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Binding of Glucocorticoid Receptors to Model DNA Response Elements

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Abstract DNA sequences were synthesized that contained the consensus 15-base pair glucocorticoid receptor binding site linked to flanking sequences of various lengths. Binding of these synthetic oligomers to glucocorticoid receptor, employing a reconstituted binding system with purified components, indicated that a minimal size of approximately 45 base pairs was necessary to bind the receptor optimally. Sequences containing multiple receptor binding sites competed more effectively for binding. These findings are consistent with recent demonstrations that multiple control elements act synergistically to affect transcriptional control by glucocorticoids and confirm that regions flanking the consensus GRE binding site are instrumental in optimizing binding interactions.

Key words: glucocorticoids, steroid-receptor family, steroid-hormone, gene regulation, transacting-factors, mouse mammary tumor virus, DNA-binding

Glucocorticoids mediate transcriptional activation of numerous eukaryotic genes via the selective interaction of glucocorticoid receptors (GR) with specific DNA sequences. Comparison of the response regions of different genes have identified a consensus 15-base pair, near-palindromic sequence, referred to as a glucocorticoid responsive element (GRE) (Strahle et al., 1987, Buetti and Kuhnel, 1986). While the presence of a consensus 15-base sequence does not predicate responsiveness, it may alter transcription rates dramatically. Synergistic effects are observed when a GRE is proximal to other hormone responsive elements (Freedman et al., 1989; Jantzen et al., 1987; Buetti et al., 1989; Cato et al., 1988; Schmid et al., 1989; Tsai et al., 1989) and distantly-spaced enhancers are known to influence glucocorticoid mediated control (Grange et al., 1989; Tur-Kaspa et al., 1988), suggesting that protein-protein interactions and conformational effects operate in transcriptional regulation (Schule et al., 1989; Kakidani and Ptashne, 1988; Ankenbauer et al., 1988), as has been observed in other systems.

The mechanisms by which various cis and trans-regulatory elements participate in regulation of transcription by glucocorticoids has been studied extensively (Mitchell and Tjian, 1989). The use of promoter-linked reporter genes, footprinting analysis, and gel shift techniques (Petersen et al., 1988; Chandler et al., 1983; Freedman et al., 1989; Payvar et al., 1983) have contributed considerable information. Although these experiments have provided information relevant to the complex environment of the cell nucleus, the initial events involved in GR:DNA interactions are incompletely understood. Recent in vitro studies have focused on defining the specific amino acids in the metal binding motif of GR responsible for binding specificity (Severne et al., 1988) and a structural model for DNA binding and for receptor dimerization has been proposed from NMR studies (Hard et al., 1990a). The importance of DNA sequences which flank the GRE has been documented (Chalepakis et al., 1990); however, the issue of whether or not the synergism of GR action seen in cellular systems can be duplicated in vitro has not been fully resolved.

To further elucidate the initial binding events, we investigated the interaction of GR with model

Abbreviations used: GR, glucocorticoid receptor; GRE, glucocorticoid responsive element; MMTV, mouse mammary tumor virus.

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elements derived from the promoter region of mouse mammary tumor virus (MMTV), a gene which is subject to well-documented glucocorticoid-dependent transcriptional regulation (Payvar et al., 1981). A preliminary account of these results has been previously presented (Benore-Parsons and Wennogle, 1989).

MATERIALS AND METHODS

Purification of Glucocorticoid Receptor

Receptor was purified from the livers of adrenalectomized rats by the method of Wrange et al. (1979), using saturating concentrations of ^{3}H-triamcinolone acetonide (New England Nuclear) (Wrange et al., 1984). The procedure was modified so that the phosphocellulose separation was carried out as a batch step rather than by column chromatography, resulting in significant time savings. Photoaffinity labeling was accomplished by UV crosslinking, using a Spectra-line transilluminator, and the steroid-receptor complex was characterized by SDS-polyacrylamide electrophoresis and autoradiography (Wrange et al., 1984). When the receptor was characterized in this manner, following UV crosslinking with ^{3}H-triamcinolone acetonide, a single band was visualized with an apparent molecular mass of 100,000 (Fig. 1). Protein determinations indicated that roughly 20% of the protein in the receptor preparation bound triamcinolone acetonide. This receptor was compared with that detailed in the original publication (Wrange et al., 1979) (sample kindly provided by Dr. J. Carlstedt-Duke) and were found to be indistinguishable in our binding assays. It should be emphasized that this receptor has been extensively characterized and its use well-documented by the authors of the original purification.

Fig. 1. SDS-PAGE of purified glucocorticoid receptor. ^{3}H-Triamcinolone acetonide:receptor complexes were crosslinked by photoaffinity labeling and subjected to 10% SDS/PAGE gel electrophoresis: **(a)** 390 ng of receptor-steroid complexes not exposed to UV light; **(b)** Complexes which were exposed to UV irradiation (Materials and Methods). Bio Rad molecular weight markers were used to determine the molecular mass of the GR (triangle) as approximately 100,000.

