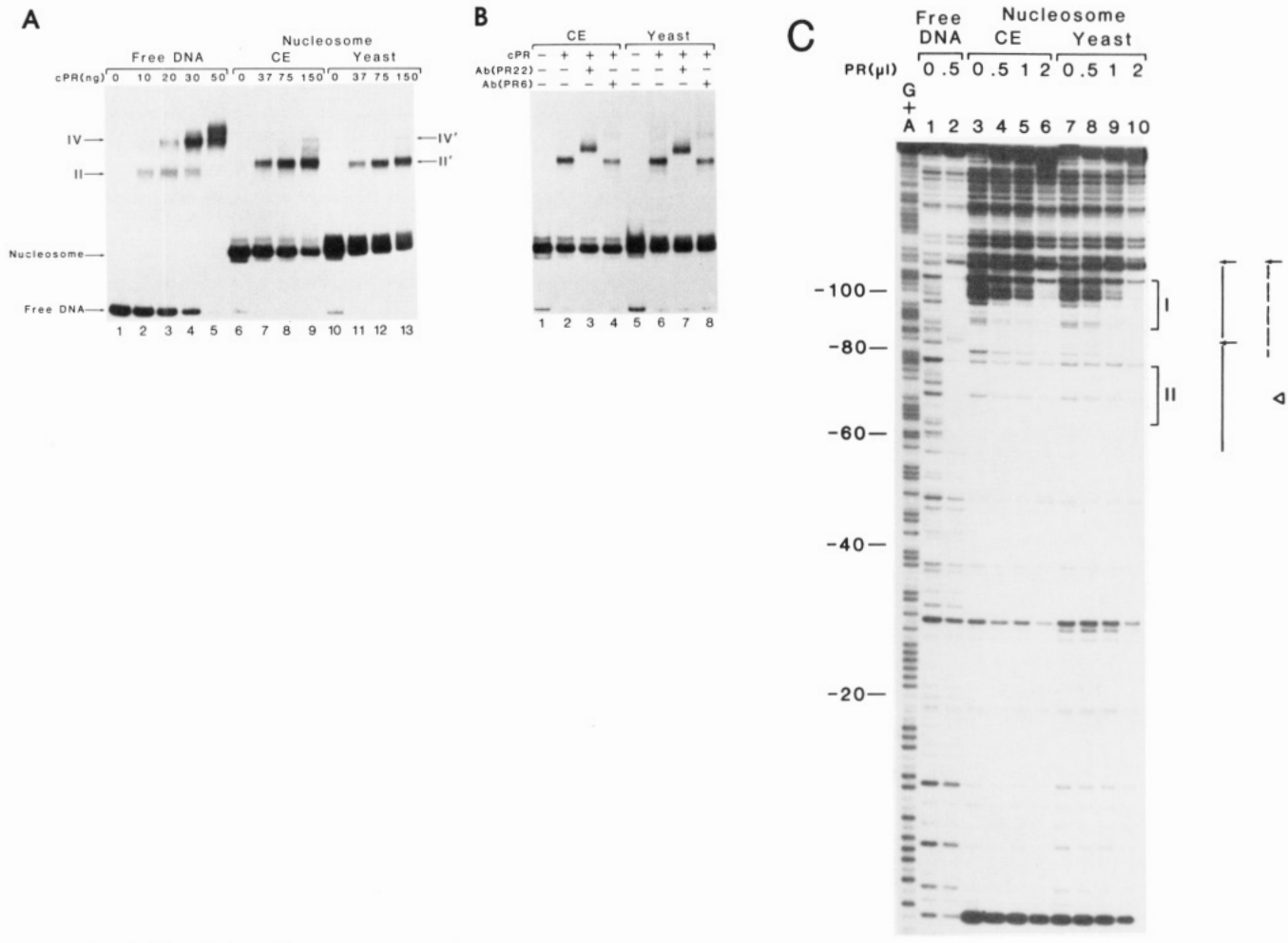

FIGURE 4: Binding of PR to fragment A reconstituted nucleosomes. (A) Gel mobility shift analysis. PR was incubated with reconstituted nucleosomes or free DNA, and the products were analyzed on a nondenaturing polyacrylamide gel. PR binding to free DNA results in the formation of two complexes, labeled II and IV. Complexes II′ and IV′ are with reconstituted nucleosomes. Chicken erythrocyte and yeast nucleosomes give rise to the same complexes with PR. (B) Antibody supershift of PR–nucleosome complexes. An antibody was added to the binding reaction to probe for the presence of receptor in the nucleosome shifted complexes. PR22 recognizes PR type A; PR6 is a control antibody. (C) DNase I footprint analysis. Different amounts of PR (140 ng/μL) were incubated with free DNA or reconstituted nucleosomes and then subjected to digestion with DNase I. The positions of the PREs are marked by brackets labeled I and II. The footprinted regions are noted by vertical lines to the right (solid line, free DNA; dashed line, nucleosome). Arrows denote hypersensitive sites; the arrowhead denotes a semiprotected band in the nucleosome samples. The samples were digested with 2 μg/mL (lanes 1, 2) or 3 μg/mL DNase I (lanes 3–10) for 25 s (lanes 1, 2) or 1 min (lanes 3–10).

identify the boundaries of the nucleosome in this analysis, and it is not clear whether all of PRE II is within the nucleosome. By performing exonuclease III analysis, it is possible to establish that the downstream boundary is at or near the end of fragment B (Figure 5B). Exonuclease III is strongly inhibited at or near the downstream end of the fragment in reconstituted samples. Thus, both PREs are well within the boundaries of the nucleosome core particle (Figure 5C). The PREs on fragment B reconstituted nucleosome are oriented outward, similar to that observed with fragment A (not shown). However, the DNase I analysis indicates that toward the downstream end the DNA is less well protected and probably interacts less strongly with the histones.

