Quantitative Analysis of the Glucocorticoid Receptor-DNA Interaction at the Mouse Mammary Tumor Virus Glucocorticoid Response Element

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Purified glucocorticoid receptor (GR) from rat liver was used for a quantitative analysis of the protein-DNA interaction at specific GR-binding segments within the 5'-long terminal repeat of the mouse mammary tumor virus. A truncated receptor was generated and used to demonstrate formation of heterodimeric GR, which further was shown to be in rapid equilibrium with receptor-monomer. The relative affinity for GR binding to specific GR sites versus random calf thymus DNA was approximately $2 \times 10^{16}$ at equilibrium a free GR concentration of $3 \times 10^{-10}$ M was required for half-maximal saturation of the two functionally important DNA sites within the mouse mammary tumor virus 5'-long terminal repeat. Although these two DNA segments act synergistically in mediating hormonal response, we did not detect cooperative GR binding to these regions in vitro. However, GR bound cooperatively within the downstream binding region. Similarly, GR was unable to facilitate factor binding to a neighboring nuclear factor 1 site, another essential element in the promoter. In contrast, nuclear factor 1 binding was inhibited slightly by GR.

The biological effects of glucocorticoid hormone are exerted via binding to the intracellular glucocorticoid receptor (GR). GR binding triggers activation of GR, which thereby gains the ability to bind specific DNA segments (glucocorticoid-response elements, GREs) in the vicinity of hormone-regulated genes (1). A GRE may have the function of a hormone-responsive enhancer (2), whereas in other genes related GR-binding sites act as hormone-dependent negative elements (3-6). The GR consensus sequence typically consists of a 15-base pair (bp) imperfect palindrome of the hexanucleotide TGTTCT, flanked by three central base pairs of non-conserved sequence. Analysis of the GR-DNA complex (7-9) has shown that a GR homodimer interacts with the 15-bp specific recognition sequence. A homodimer is formed also in the absence of DNA (7), as demonstrated by chemical cross-linking experiments. The unbound receptor seems to be sequestered by complex formation with a dimer of the 90-kDa heat-shock protein (hsp90) (10-12). Binding of hormone leads to dissociation of hsp90 from GR, and after activation the receptor becomes tightly associated with the nucleus. Other events, e.g. phosphorylation of GR (13), may also occur during activation.

The carefully analyzed mouse mammary tumor virus (MMTV) promoter is stimulated transcriptionally by glucocorticoid hormone (14). Two regions which bind purified GR in vitro (15) and confer hormone induction in vivo (16) are located within the MMTV LTR. Binding of GR to these regions clearly is a fundamental step in the process leading to GR-induced transcription. Here we have used quantitative DNase I footprinting (17) and other methods to analyze the GR-DNA interaction at the MMTV-GRE.

MATERIALS AND METHODS

Buffers—The buffers used were all derived from ETG buffer (20 mM Tris-Cl, pH 7.8, 1 mM Na$_2$EDTA, 10% (v/v) glycerol). Additional components are indicated in each case.

GR and Nuclear Extract—GR-[3H]triamcinolone acetonide complex was purified from rat livers as described previously (18), with the exception that the last DEAE-Sepharose step was replaced by chromatography on a 5-ml fast liquid chromatography Mono Q column (Pharmacia, Uppsala) (19). GR-containing fractions, 60-95% pure according to SDS-polyacrylamide gel electrophoreses, were pooled. Porcine insulin (a gift from Nordisk Gentofte, Denmark), dithiothreitol (DTT), and glycerol were added to final concentrations of 0.1 mg/ml, 10 mM, and 40% (v/v), respectively, and stored at -113 °C. The 38 kDa receptor fragment was prepared as described previously (9). The eluate from the Mono Q column (see above) was incubated with 0.3 μg of α-chymotrypsin (Sigma, Type VII, 1-chloro-3-tosylamido-7-aminot-2-heptanone-treated, 57 units/μg) per μg of receptor protein for 30 min at 10 °C. The sample was then diluted with 2 volumes of ETG buffer (20 mM Tris-HCl, pH 7.8, 1 mM Na$_2$EDTA, and 10% (v/v) glycerol) containing 4.5 mM DTT, 0.1 mg/ml insulin, and 3 mM phenylmethanesulfonyl fluoride. The receptor was eluted by a linear 0–5 M NaCl gradient, thus separating the 39-kDa receptor fragment, which is eluted around 0.15–0.2 M NaCl, from undigested receptor, protease, and other degradation products. Analysis of purified receptor fragment by this technique showed no residual full-length 94-kDa receptor in silver-stained SDS-polyacrylamide gel electrophoresis. GR was quantitated by analysis of bound [3H]triamcinolone acetonide (specific radioactivity: 2.25 Ci/mmol), because it was demonstrated previously that one hormone ligand is bound per GR polypeptide (7). The purified GR is competent to bind DNA only if the hormone receptor complex is intact, whereas a preformed GR-DNA complex is maintained also in the absence of ligand (7). A quantitative DNase I footprinting assay (see below), revealed that the GR-hormone complex was significantly less stable at temperatures above 25 °C (not shown) and thus our GR-DNA binding experiments have been conducted at 25 °C. The same assay showed that equilibrium was reached after 40 min at this temperature (not shown).

