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Glucocorticoid receptor DNA-binding specificity is increased by the organization of DNA in nucleosomes

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ABSTRACT **A DNA fragment containing glucocorticoid receptor binding sites in the mouse mammary tumor virus promoter was reconstituted *in vitro* with histones to form nucleosome cores, which become positioned on the DNA fragment in a sequence-specific manner. Glucocorticoid receptor binding to specific DNA sequences was analyzed by quantitative DNase I footprinting. The receptor interacted with surprisingly high affinity with one of the binding sites in the reconstituted promoter, although it was reduced by a factor of ≈2 compared with the same site in protein-free DNA. By contrast, the affinity for random genomic nucleosomal sites was drastically reduced compared with histone-free DNA. Thus, reconstituting the promoter *in vitro* resulted in a 60- to 70-fold increase in binding specificity. Such an increase in selective binding may help to explain the ability of glucocorticoid receptor to effectively locate its target sites in chromatin.**

Glucocorticoids act by associating with an intracellular receptor protein that belongs to the large family of ligand-dependent nuclear receptors (1). Several genes are activated or repressed at the level of transcription initiation by the ligand-associated glucocorticoid receptor (GR) (2). Activation involves hormone-dependent binding to specific DNA recognition sequences, termed glucocorticoid response elements, in the vicinity of glucocorticoid-regulated genes. One of the best characterized promoters induced by GR is situated in the long terminal repeat of the mouse mammary tumor virus (MMTV); several GR-specific recognition sequences are located within 200 base pairs (bp) upstream of the transcription start site. In addition to these sequences and a TATA box, binding sites for nuclear factor 1 (NF-1) and octamer-binding proteins are also important for efficient hormone responsiveness (3).

Activation is paralleled by local chromatin alterations, as evident from the appearance of a DNase I-hypersensitive site over the MMTV promoter in hormone-treated cells (4). Such sites have also been demonstrated in several other genes regulated by glucocorticoids (5, 6). The hypersensitivity appears to be the result of a local disruption of a nucleosome specifically positioned over the regulatory region in the MMTV long terminal repeat (7). An *in situ* detection assay showed that the establishment of transcription factors in the promoter was hormone-dependent (8). Taken together, the results suggest that transcription is repressed by an intact chromatin structure and that one role for GR may be to alter the array of positioned nucleosomes, thereby allowing the binding of additional transcription factors. Such a direct role for histones as regulatory components of transcription is further supported by other studies, using both *in vitro* and *in vivo* strategies (9–12). The proposed mechanism for promoter activation suggests that GR is the initial factor to interact with the uninduced promoter. GR, therefore, has to localize and bind to its recognition sequences within nucleosomes. Thus, it is important to understand how GR and other transcription factors locate and interact not only with binding sites in free DNA but also with recognition sequences in a chromatin context.

In histone-free DNA, GR binds to the MMTV glucocorticoid response element with relatively high affinity; it also recognizes its cognate binding sites within reconstituted nucleosomes (13–15). The question addressed here is how the organization of DNA in nucleosomes affects the affinity and specificity for its target sites. Quantitative DNase I footprinting was used to analyze the binding between purified rat GR and MMTV DNA fragments assembled with core histones to form nucleosomes *in vitro*. GR showed surprisingly high affinity for nucleosomal binding sites, only moderately lower than that for sites in free MMTV DNA. The affinity for random genomic sites within nucleosome cores was drastically reduced, compared with free random DNA sites; the net result was a 60- to 70-fold increase in the selective binding of GR to one of the specific nucleosomal binding sites. This is probably important for the ability of GR to discriminate between specific and random DNA sites within the genome.

MATERIALS AND METHODS

GR Preparation. GR–[^{3}H]triamcinolone acetonide complex was purified from rat livers (16, 17) and was 60–95% pure by SDS/PAGE. The fraction of purified GR that was competent to bind to DNA was established as described (17).