Binding of PR to Fragment B Reconstituted Nucleosomes. Figure 6A shows an analysis of PR binding to the fragment B reconstituted nucleosome by a gel mobility shift assay. Both the free DNA and reconstituted samples are shifted upon PR addition. As before, two complexes are formed representing the occupation of one or both PREs. Whereas PR forms complex IV efficiently even at low PR levels, the analogous complex on nucleosomal DNA (IV′) is formed less efficiently, suggesting a loss of cooperative binding upon nucleosome formation. It is highly unlikely that complex IV′ (Figure 6A, lanes 6–8) could have resulted from PR binding to residual free DNA in reconstituted samples since the residual free DNA disappears upon addition of low levels of PR. In contrast, complex IV′ is formed in appreciable amounts only when high levels of PR are present, suggesting that this complex is a lower affinity species distinct from complex IV. As we have shown that both PREs are well within the boundaries of the nucleosome, these results suggest that PR can bind to nucleosomal DNA, but with altered affinity.

To examine whether PR binds preferentially to one PRE, DNase I footprint analysis was performed. As shown in Figure 6B, PR binds preferentially to PRE II in the reconstituted nucleosome, footprinting PRE II clearly but not PRE I. Both PREs can be footprinted on free DNA. Greater concentrations of PR are required to footprint PRE II on nucleosomal DNA, compared to free DNA. Note that PRE II occupies a region in the reconstituted nucleosome more sensitive to DNase I compared to PRE I. As both PREs lie on the same surface of the DNA, these results suggest that PR has a preference for nucleosomal DNA that is less constrained by histones.