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2 The abbreviations used are: GR, glucocorticoid receptor; GRE, glucocorticoid-response element; bp, base pair(s); SDS, sodium dodecyl sulfite; DTT, dithiothreitol; MMTV, mouse mammary tumor virus; LTR, long terminal repeat; BS, bis(sulfosuccinimidyl)suberate; NF-1, nuclear factor 1; UBR, upstream binding region; DBR, downstream binding region.
Glycerol gradient centrifugation analysis, both in the presence and absence of excess DNA, was used routinely to determine the fraction of GR which bound hormone and was competent to bind DNA, respectively. DNA-binding activity was usually around 50% and was always directly correlated to the fraction of bound ligand. Similar experiments showed that the GR-ligand complex was stable under the conditions employed in this work (not shown).

The nuclear extract used in the DNase I footprint in Fig. 8 was prepared from rat liver as described by Gorski et al. (20). The protein concentration in these extracts was usually around 4-6 mg/ml.

Construction and Preparation of DNA Fragments—Labeled fragments used for footprinting were prepared from pMTVwt, pMTV-(−198/+19), pMTV-(−151/+155), and pMTV-(−125/+116), which are derivatives of linker-scanning mutants in the MMTV LTR (16). Plasmids were cleaved with BamHI, treated with calf intestinal alkaline phosphatase (Boehringer), and 5' end-labeled using [γ-32P]ATP and T4-polynucleotide kinase (21). The fragments were generated by cleavage with PvuII, separated from the vector on 5% (w/v) native polyacrylamide gels, localized by autoradiography, and purified on Schleicher and Schuell DEAE membrane by electrophoresion, using the procedure recommended by the manufacturer. DNA fragment concentration was determined by spectrophotometric absorbance measurement at 260 nm, assuming that 1 absorbance unit = 50 μg of DNA. Radioactivity was calculated by liquid scintillation counting (see below). The fragment used in the experiment in Fig. 2 was from pMTV-(−151/+155), also a derivative of linker-scanning mutants (16), and was generated by cleavage with BamHI and HindIII. The fragment ends were phosphatase-treated, 32P-labeled, and isolated as described above. An 80-bp DNA fragment derived from pMTV-(−192/+164) by cleavage with EcoRI and HindIII (9) was used in the gel retardation assay in Fig. 3. The DNA fragment used in the experiment displayed in Fig. 8 was derived from pMTV-(−198/+19). The plasmid was constructed by annealing two complementary synthetic oligonucleotides containing MMTV LTR sequences to +1, flanked by an upstream BglII site and a downstream BamHI site. The resulting DNA fragment was ligated into the unique BamHI site of pMTV-(−198/+58). The plasmid thus contains MMTV sequences from position −198/+19 with the −57/−52 segment replaced with a mixed BamHI-BglII site. The fragment was 32P-end-labeled and isolated as described for the other fragments used for DNase I footprinting.