Construction and Preparation of DNA Fragments. Labeled fragments were prepared from the plasmid pB/b-wt, which contains sequence from −51 to −241 bp upstream of the transcription start site in the MMTV long terminal repeat. The MMTV sequence was flanked by a *Bam*HI site at −51 and a *Bgl* II site at −241. The plasmids were constructed by amplifying the −51 to −241 sequence by PCR using pLSwt (18) as a template. The two primers were each 30 nucleotides long including 20 nucleotides of complementary sequence: one was tailed with a *Bam*HI site, and the other with a *Bgl* II site. The resulting amplified DNA was digested with *Bam*HI and *Bgl* II and cloned into pMTV(−200/−148)[1] (19), which had been opened with *Bam*HI and *Bgl* II and treated with calf intestinal phosphatase (Boehringer Mannheim) before ligation. DNA fragments for nucleosome reconstitution and GR binding were labeled by cleaving pB/b-wt with either *Bam*HI or *Bgl* II (see below). DNA was treated with calf intestinal phosphatase and 5′-end-labeled by using [γ-^{32}P]ATP and T4 polynucleotide kinase (20). Fragments were generated by secondary digestion with *Bam*HI or *Bgl* II and isolated by electrophoresis in nondenaturing 5% polyacrylamide gels; they were then electroeluted onto Schleicher & Schuell DEAE membranes according to the manufacturer's recom-

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Abbreviations: GR, glucocorticoid receptor; MMTV, mouse mammary tumor virus; UBR, upstream binding region; DBR, downstream binding region.

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mendations. In Figs. 2A, 3A, and 4B, the fragments were labeled at the *Bam*HI site at −51; the fragments in Fig. 2B were labeled at the *Bgl* II site at −241. Specific radioactivity was determined as described (17); 2–5 fmol of radioactive fragment was used in each binding reaction.

Core Nucleosome Reconstitution. Nucleosomes were reconstituted (13) by a high-salt histone exchange method (21). The histones were derived from histone H1-depleted chromatin prepared by micrococcal nuclease (Boehringer Mannheim) digestion of rat liver nuclei (22, 23). This preparation yields pieces of histone H1-depleted chromatin that are 30–60 nucleosomes long (23).

DNase I Footprinting. GR binding and DNase I footprinting, densitometry, and quantitation of the degree of saturation in protected segments followed the procedures described previously (17). Autoradiograms were obtained on Fuji RX film.

Competition with Random DNA. In the competition experiments, a constant amount of GR was added to labeled MMTV fragments so that the UBR footprint was fully saturated when no competitor was added. The decrease of DNase I protection with increasing amounts of competitor was determined by densitometry (17). The relative affinity for the nonspecific competitors was calculated as described (24) (see also legend to Fig. 4). Chromatin used as competitor was derived from histone H1-depleted material used also for nucleosome reconstitution (see above). Mononucleosome cores used as competitor were prepared from histone H1-depleted chromatin as follows: $CaCl_2$ (final concentration, 3 mM) was added to 300 μl of rat liver chromatin (≈450 μg of DNA), and the chromatin was digested with 150 units of micrococcal nuclease (Boehringer Mannheim) at 37°C for 16 min. The reaction was terminated by addition of Na_2EDTA to 6 mM. The digestion mixture was layered on top of a 5–30% (vol/vol) glycerol gradient in 50 mM Tris·HCl, pH 7.5/1 mM Na_2EDTA/0.01% porcine insulin and centrifuged in a Beckman 50.1 rotor at 35,000 rpm for 15 hr at 4°C. Nucleosome cores were located by running a parallel gradient with ^{32}P-labeled reconstituted nucleosomes. Relevant fractions were pooled and divided into two equal parts. One was used directly in competition experiments (see Fig. 5). The other was treated with proteinase K (Boehringer Mannheim) at 37°C for 1 hr and carefully extracted twice with phenol/chloroform; the DNA was recovered by ethanol precipitation. DNA was dissolved in a small volume of water, and the DNA concentration was established from A_{260}. An aliquot was analyzed by electrophoresis in a nondenaturing 5% polyacrylamide gel in 40 mM Tris acetate, pH 7.6/1 mM Na_2EDTA; the DNA bands were located by staining with ethidium bromide. To achieve similar conditions in the two samples, DNA was diluted to the original volume (i.e., identical volume as the sample containing nucleosome cores) in buffer from a gradient run in parallel with no chromatin or DNA. The final DNA concentration after dilution was 0.99 μg/μl.