DISCUSSION

The need to compact cellular DNA via association with

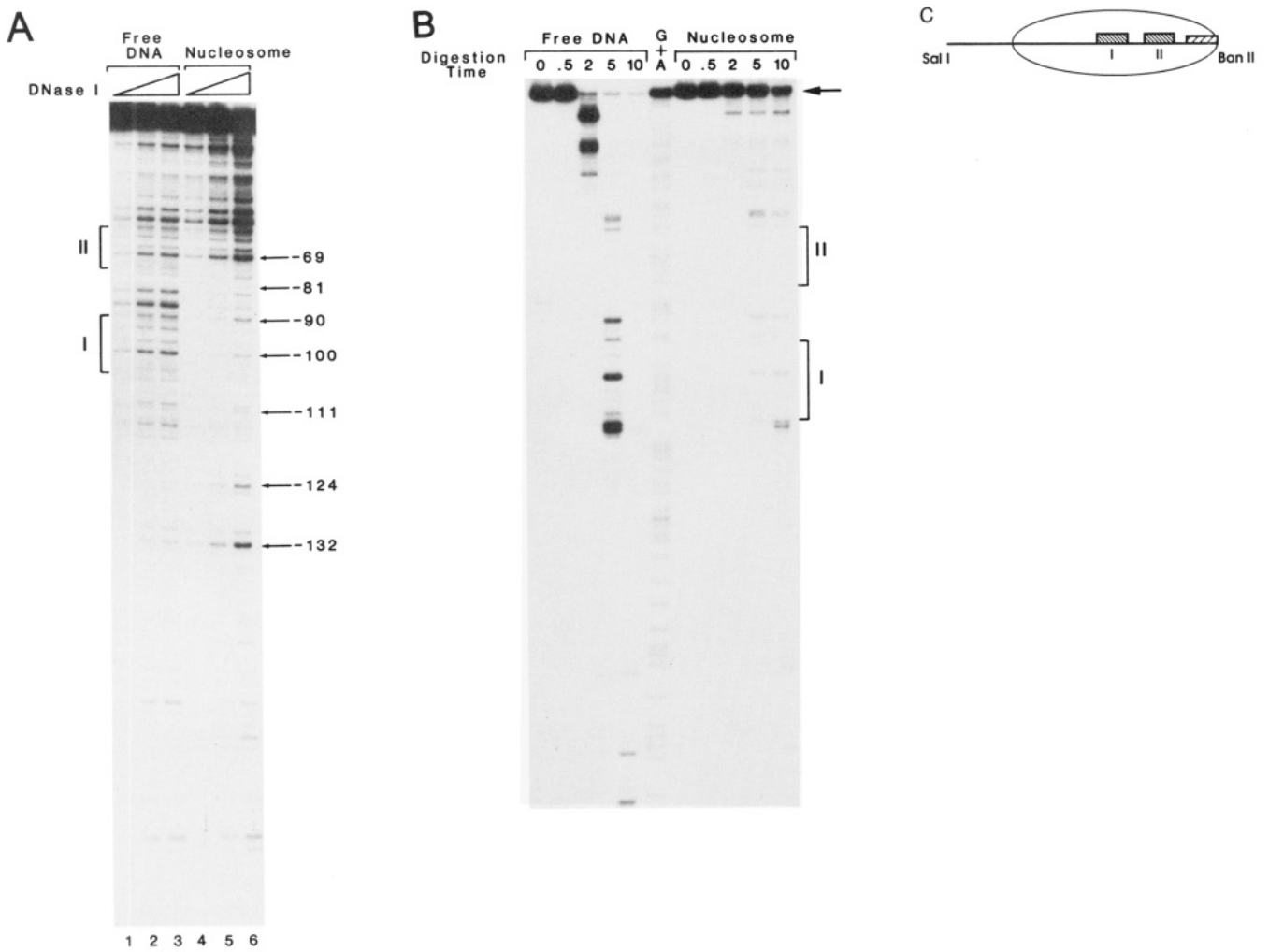

FIGURE 5: Characterization of nucleosomes reconstituted on fragment B. (A) DNase I digestion analysis. The top strand of fragment B was end-labeled and reconstituted with chicken erythrocyte histones. Reconstituted nucleosomes and control free DNA were digested with different concentrations of DNase I and analyzed on a sequencing gel. Arrows denote sites enhanced on nucleosomal DNA relative to free DNA. DNase I digestion conditions used are as follows: DNase I concentrations of 0.5 μg/mL (lanes 1 and 4), 1 μg/mL (lanes 2 and 5), 2 μg/mL (lane 3), and 3 μg/mL (lane 6) and digestion times of 15 s (lanes 1–3) or 1 min (lanes 4–6). (B) Exonuclease III analysis. The reconstituted nucleosome used in panel A was analyzed by exonuclease III digestion. The strong exonuclease III stop is indicated by the arrow. The positions of the PREs are marked by brackets. (C) Schematic diagram of the translational positioning of the reconstituted nucleosome on fragment B.

histones presents eukaryotic cells with a formidable problem: how to access this DNA for transcription and replication. It has become apparent that DNA and RNA polymerases can elongate through nucleosomal DNA, even without displacement of nucleosomes from the template (Lorch et al., 1987; Losa & Brown, 1987; Bonne-Andrea et al., 1990). However, at least for transcription, initiation appears to require a nucleosome-free region of DNA (Lorch et al., 1987). A related but distinct issue concerns how sequence-specific DNA binding proteins that direct RNA polymerase to promoters access target sites sequestered in chromatin. Clearly, the association of cellular DNA with histones is of major importance in modulating transcription factor access to regulagory sequences [for reviews, see Grunstein (1990) and Elgin (1988)]. Although most regulatory proteins may be excluded from the DNA by the presence of nucleosomes, some may be unaffected or even have enhanced affinity for nucleosomal DNA.