Glycerol Gradient Centrifugation—GR DNA binding was performed in a total volume of 100 μl of ETG buffer containing 5 mM DTT, 75 mM NaCl, and 0.1 mg/ml of insulin. GR and/or DNA were kept on ice until centrifugation. Four ml of 12-25% (w/v) glycerol gradients were prepared by diffusion with the ETG buffer containing 5 mM DTT, 0.1 mg/ml of insulin, and 25 mM NaCl, by diffusion with the tubes in horizontal position. Samples were centrifuged for 18-22 h at 0 °C at 53,000 rpm in a Sorvall TST 60.4 rotor, followed by fractionation from the bottom of the tube into scintillation vials. Four ml of Emulsifier-Safe (Packard Instrument) was added, and radioactivity was determined by liquid scintillation counting (see below). The approximate sedimentation coefficient for GR was determined previously (22) and used in this study as a standard for calculating sedimentation coefficients of other components (23).

The half-life of the GR-hormone complex during glycerol gradient centrifugation was 130 h at 0°C, as determined by analysis of GR-bound 3H-labeled ligand after 15 and 24 h of centrifugation in duplicates (not shown). Thus, approximately 10% of the hormone dissociates from GR during glycerol gradient centrifugation analysis.

Chemical Cross-linking—Purified GR was cross-linked by incubation with 1 mM cross-linking agent [bis(sulfosuccinimidyl)suberate (BS3, Pierce) for 5 min at 25 °C in EP buffer (1 mM Na2EDTA, 20 mM phosphate, pH 7.0) containing 0.1 mg/ml of insulin, 5 mM DTT, and 300 mM NaCl. The cross-linking reaction was stopped by adding 10 μl of 3 M lysine/100 μl. The products were analyzed by glycerol gradient centrifugation or Sephacryl S-300 gel filtration chromatography. ETG buffer containing 1 mM DTT, 300 mM NaCl, and 50 μg/ml of purified human serum albumin (a gift from Kabi, Stockholm) was used for the chromatography.

Mobility Shift Assay—GR DNA binding was performed in a total volume of 20 μl of ETG buffer containing 5 mM DTT, 0.1 mg/ml of insulin, and 0.1 mg/ml of NaCl. All binding reactions also contained 50 ng of unlabeled Sau3AI-digested pGEM-1 to reduce unspecific binding (9). Samples were applied on a 3.5% (w/v) polyacrylamide/bisacrylamide gel (55:1) in 6.7 mM Tris-HCl, pH 7.5, 3.3 mM sodium acetate, and 1 mM Na2EDTA (24). The gel also contained 0.1% (v/v) Triton X-100 to avoid GR aggregation (7). Electrophoresis (30 mA) was performed at ambient temperature using a recirculating buffer system. Approximately 5 min were required for the samples to enter the gel.

Radioactivity Analysis—Tritium and 32P were quantitated in fractions after glycerol gradient centrifugation and gel filtration chromatography using a Packard Tri-Carb 300. An efficiency of 31% for tritium and 100% for 32P was determined by use of tritiated tolune (Du Pont) and 32P-labeled DNA as internal standards. The GR and DNA in the peak fraction was estimated by subtracting the radioactivity background, correcting for spillover of 32P into the H channel (1.2-2.5%), and correcting for any quench by use of internal standards. Radioactive decay of the labeled DNA was corrected for, assuming a 3P half-life of 14.3 days.

DNase I Footprinting—Quantitative DNase I footprinting was performed as described previously (17), with the modification that we also determined the amount of DNA in the binding reactions (7). In the experiment displayed in Fig. 8, 400 ng of pGEM-1 vector DNA was added to the binding reactions prior to addition of nuclear extract. Autoradiograms were obtained using Fuji RX film, which was exposed without a screen. There was a linear relationship between density and amount of 32P in each band, as demonstrated by measurements of standards (not shown). Two-dimensional densitometry of autoradiograms (cf. Fig. 7A) were conducted using a Photoscan system P-1000 (Optronics International Inc., Chelmsford, MA) connected to a VAX 11/750 computer (Digital, Boston, MA). The autoradiogram was thus digitized, and indicated bands were selected for densitometry using a Spectragraphics 1500 (Spectragraphics, CA) display (7, 9).