DNA Fragment Isolation and Labeling

The MMTV promoter fragment referred to as MMTV(−644:−103) contains a 541 bp section located at a site 644 to 103 bp from the transcription start site (sample provided by Dr. Michael Uhler). The fragment was ligated into the BamH 1/Sac 1 site in the plasmid Bluescript (Stratagene) and transformed into XL1-BLUE cells (Stratagene). Plasmid was isolated by the cesium chloride gradient method, digested with BamH 1/Sac 1 restriction enzymes, and the promoter fragment was purified from low melt agarose following electrophoresis. MMTV(−644:−103) was end-labeled with ^{32}P-dGTP using the Klenow fragment of *E. coli* DNA polymerase. Free nucleotides were removed by Sephadex G-50 spun column chromatography. Fragment pBR (1:651), used as a nonspecific competing DNA fragment, was obtained similarly by EcoR 1/Sal 1 digestion of pBR322 (Boehringer Mannheim). This fragment does not contain a recognizable glucocorticoid response site. Synthetic oligomers (described below) were ligated into either the Sma 1 (40 bp fragment) or Sal 1 (30 bp fragment) sites in the multiple cloning

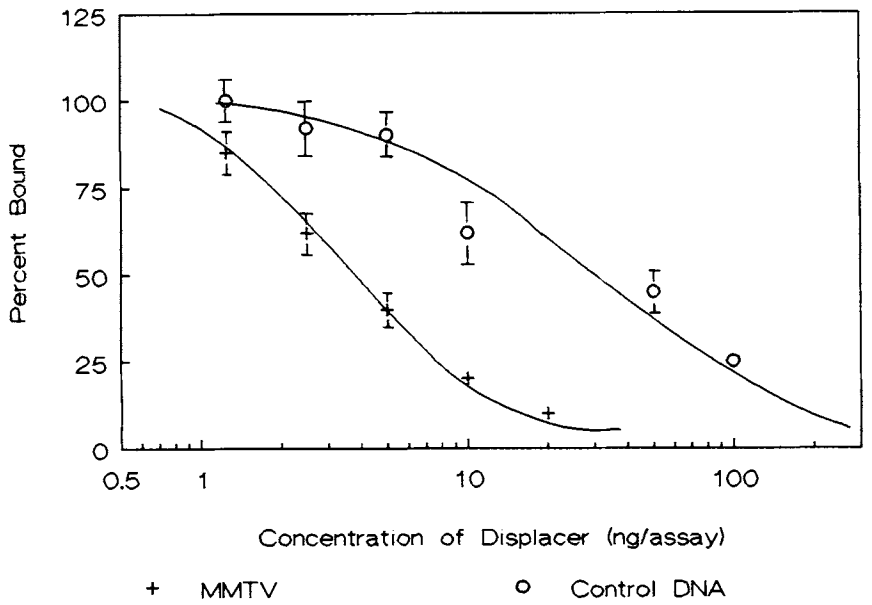

Fig. 2. Competition for binding to glucocorticoid receptor. Increasing amounts of unlabeled MMTV(−644:−103) or calf thymus DNA were included in the assay at fixed concentrations of receptor. This plot is a compilation of three assays.

region of phagemid Bluescript II and transformed into XL1-Blue cells (Stratagene), using standard transformation methods. Plasmid was isolated and fragments obtained by Kpn 1/Sac 1 digestion followed by purification on a GENFAX HPLC column (DuPont). Oligomers were synthesized using a Pharmacia Gene Assembler. Calf thymus DNA was obtained from Sigma. Concentrations were determined by UV absorption at 260 nm and by fluorescence spectrophotometry using Hoechst dye 33358 and the Hoefer Mini-Fluorometer. Concentrations of ^{32}P-MMTV were determined prior to labeling and should be considered approximate, as they do not account for losses during labeling. Restriction enzymes were obtained from Boehringer Mannheim.

Nitrocellulose Filter Binding Assay

Unless noted otherwise, for displacement experiments 10 ng of ^{32}P-MMTV(−644:−103) was incubated with 8 ng of GR (with saturating triamcinolone acetonide bound to the receptor) and varying concentrations (0 to 300 ng) of competing DNA fragments in 250 μl of assay buffer (10 mM Tris pH 7.5, 1 mM magnesium acetate, 0.1 mM EDTA, 10 mM DTT and 5% glycerol) supplemented with 150 mM NaCl and 80 μg/ml insulin. All manipulations were performed at 0°C. After a 45-min incubation, the mixtures were diluted with 2 ml of cold assay buffer, immediately filtered on prewet nitrocellulose filters (Schleicher & Schuell), and rinsed with 2 ml of buffer using filtration rates of 2 ml/min. Radioactivity was determined by scintillation counting. Up to 100% of the radioactivity was bound to filters at high concentrations of receptor, whereas less than 2% of the labeled ^{32}P-MMTV(−644:−103) was retained in its absence. To determine cooperative effects of MMTV binding to GR, 0–100 ng GR was used per assay at a fixed DNA concentration of 10 ng (Ptashne, 1987).