RESULTS

Affinity for Nucleosomal GR Binding Sites. A 196-bp DNA fragment harboring MMTV sequence from position −241 to −51, relative to the transcription start site, was used for nucleosome core reconstitution and GR binding experiments. Fig. 1 illustrates the regulatory region of the MMTV promoter. Two segments protected by GR in DNase I footprinting experiments have been shown to be important for GR responsiveness (3, 18, 25). These regions are here referred to as UBR and DBR. Both regions contain the conserved GR binding motif TGTTCT; in UBR it is part of an imperfect inverted repeat, and in DBR, there are three copies of the motif in a more complex configuration. In addition to these

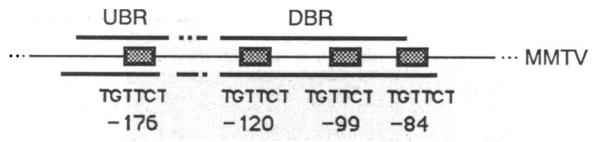

FIG. 1. Regulatory region of the MMTV long terminal repeat. Regions protected by GR from DNase I digestion in each strand are indicated by solid lines. The upstream binding region (UBR) and the downstream binding region (DBR) are of functional importance for the hormone response. Boxes indicate positions for the conserved TGTTCT GR-binding motifs. Numbers represent position relative to the transcription start site and correspond to the first T in the TGTTCT motifs.

two regions, a segment situated between UBR and DBR is protected from DNase I digestion by higher levels of GR; however, this region has failed to show any function in the promoter and was not analyzed in the present study. Other factors, such as NF-1 and octamer-binding proteins, also bind to the promoter, between the GR binding sites and the TATA box, and are important for hormone responsiveness (3, 18, 25, 26).

Core nucleosomes were reconstituted by high-salt histone exchange (21), in which end-labeled MMTV fragments were mixed, in the presence of 1 M NaCl, with long pieces of histone H1-depleted chromatin (23) isolated from rat liver nuclei (22). The mixture was diluted stepwise to 0.1 M NaCl, and the reconstituted nucleosome cores were separated from chromatin and free labeled MMTV fragments by glycerol gradient centrifugation (13). Reconstituted and native rat liver core nucleosomes sedimented identically in glycerol gradients and comigrated in nondenaturing polyacrylamide gels (data not shown). In protection experiments using either DNase I (see below) or exonuclease III (data not shown), ≈145 bp were protected by the histones in both native and reconstituted nucleosomes. Thus, reconstituted and native core particles were indistinguishable by several criteria.

The reconstituted nucleosomal DNA was sensitive to DNase I attack, at intervals of ≈10 bp (Fig. 2 A and B), which is typical for DNA on the surface of a precisely positioned histone octamer (23). DNase I-sensitivity maxima were in identical positions as observed in a previous study, in which a similar fragment was used for nucleosome reconstitution (13). The histone borders, as determined by exonuclease III protection analysis (data not shown), were also in similar positions, placing the nucleosome dyad approximately at position −150.

The binding affinity of GR for the various binding regions within the reconstituted nucleosome was analyzed at equilibrium by quantitative DNase I footprinting (17, 27). Increasing amounts of GR were incubated with a fixed amount of either free or nucleosome-reconstituted ^{32}P-labeled MMTV DNA fragments in parallel experiments. Except for the presence of histones on the reconstituted fragments, the conditions for the GR binding assays were identical; thus, the affinity of GR for nucleosomal sites versus free sites could be directly compared. The resulting autoradiograms were used to plot the degree of saturation, and the amount of GR required for half-maximal saturation was determined.

In the experiment shown in Fig. 2 A and C, the binding to UBR was analyzed. The affinity for UBR in nucleosomes was surprisingly high; 2.4-fold more GR was required for half-maximal saturation of nucleosomal UBR, compared with the histone-free binding site. In two additional experiments, the difference in affinity between reconstituted and free UBR was 2.1-fold and 2.4-fold (data not shown).

DBR is a more complex region, with three TGTTCT binding motifs. Binding to this region in nucleosomal DNA is demonstrated by the hypersensitivity at position −126, which appears close to the TGTTCT motif at −120 in the nucleo-