RESULTS

DNA Construct—A 199-bp restriction fragment (MTVwt) which harbors the segment −198/−58 bp, relative to the transcription start site (+1) in the MMTV promoter, was used in most experiments. MMTV sequences protected by GR in DNase I footprinting experiments are indicated in Fig. 1A, as well as the binding site for nuclear factor 1 (NF-1), another essential element in the promoter (16, 25). There are three separate regions which interact with GR in vitro according to DNase I footprinting (15, 19) (see Fig. 1A): (a) The upstream binding region (UBR) is a 24-bp segment at −190/
—167. UBR harbors an imperfect inverted repeat which includes the GRE consensus hexanucleotide TGTTCCT (15, 26). A GR homodimer has been shown to interact with the UBR, making specific contacts in two consecutive major grooves, on the same side of the DNA double helix (9, 28). (b) Three tandemly arranged TGTTCCT motifs are also found in the downstream binding region (DBR) within the −134/−76 footprint. (c) In addition to UBR and DBR, a DNA segment between these regions, −161/−143, show a weak and less distinct GR-induced DNase I protection (15, 19, cf. Fig. 5A). Mutations in this region have failed to affect the hormone response of the promoter (16). In contrast, mutations altering the GR-recognition sequence within the UBR and the DBR have been shown to reduce drastically the hormone inducibility (16, 27).

Quantitative GR: GRE Binding Analysis

GR-DDBR Stoichiometry—We have established previously that two GR subunits interact with UBR in vitro (7, 9). To determine how many GR entities that interact with DBR, a 32P-labeled 95-bp DNA fragment, harboring the MMTV segment −149/−58, was allowed to form a complex with GR and was separated subsequently from unbound GR and DNA by glycerol gradient centrifugation (Fig. 2). In the presence of excess DBR DNA, a 32P-labeled DNA peak with a sedimentation rate corresponding to free DNA was formed, in addition to a broad peak of GR-DNA complex. Radioactivity analysis from four experiments, where either GR or DNA was added in excess, showed that four to six 3H-labeled hormone ligands co-sedimented with each DNA fragment, indicating that four to six GR entities were bound to the DBR. Consequently, 6 to 8 GR subunits are altogether associated with UBR and DBR at full occupancy.

Interestingly, the addition of excess DBR DNA to the GR incubation resulted in two populations of DBR DNA (Fig. 2): one sedimenting as free DNA (4.6 S) and another co-sedimenting with the tritium-GR peak at 15 S. Thus, a fraction of the DBR fragment was apparently free of GR, whereas another bound 6 GR molecules; this indicates that GR binds cooperatively to the DBR.

GR Dimerization—The GR dimer which was shown previously to interact with the UBR, formed also in the absence of DNA as demonstrated by chemical cross-linking of GR, using either glutaraldehyde (7) or BS3 (data not shown). BS3 treatment shifted the GR sedimentation coefficient from 4 to 6.9 S, according to glycerol gradient centrifugation analysis. This is similar to the sedimentation rate of dimeric GR bound to a specific 35-bp DNA site (7). Furthermore, GR cross-linking with BS3 resulted in an increase in size (from a Stokes radius of 5.9–6.8 nm, according to gel filtration chromatography, data not shown). The increase in the Stokes radius and in sedimentation coefficient corresponds roughly to a 2-fold increase in molecular mass (28).

An alternative procedure to demonstrate dimer formation is by mobility shift assay. If a full-length and a truncated variant of GR are incubated with DNA, a putative heterodimer-DNA complex will migrate at an intermediate rate in the gel, compared to homodimeric complexes (24). Here we have used a chymotryptic 39-kDa GR fragment (9), which has been shown to retain the C-terminal half of the full-length 84-kDa GR. The 39-kDa fragment binds specifically to DNA as a dimer (9), albeit with reduced specificity (29). When the full-length and the truncated GR fragments were incubated together, a heterodimer was bound to the 32P-labeled DNA fragment, as evident from the appearance of a band with intermediate electrophoretic mobility in the gel (Fig. 3). Dimer formation seems to be a rapid process, because no decrease in the amount of heterodimer was detected, even if the two GR entities were incubated together less than 5 min prior to entry into the gel (Fig. 3, compare lanes 2 and 3).