RESULTS

Competition of DNA Fragments for Binding to Glucocorticoid Receptor

Nitrocellulose filter binding assays were employed to compare the relative efficacy of various sequences in competing with ^{32}P-MMTV(−644:−103) for binding to receptor. Unlabeled MMTV(−644:−103) efficiently displaced the binding of ^{32}P-MMTV(−644:−103) to GR as shown in Figure 2. Calf thymus DNA, used to define nonspecific interactions, required 10-fold greater concentrations to displace 50% of the labeled fragment. Similar results were obtained when pBR(1:651) was used as the competing fragment (data not shown). As described by von Hippel and Berg (1986), a 540 bp DNA fragment

TABLE I. Effect of Fragment Length on the Ability of Synthetic Oligomers to Compete With ^{32}P-MMTV (−644:−103) for Binding to GR*

Displacer fragment	Percentage radiolabel bound
MMTV(−194:−170) → TTTTGGTTACAAACTGTTCTTAAAA AAAACCAATGTTTGACAAGAATTTT	93.5 ± 9.0
MMTV(−204:−160) → TTAAGTAAGTTTTTGGTTACAAACTGTTCTTAAAACGAGGATGTG AATTCATTCAAAAACCAATGTTTGACAAGAATTTTGCTCCTACAC	39.1 ± 7.7
MMTV(−214:−150) → TTCCCAGGGCTTAAGTAAGTTTTTGGTTACAAACTGTTCTTAAAACGAGGATGTGAAGACAAGTG AAGGGTCCCGAATTCATTCAAAAACGAATGTTTGACAAGAATTTTGCTCCTACACTTCTGTTCAC	35.2 ± 8.4

*Binding of radiolabeled ^{32}P-MMTV(−644:−103) to GR was performed in the presence of 246 ng of competitor DNA. Results are expressed as the percentage of cpm bound in the absence of displacer (mean ± SEM), using triplicate determinations. GR binding sites are denoted by arrows.

TABLE II. Ability of Oligomeric Sequences to Compete With ^{32}P-MMTV for Binding to Receptor*

Insert	Orientation	Length	^{32}P-MMTV bound
1 × 40mer	→	138 bp	49.3 ± 8.6
1 × 40mer	←	138 bp	53.4 ± 9.6
3 × 40mer	←→→	218 bp	25.4 ± 1.9
2 × 30mer	→→	158 bp	29.6 ± 4.4
MMTV(−644:−103)		542 bp	17.7 ± 1.2

*Inserts of 30 and 40 bp were synthesized and inserted into Bluescript II, excised with restriction enzymes and the resulting fragments tested in the binding assay as described in Methods. Binding of ^{32}P-MMTV(−604:−103) was performed in the presence of 75 ng of competitor DNA. Results are the averages of three assays in quadruplicate. Each fragment contained flanking sequences from the plasmid, accounting for the extra size. Shown below are the inserted sequences referred to as 30mer and 40mer. As written, these sequences correspond to the leftward pointing arrows listed above. The GRE consensus sequence is in bold letters.

30mer TCGACCTAAGTTTTT**GGTACAATATGTTCT**G
GGATTCAAAAA**CCATGTTATACAAGA**CAGCT

40mer GGTCGACGTTTTT**GGTACAATATGTTCTCT**AGAGTCGACC
CCAGCTGCAAAAA**CCATGTTATACAAGAGA**TCTCAGCTGG

represents 525 independent 15 bp binding sites. Because all but four such sites in MMTV(−644:−103) correspond to nonspecific sites, the affinity of GR responsive elements versus nonspecific DNA is significantly greater than 10-fold. The flatter shape of the calf thymus curve may reflect the fact that multiple nonspecific sites exist.

The effects of DNA size and flanking sequences were investigated, using a series of synthetic oligomers identical to a site in MMTV (−644:−103), designated by footprinting experiments as site 1.3 (Payvar et al., 1983). The larger (45 and 65 bp) fragments competed for binding effectively, while a 25 bp fragment was ineffective, as outlined in Table I. In addition, fragments of 30 and 40 bp (listed at the bottom of Table II) were unable to compete effectively for binding (results not shown).