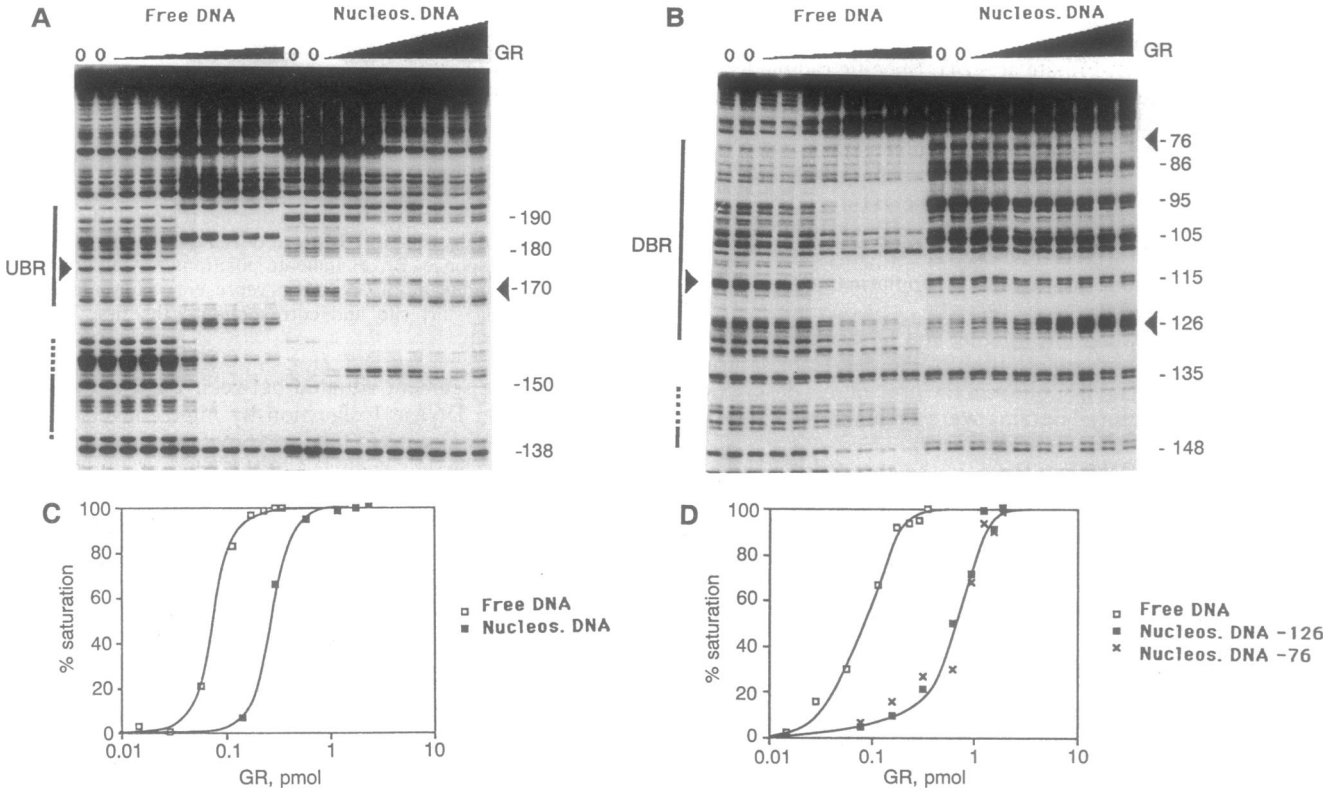

FIG. 2. Quantitative DNase I footprinting analysis of UBR and DBR. (*A*) Autoradiogram showing UBR and surrounding regions. Increasing amounts of GR were added to free MMTV DNA in the lanes to the left (free DNA), and to reconstituted MMTV DNA in the lanes to the right (nucleos. DNA). The first two lanes in each series are from incubations without GR. Regions protected from DNase I are indicated at left. Numbers to the right refer to the positions of DNase I maxima, which occur at 10-bp intervals, in reconstituted DNA. Arrowheads indicate the bands selected for optical density measurements. Due to nucleosome-specific effects in the DNase I pattern, different bands had to be chosen for optical density measurements. (*B*) Autoradiogram as in *A*, showing DBR and surrounding regions. (*C*) Degree of saturation in UBR as a function of the total amount of added GR. □, free MMTV DNA; ■, reconstituted MMTV DNA. (*D*) Degree of saturation in DBR, as in *C*.

somal DBR (Fig. 2*B*). DNase I hypersensitivity gradually increased with increasing amounts of GR; 6.9-fold more GR was required for half-maximal saturation in the nucleosomal DBR, compared with this region in free DNA (Fig. 2*D*). In two additional experiments, the decrease was 7.5-fold and 9-fold. The most proximal segment of DBR at −84 was also protected by GR and was half-maximally saturated at a similar GR concentration as the −120 region (Fig. 2*D*).