Specificity of the GR-DNA Interaction—The binding spec-

![Fig. 2. Glycerol gradient centrifugation of the GR-DBR DNA complex after incubation of 0.3 pmol of GR with 50 fmol of a 32P-labeled 95-bp DNA fragment harboring the −144/−58 MMTV segment. An empty arrowhead at fraction 15 indicates the position of free DNA (4.6 S) in a control gradient where no GR was added. Free GR (4 S) was not resolved from unbound hormone ligand at the top of the gradient. GR contained 3H-labeled hormone of 86 dpm/fmol specific radioactivity. DNA was 32P-labeled to a specific radioactivity of 137 cpm/fmol. These incubations and gradients also contained 0.002% (v/v) of Triton X-100 to reduce unspecific aggregation of GR (7).](image1)

![Fig. 3. Mobility shift assay demonstrating heterodimer formation. Lane 1, 0.18 pmol of full-length GR was incubated with an 80-bp 32P-labeled UBR fragment. Lanes 2 and 3, 0.18 pmol of full-length receptor and 0.32 pmol of 39-kDa receptor fragment were incubated together (lane 2) or separately (lane 3) for 30 min at 25°C, before mixing the two GR entities with the 80-bp 32P-labeled DNA fragment and followed by immediate loading on the gel. Lane 4, 0.32 pmol of 39-kDa receptor incubated with UBR fragment. An arrow indicates the heterodimeric complex.](image2)
ificity was evaluated by incubating a saturating concentration of GR with \(^{32}\)P-end-labeled MTVwt and increasing concentrations of calf thymus DNA. This resulted in a gradual and parallel loss of protection at both the UBR and the DBR in DNase I footprinting experiments. Competitor concentrations required to reduce the protection at GR-induced footprints by 50% were thus established. In two experiments the relative affinity (30) for specific sites compared to random sites in calf thymus DNA was 1.2 \(\times\) \(10^2\) and 3.4 \(\times\) \(10^3\), respectively (Fig. 4). Poly(dl-dC)-poly(dl-dC) (Pharmacia, Uppsala, Sweden) as competitor, failed to compete for GR even at 12-20-fold higher concentrations than required for 50% competition with calf thymus DNA. The relative affinity of GR for the MMTV GRE, compared with poly(dl-dC)-poly(dl-dC), is consequently higher than 2 \(\times\) \(10^4\) (data not shown).

Equilibrium Affinity Constants—The affinity of GR for the UBR and DBR was analyzed at equilibrium. In these experiments, the gradual appearance of a DNase I footprint was quantitated by incubating increasing amounts of GR with a fixed amount of \(^{32}\)P-labeled MTVwt fragment. Saturation diagrams were constructed, and the concentration of free GR at 50% occupancy of each binding site, here referred to as \([GR]^*\), was determined. Incubations were in large volumes (400 \(\mu\)l), and with smallest possible amount of DNA probe, ensuring that the concentration of free GR was considerably larger than the concentration of GR bound to DNA. In these calculations we assumed that altogether four GR homodimers can bind to UBR and DBR at full occupancy. In the displayed experiment (Fig. 5), the calculation of the free GR concentration at 50% saturation required the subtraction of 21% of total GR. The degree of saturation in the \(-161/-143\) region was less than 15% and did not significantly influence the amount of free GR at half-maximal occupancy of UBR and DBR. Similarly, correction for a 2 \(\times\) \(10^9\)-fold relative affinity for specific versus random DNA binding was not required, because it corresponded to less than 1% of total GR at half-maximal saturation (cf. Fig. 4). In the experiment displayed in Fig. 5, the \([GR]^*\) was estimated to be 2.5 \(\times\) \(10^{-10}\) M for GR binding to both the UBR and the DBR. Measuring densities from various bands within the protected binding regions gave similar results. In two additional experiments, the \([GR]^*\) was found to be 4 \(\times\) \(10^{-10}\) and 2.6 \(\times\) \(10^{-10}\) M for GR binding to the UBR, and 4 \(\times\) \(10^{-10}\) and 2.7 \(\times\) \(10^{-10}\) M for GR binding to the DBR. These three experiments gave a mean \([GR]^*\) of 3 \(\times\) \(10^{-10}\) M for both UBR and DBR.

Dissociation Rate—The dissociation rate from the UBR was measured by adding a 60-fold molar excess of unlabeled DNA to preformed GR-MTVwt complex at time zero. Samples were withdrawn subsequently for DNase I footprinting at different time points. The 60-fold excess of specific unlabeled DNA assured that the influence of GR reassociating with the labeled DNA probe was negligible; this was confirmed by adding the 60-fold excess of unlabeled DNA before GR and incubating for various time intervals. No footprints were detected in these controls (not shown). The data from one UBR:GR dissociation experiment is plotted, assuming first-order dissociation kinetics (Fig. 6). The dissociation constant was found to be 1.1 \(\times\) \(10^{-4}\) s\(^{-1}\), and the complex half-life was \((\lambda_0)\) 108 min.