To further understand how nucleosomes can modulate the binding of regulatory factors to DNA target sites, we have characterized the binding of PR to PRE sequences on nucleosomes assembled in vitro. As with the GR (Perlmann & Wrange, 1988; Pina et al., 1990; Archer et al., 1991), we find that PR is able to bind to nucleosomal DNA. However, our results indicate that PR has decreased affinity for nucleosomal DNA and binds efficiently only where the DNA is least constrained by histones; this is toward the edge of the nucleosome. It is thought that the DNA near the boundaries of the nucleosome core interacts primarily with histones H2A and H2B, whereas the central region is stabilized by interaction with histones H3 and H4 [reviewed by van Holde (1989)]. It is likely that this preferential binding applies also to GR, a highly related steroid receptor that recognizes the same DNA target site. Previous reports of GR binding to MMTV sequences on nucleosomes are not inconsistent with a restriction of GR binding to GREs located near the nucleosome boundary. Note that by changing nucleosome positioning, the preference of PR binding could be changed from PRE I (fragment A) to PRE II (fragment B). These results suggest that the position of the receptor binding site within the nucleosome is likely to affect the degree of hormone-responsiveness of potential target genes in vivo.

It is striking that the nucleosomes are positioned so precisely on fragments A and B. It has been shown that nucleosome positioning on short fragments is DNA sequence-dependent (Schrader & Crothers, 1989; Drew & Travers, 1985), presumably determined by the physical characteristics of the DNA. We have not analyzed the determinants of nucleosome positioning on our fragments. However, we suspect that the

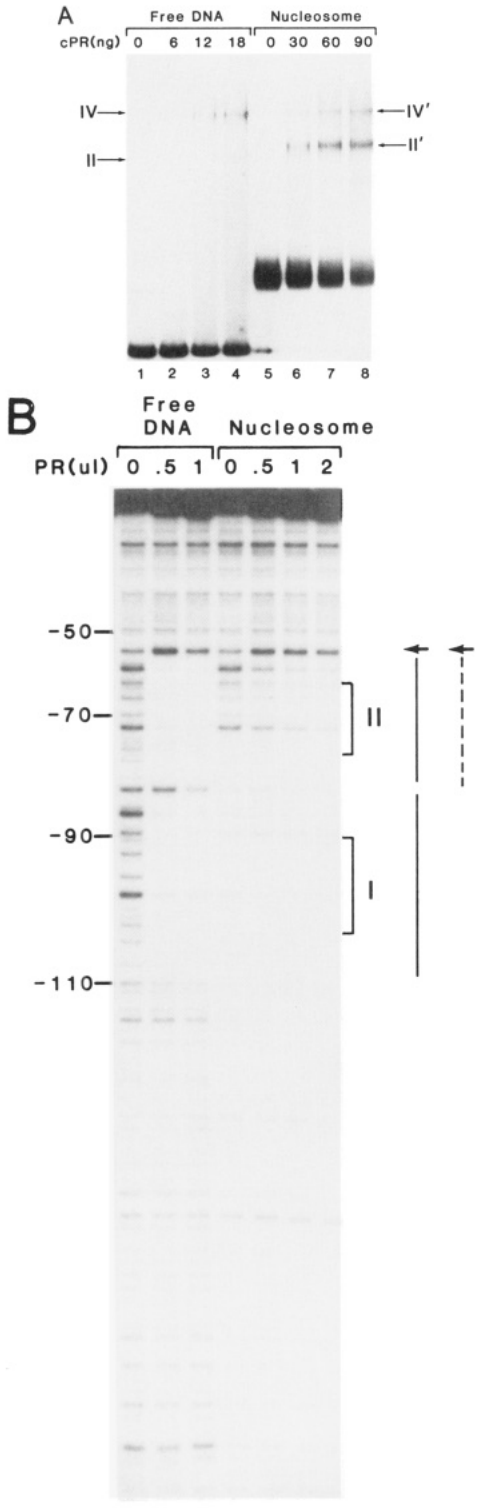

FIGURE 6: Binding of PR to fragment B reconstituted nucleosomes. (A) Gel mobility shift analysis. Different amounts of PR were added to free DNA and reconstituted nucleosomes (same as in Figure 5) and separated on a nondenaturing polyacrylamide gel. PR binding to free DNA generates complexes II (barely visible) and IV; equivalent PR–nucleosome complexes are II′ and IV′. (B) Dnase I footprint analysis. Purified chicken PR (140 ng/μL) was incubated with free DNA or reconstituted nucleosome and then digested with DNase I. Regions of protection are indicated by lines to the right (solid line, free DNA; dashed line, nucleosome). Arrows denote hypersensitive sites. Brackets mark the positions of the PREs. The digestion conditions used are as follows: 2 μg/mL DNase I for 25 s (free DNA) or 3 μg/mL for 1 min (nucleosome).

presence of bacterial vector DNA (which is not naturally nucleosomal) at the upstream region may have forced the nucleosome to occupy the downstream end.