In summary, the affinity for GR binding to the nucleosomal UBR was moderately reduced (≈2.3-fold); GR binding to the distal −120 region and proximal −84 region of DBR was further reduced (≈8-fold). In a previous study, in which the interaction with naked MMTV DNA was quantitated, the concentration of free GR at half-maximal saturation was ≈0.3 nM for both UBR and DBR (17). The corresponding GR binding constants for nucleosomal binding sites can thereby be estimated to be 0.7 nM for UBR and 2.4 nM for the −120 and −84 regions of DBR.

Specificity of the GR–Nucleosome Interaction. Previously, the relative affinity for specific GR binding sites in MMTV was found to be 2–3 × 10^3-fold higher than that for random nonspecific sites in calf thymus DNA (17). Considering the vast excess of random targets in the nuclei of mammalian cells, the calculated selectivity appears to be too low to explain frequent occupancy at target binding sites *in vivo* (28).

A possibility for high specificity might be that GR binds with increased selectivity to specific sites in nucleosomal DNA. In the initial attempts to analyze this possibility, the affinity for histone H1-depleted rat liver chromatin was determined. In one such experiment (Fig. 3), histone H1-depleted chromatin or rat liver DNA was added as competitor in binding reactions containing a constant amount of GR and naked ^{32}P-labeled MMTV fragments. The experiment did not demonstrate any drastic decrease in the ability of chromatin to compete. The difference was only about 2-fold, and since the specific binding was also reduced similarly in nucleosomes, no net increase in selectivity was demonstrated. However, >30% of the added histone H1-depleted chromatin competitor was linker DNA, free of histones (data not shown). Consequently, a low affinity for random nucleosomal binding sites would have been masked by the relatively high affinity for histone-free DNA.

To avoid the possible influence from linker regions, nucleosome cores were prepared by extensive micrococcal nuclease digestion of rat liver chromatin. After isolation by glycerol gradient centrifugation, the material was divided, so that core nucleosomes and naked core DNA could be used in parallel competition experiments. To determine the length of the DNA in the core nucleosomes, an aliquot of the deproteinized sample was analyzed by electrophoresis in a polyacrylamide gel. The DNA fragments were ≈145 bp long (Fig. 4*A*), which corresponds to the expected length of DNA protected from micrococcal nuclease in native nucleosomes (23).

The affinity of GR for random mononucleosome cores was considerably lower than for all previously tested competitors (Fig. 4 *B* and *C*). Relative to the corresponding histone-free DNA, the affinity for the nucleosome-organized random DNA was reduced ≈150-fold. In two additional experiments, 1.2 μg (DNA content) of nucleosome cores failed to show any competition. Attempts to further increase the amount of nucleosome core competitor resulted in smearing of the DNA under electrophoreses. Considering the moderate reduction

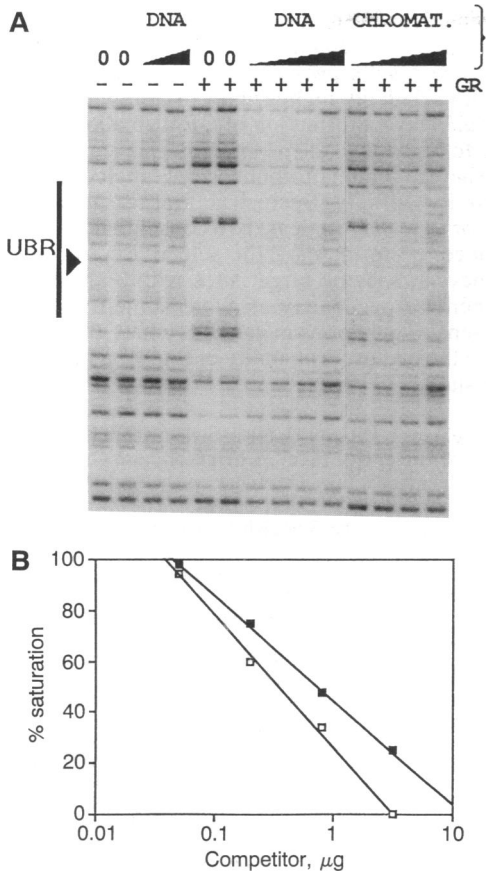

FIG. 3. Chromatin competition experiment. (*A*) Autoradiogram showing UBR and surrounding regions. A constant amount of GR (+) was added to binding reactions containing free ^{32}P-labeled MMTV fragments and increasing amounts of competitor. The added competitor was either deproteinized histone H1-depleted chromatin (DNA), or histone H1-depleted chromatin (CHROMAT.). Position of UBR is indicated at left. Arrowhead indicates the band selected for densitometry. (*B*) Degree of saturation of UBR as a function of the amount of added competitor (DNA). The amount of competitor DNA required to reduce saturation to 50% was determined for both competitors. □, DNA; ■, chromatin.