Cooperativity—GR acts synergistically at the UBR and the DBR in vivo (16, 27). Thus, almost all hormone inducibility is lost by mutating either the UBR or the upstream part of the DBR (16), demonstrating that each region depends on the
Fig. 5. A, quantitative DNase I footprinting analysis of UBR and DBR. A concentration of $1.85 \times 10^{-11}$ M $^{32}$P-labeled MTVwt was incubated with $0.7 \times 10^{-16}$ to $7 \times 10^{-13}$ M GR. The first two lanes are from incubations without GR. Protected (Prot.) and reference (Ref.) bands used for optical density measurements are indicated, as well as the positions for UBR and DBR. B, degree of saturation in the UBR footprint plotted against the concentration of free GR. Free GR concentration ($[GR]_0$) at 50% saturation was estimated from this plot. C, degree of saturation in the DBR footprint plotted as in B.

Fig. 6. Dissociation rate of the GR-UBR complex at 25 °C. Time of dissociation is plotted against % saturation (%), assuming first-order kinetics (31). The $k_{diss}$ and half-life ($t_{1/2}$) was determined from the plot.

other for intact promoter function. The mechanism for this synergism is not understood, but may reflect cooperative GR binding to these regions. To test this possibility, we performed binding experiments with a fragment similar to MTVwt, but which harbors a mutation in the UBR previously shown to reduce hormone inducibility to 5% of wild type (16) and to disrupt GR binding in vitro (19). The $[GR]_0$ for GR binding to DBR on this fragment was $3.4 \times 10^{-10}$ M (Fig. 7A), which is not significantly different from the $[GR]_0$ of DBR estimated for the MTVwt fragment in a parallel experiment ($2.6 \times 10^{-10}$ M, Fig. 5C). In another experiment, a fragment mutated in the first TGTTCT hexanucleotide in the DBR (position -125 to -116, Fig. 7B) revealed a $[GR]_0$ of $2.7 \times 10^{-10}$ M for both the UBR and the unmutated region of the DBR. The latter experiment is shown in Fig. 7C. We conclude that both mutations fail to affect the affinity of GR for surrounding binding sites. That GR binds to UBR independently of DBR was substantiated further by the observation that the affinity of GR for a fragment harboring only one copy of UBR ($[GR]_0 = 2.2 \times 10^{-10}$ M), was similar to the $[GR]_0$ obtained for the UBR on the MTVwt fragment (data not shown).

Mutations in the NF-1 binding site in the MMTV promoter have similarly been shown to impair hormone response (16, 27), demonstrating functional cooperativity also between NF-1 and GR. We wished to test whether this synergism reflected cooperative binding of NF-1 and GR to the promoter. Here, a $^{32}$P-labeled DNA fragment which contains MMTV sequences -198/+19 relative to the transcription start site was incubated with or without GR. Protection of the NF-1 site, using nuclear extract (20) as a source of NF-1 binding activity, was analyzed by DNase I footprinting. As seen in Fig. 8, lane 7, the NF-1 binding site becomes fully protected by 5 μl of added extract. With GR prebound to the fragment, NF-1 binding was partly inhibited, and higher amounts of extract were required for full NF-1 occupancy (Fig. 8, compare lanes 7 and 8 and lanes 9 and 10). This experiment does not support binding cooperativity between GR and NF-1 as the basis for the observed in vivo synergism (16, 27). In contrast, prebound GR seems to somewhat inhibit NF-1 binding.