Another potential factor that could affect the interactions of PR with nucleosomal DNA is nucleosome stability. Nucleosomes of lower stability may more easily undergo structural alterations that facilitate high-affinity binding to PR. Such structural alterations may very well underly changes in chromatin structure observed in steroid-induced transcription. It should be noted that DNA sequence-dependent nucleosome stability appears to determine whether nucleosomes are displaced during elongation of transcription in vitro (Lorch et al., 1987, 1988; Losa & Brown, 1987).

We have attempted to alter nucleosome stability by forcing the nucleosome to adopt a more unfavorable position on the fragment. By removal of a large portion of the downstream end of fragment A sequences incorporated in the positioned nucleosome, we were able to shift the position of this nucleosome. This shift in position alters the position of the PREs with respect to the nucleosome center. Our results suggest that the original positioning on fragment A is less conducive to PR binding compared to the subsequent positioning on fragment B. Note that with fragment A, only the PRE located on the nucleosome boundary is able to bind PR, whereas with fragment B both PREs are completely inside the nucleosome, but nonetheless appear to bind PR relatively well. This facilitation of PR binding to fragment B nucleosomes may be a result of lowered nucleosome stability. A contributing factor could be the lack of "linker" DNA at the downstream end, which may cause the DNA toward this end of the nucleosome to be less tightly constrained. There is evidence that H2B makes contacts with linker DNA (Hill & Thomas, 1990).

The properties of histones unique to transcriptionally competent chromatin may also affect nucleosome stability. To examine whether these histones may facilitate productive interactions of PR with nucleosomal DNA, we reconstituted nucleosomes with yeast histones as well as with inactive chicken erythrocyte histones. The use of yeast histones is particularly appropriate for these studies since steroid hormone receptors can function in yeast (Metzger et al., 1988; Mak et al., 1989). In fact, gene activation by the human estrogen receptor in yeast is accompanied by chromatin structure disruption not unlike that observed in animal systems (Pham et al., 1991a). Yeast nucleosomes are thought to be more loosely folded compared to inactive mammalian nucleosomes (Chen et al., 1991), are less stable (Lee et al., 1982), and are unable to constrain their DNA from thermal untwisting (Saavedra & Huberman, 1986; Morse et al., 1987). In light of these differences between yeast and mammalian nucleosomes, it is somewhat surprising that we find only minor differences between reconstituted yeast and chicken erythrocyte nucleosomes toward DNase I digestion, and virtually identical interactions with PR. It is highly unlikely that our yeast histones have somehow been altered during isolation, as the histones are intact and are properly acetylated (data not shown). Although not evident here, the documented differences between yeast and bulk mammalian nucleosomes may be more significant in the context of higher order chromatin organization. Nonetheless, the use of histones from an active chromatin, such as yeast, may be desirable in future reconstitution experiments that attempt to mimmick in vivo patterns of steroid-induced chromatin structure changes.

Our results, as well as that of others (Perlmann & Wrange, 1986; Pina et al., 1990), suggest that the binding of receptor to nucleosomes in vitro has little impact on nucleosome structure. This raises the possibility that receptor binding in itself may be insufficient to disrupt chromatin structure in vivo. Recent evidence from our lab supports this idea. Using an estrogen receptor system reconstituted in yeast, we recently

showed that estrogen receptor derivatives could bind to estrogen-responsive elements in vivo without inducing significant alterations in chromatin structure (Pham et al., 1991b). We found that the ability to disrupt chromatin structure requires the presence of transactivation functions on the receptor and correlates with transcriptional competence. Thus, the in vivo process by which nucleosome structure is altered by steroid appears to be complex, and most likely requires accessory factors.

In summary, the results of this study show that the binding of PR to PRE sequences is modulated by nucleosomes. PR can bind to nucleosomal DNA but has a definite preference for sites near the nucleosome boundary, and for DNA regions least constrained by histones. Thus, nucleosome positioning may affect the hormonal regulation of potential target genes in vivo. It is becoming clear that histones can profoundly affect transcriptional regulation of genes and that native nucleoprotein organization is an issue that must be taken into consideration in studies of transcription factor–DNA interactions.

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