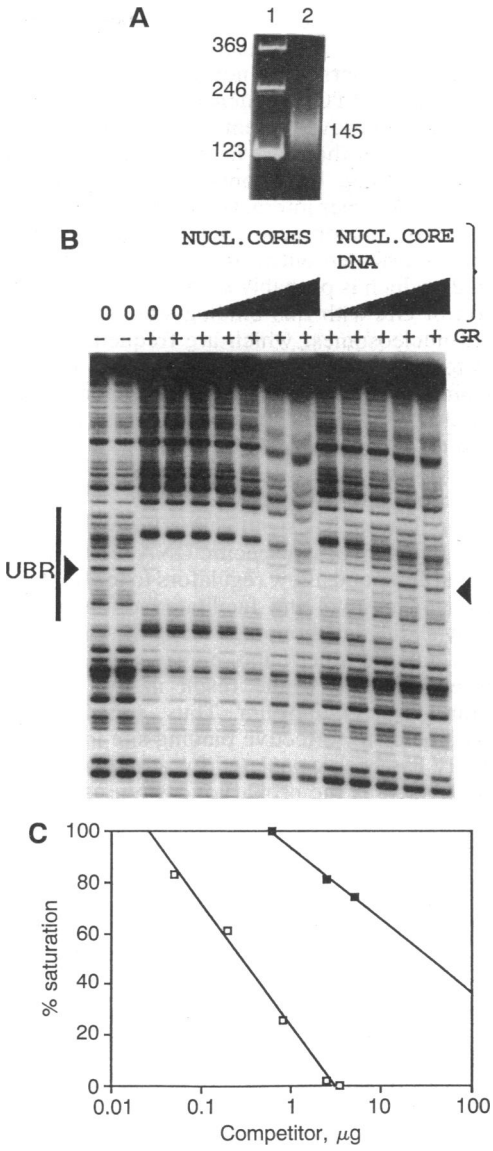

FIG. 4. Random nucleosome core competition experiment. (*A*) Ethidium bromide-stained polyacrylamide gel. Lane 1, molecular size markers; lane 2, deproteinized isolated nucleosome cores yield fragments of ≈145 bp. (*B*) Autoradiogram showing UBR and surrounding regions. A constant amount of GR was added to binding reactions containing free ^{32}P-labeled MMTV fragments and increasing amounts of competitor. The added competitor was either core nucleosomes (NUCL. CORES), or deproteinized core nucleosomes (NUCL. CORE DNA). (*C*) Degree of saturation of UBR as a function of amount of added competitor DNA. □, Nucleosome core DNA; ■, nucleosome cores. The amount of competitor required to reduce saturation to 50% was determined for both competitors. This required extrapolation to calculate the amount of nucleosome core competitor added at 50% saturation. At 50% saturation, $K_{d(random)}/K_{d(specific)} = [D]/[D{\cdot}GR]$, where $K_{d(random)}$ is the dissociation constant for the GR–random site complex, and $K_{d(specific)}$ is the dissociation constant for the GR–UBR complex (24). [D] is the concentration of free random sites (assumed to correspond to the total concentration of base pairs), and [D·GR] is the concentration of random sites in complex with GR at 50% saturation. The relative affinity for UBR in naked MMTV DNA versus random sites in free core DNA was 2.98×10^3, in good agreement with a previous study (17). The relative affinity for free UBR versus random sites in core nucleosomes was 4.4×10^5.

in affinity for the nucleosomal UBR (about 2.3-fold) and the drastic reduction in affinity for random nucleosomal sites, the overall selectivity for nucleosomal-specific binding sequences was increased 60–70-fold.

DISCUSSION

Gene regulatory sequences in nuclease-resistant chromatin usually become sensitive upon gene activation, as evident from the appearance of DNase I-hypersensitive sites. This indicates that many activating factors either bind to their cognate binding sites within nucleosomes or compete with histones for DNA binding sites during replication. Gene activation by GR is a rapid process and does not require ongoing replication. Thus, at least this class of regulators seems to have the ability to locate their cognate binding sites within nucleosomal DNA.