DISCUSSION

GR is a homodimer when bound to a specific DNA site (7, 9); the receptor can also form dimers in the absence of DNA as evident from chemical cross-linking (Ref. 7 and above). We did not detect any GR monomers in specific GR-DNA complexes formed on a complete GR-binding site, neither by mobility shift assay nor glycerol gradient centrifugation analysis. Our experimental data indicate that either the preformed GR dimer binds to DNA or that GR binds to DNA as a monomer and that strong cooperativity in binding of the second GR subunit results in undetectable levels of monomer-DNA complexes. In fact both these alternatives may operate concomitantly. The overall two-step binding reaction can thus be written as:

$$\text{GR:D + GR} \rightarrow \text{2GR + D} \rightarrow \text{GR}_2 + D$$

where $D$ is a DNA-binding target for a GR dimer. This infers that the experimentally determined $[GR]_0$ in square (i.e. $([GR]_0)_0 = 9 \times 10^{-29}$ M$^2$) is equivalent to the equilibrium dissociation constant for the overall binding reaction and to the product of the two dissociation constants of the two-step reaction (33, 34).
Under the binding conditions we have used, a majority of the free receptor is monomeric. This is based on our previous observations that GR behaves as a monomer during gel filtration (Ref. 7 and BS1 experiments described above) and in glycerol gradient centrifugations (7), unless GR is first exposed to cross-linking agents or bound to a specific DNA-binding site which in both cases results in dimeric GR. Since free receptor in concentrations of up to $1 \times 10^{-6}$ to $2 \times 10^{-8}$ M were used in these experiments, the equilibrium dissociation constant for the GR dimer formation is probably within that range or higher (33). The mobility shift assay displayed in Fig. 3 would also fit with GR existing largely in monomeric form, because no difference in heterodimer formation was detected as a consequence of variation in incubation times (see above). Consequently, the receptor monomer seems to be in equilibrium with the dimer, having a dimerization constant which is considerably higher than the [GR]° for the binding of the receptor to DNA; similar observations have been made for the $\lambda$ (33) and P22 repressors (34). A monomer-dimer equilibrium, in which two GR subunits cooperate by protein-protein contacts either before or during the DNA-binding event, is a probable explanation for the sigmoidal shape of the saturation curves in the DNA-binding experiments (33, 34).

The affinity of GR for specific DNA sequences was quantitated by DNase I footprinting. This technique allows a simultaneous analysis of multiple binding sites within a DNA fragment (17). We have thus been able to quantitate the binding of GR to DNA in the wild-type MMTV promoter context. The technique has also been shown to give values for DNA-binding affinity, which are in good agreement with those obtained by other methods (17, 32).

Sequence-specific DNA-binding proteins also have affinity for nonspecific DNA sites (30). We found that the selectivity with which GR bound to specific versus nonspecific sites differed somewhat for different types of DNA competitors. The affinity was approximately 2000 times higher for UBR, compared to an average binding site in calf thymus DNA, whereas synthetic poly(dl-dC).poly(dl-dC) failed to compete for binding under the conditions tested. Compared with other DNA-binding proteins, a relative affinity of $2 \times 10^5$ for the specific GR-DNA interaction is rather low. Thus, the lac-repressor (35) and the catabolite gene activator protein (30) bind specifically to DNA with a relative affinity which is approximately 10–100 times higher. The relative affinity of GR was tested at buffer conditions which are optimal for general DNA-binding affinity, rather than for selective binding at specific sites. At higher ionic strength, however, the
The [%P-labeled fragment was incubated alone (lanes 3 and 4), with +19 relative to the transcription start site in the MMTV LTR. By a recent report (36) using the 115-amino acids-long DNA-binding domain derived from GR. Our trials to use higher increasing amounts of nuclear extract (5 ~1, lane 7; 10 ~1, lane 8). The UBR and the DBR, as well as the binding region for NF-1, are indicated. The [GR]" of the unmutated region of DBR (cf. Fig. 5C). We demonstrated that GR interacted with the regulatory region of an MTVwt fragment reconstituted with histones to form a nucleosome in vitro. GR bound with approximately the same affinity to nucleosomal DNA as to histone-free DNA (Ref. 8). An interesting possibility is that a close contact between the two binding regions, due to the winding of the DNA on the surface of a nucleosome, will favor cooperative DNA binding. Sequence-specific positioning of a nucleosome at -250/-50 within the MMTV promoter (42) will indeed place UBR and DBR on the same side of the histone octamer. Besides the possibility of binding cooperativity in a nucleosomal context, there are other possibilities for functional synergism. Thus, according to one model for GR activation of the MMTV promoter, a nucleosome is destabilized by the action of glucocorticoids and opens the promoter for NF-1 binding, leading to promoter activation (43).

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Quantitative GR: GRE Binding Analysis

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