Previously, GR binding to specific sites in protein-free MMTV DNA was analyzed by quantitative DNase I footprinting. GR bound with similar affinities to UBR and DBR (17). In contrast, the affinities for UBR and DBR varied when they were present in reconstituted nucleosomes. GR bound with only a 2-fold reduced affinity to UBR, whereas the affinity for the −120 and −84 regions of DBR (Fig. 1) was reduced 7- to 8-fold (Fig. 2). These variations are most likely the result of the helical orientations of the various binding motifs in the positioned nucleosome. The DNase I maxima seen in nucleosomal footprints (as in Fig. 2) establish the

helical orientation of the MMTV sequence on the histone surface. Exonuclease III protection data give the positions for the histone borders (data not shown). These data show that two of the TGTTCT sequences (at −176 and −84) are situated in major grooves oriented outward, away from the histone surface, and therefore in apparently favorable orientations for GR binding. UBR consists of an imperfect inverted repeat, and a GR dimer interacts with this sequence through two consecutive major grooves (19, 29, 30). The critical sequences are oriented outward away from the histone surface (Fig. 2), which is probably essential for the high-affinity interaction of GR with this binding site. Thus, specifically positioned nucleosomes, which are frequently observed in the regulatory regions of many genes (7, 31, 32), may modulate the binding strength for regulatory factors. Factors that disrupt or alter such nucleosomes can thereby modulate gene expression (33–36).

How do sequence-specific transcription factors effectively locate their cognate binding sites? GR, as well as other transcription factors, has significant affinity also for nonspecific DNA. Fast kinetics in specific DNA recognition depend on such an affinity and allow regulators to locate their targets by a mechanism involving facilitated diffusion; sites are searched by sliding or "hopping" along the DNA (for review see ref. 37). On the other hand, an excessively high affinity for nonspecific DNA would result in low occupancy at specific targets at equilibrium. Thus, specificity is also a crucial parameter for effective binding-site localization.

Specificity has been calculated for a number of transcription factors, such as the prokaryotic λ repressor and catabolite activator protein, both of which are highly selective and bind 10^5-fold more efficiently to their respective specific sequences than to nonspecific random sites (24, 38). In contrast, some eukaryotic transcription factors, such as Sp1 and the progesterone receptor, bind with much lower specificity (1.2×10^3- and $<10^2$-fold, respectively) (39, 40). In a previous study, GR was found to bind $2–3 \times 10^3$-fold less strongly to nonspecific sites in calf thymus DNA than to UBR and DBR in the MMTV promoter. The degree of specificity required for frequent target site occupancy can be estimated and appears to be at least 2 orders of magnitude too low to account for the frequent GR occupancy at target binding sites *in vivo* (28). It is therefore intriguing to find the selectivity so drastically increased in nucleosomal DNA (Fig. 4); this selectivity could significantly improve effective target-site localization.

What is the structural explanation for the drastic decrease in affinity for random, but not specific, binding sequences in nucleosomes? The DNA-binding domains of GR and other transcription factors are enriched in basic amino acids. Basic residues can establish contacts with negatively charged phosphates in the DNA backbone and thereby contribute to the binding affinity for nonspecific DNA. Specific binding, on the other hand, is more dependent on hydrogen-bond interactions between the protein and its DNA binding site. How does the histone organization influence these interactions? Clearly, DNA phosphates are directly occupied in electrostatic interactions with basic residues in histones, which should influence the nonspecific binding more than the specific (41). For steric reasons, even more of the phosphates than are directly involved in histone–DNA interactions may be unavailable in nucleosomes. The greater flexibility of unconstrained free DNA may also allow more nonspecific ionic contacts than are possible with the more rigid nucleosomal DNA.

It is commonly assumed that histones "hide" nonspecific sequences from interaction with regulators; this would presumably allow the regulators to locate their targets more effectively. However, this argument raises the question of how specific sites could be excluded from such masking effects. The results presented in this study show that GR binds with relatively high affinity to non-nucleosomal DNA in general. The conformation of GR also allows high affinity binding to specific nucleosomal sites in certain configurations, whereas binding to nonspecific nucleosomal sites occurs with low affinity. Such structural features of transcription factors may have at least two consequences of functional importance: (*i*) to increase the selectivity, and thereby the occupancy, at specific target sites within nucleosomes and (*ii*) to increase occupancy at nucleosome-free (i.e., DNase I-hypersensitive) regions in general, where transcription factors have the highest probability of finding a functional target binding site.

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