A second series of oligomers containing GREs were synthesized and used to determine whether or not fragments containing multiple GRE's

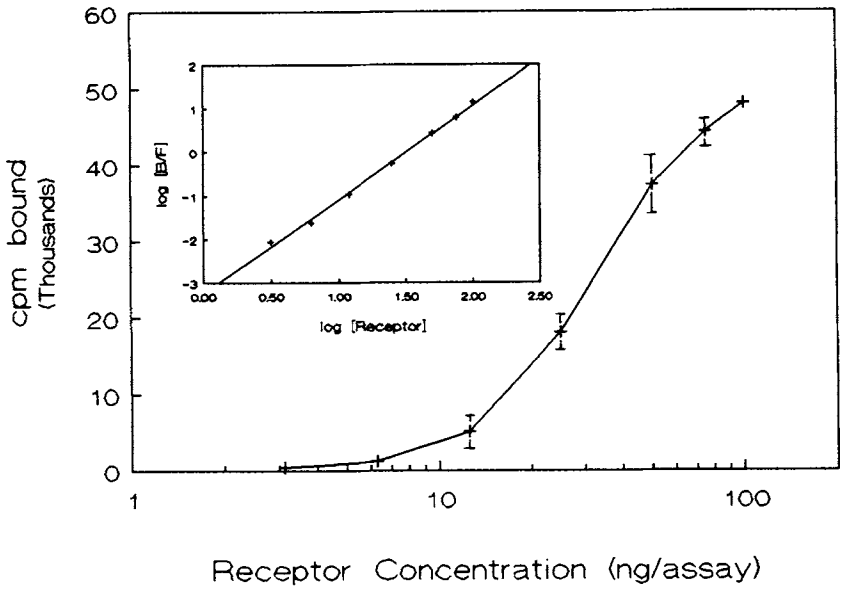

Fig. 3. Cooperativity of MMTV(−644:−103) binding to glucocorticoid receptor. Ten nanograms of ^{32}P-MMTV(−644:−103) was incubated with 0–100 ng of receptor in the binding assay. When replotted as a function of bound/free ^{32}P-MMTV(−644:−103) vs. receptor concentration, a Hill coefficient is obtained **(inset)**. This plot is representative of typical curves obtained in seven independent experiments.

would have an enhanced ability to compete with MMTV(−644:−103) for binding to GR. Two fragments, containing 30 and 40 bp respectively, were synthesized, each containing a single consensus sequence site with flanking sequences designed to allow incorporation into plasmid vectors (Table II). Although unable to compete effectively for binding as small oligomers, when ligated into plasmid and larger fragments which contained the inserts were excised and tested, competition with ^{32}P-MMTV(−644:−103) was observed. Fragments containing two insert copies were more effective competitors than those with single inserts, displacing labeled substrate nearly as effectively as MMTV(−644:−103) (Table II).

Cooperativity of GR Binding to DNA

Experiments in which ^{32}P-MMTV(−644:−103) was incubated with increasing receptor concentrations resulted in a sigmoidal binding curve (Fig. 3). When plotted as a function of bound/free ^{32}P-MMTV(−644:−103) versus receptor concentration, a Hill coefficient of 2 was obtained.

Discussion

Glucocorticoid receptors are among the best characterized eukaryotic trans-acting factors, and constitute a prototype for the family of steroid nuclear receptors. Members of this hormone receptor family, which include progesterone, estrogen, and thyroid receptors, bear striking similarities to one another in their primary amino acid sequence, as well as in the cis-acting responsive elements to which they bind (Mader et al., 1989; Gustafsson et al., 1987; Mitchell and Tjian, 1989). Site-directed mutagenesis, transcription assays, and protein mapping have functionally defined several domains of steroid hormone receptors, including carboxy-terminal hormone-binding and mid-sequence DNA-binding domains (Payvar and Wrange, 1983; Carlstedt-Duke et al., 1987; Reichman et al., 1984). Two trans-activating domains have been mapped in the glucocorticoid receptor, one sequence near the amino-terminal and another between the DNA-binding and hormone-binding domains (Hollenberg and Evans, 1988). The function of these trans-acting domains has not been fully elucidated, but they apparently act to promote protein-protein interactions.

Protein-protein interactions are essential to the action of GR. It is well documented that GR dimers bind to a single consensus GRE (Tsai et al., 1988; Chalepakis et al., 1988), although it is unclear whether or not dimer assembly occurs prior to or during binding. The cooperative binding behavior reported in this study is consistent with a stimulatory interaction between GR monomers, as predicted by either model (Hard

et al., 1990b; Tsai et al., 1988). In addition, a higher order of cooperative effects is suggested by the ability of constructs containing two GRE's to compete more effectively for binding than comparable constructs containing a single site. Unlabeled MMTV(−644:−103) which contains multiple consensus elements was the most potent competitive agent found and its effects are most likely due to a combination of fragment size and to the arrangement of multiple response elements in its promoter region. Evidence suggests that GR transacting domains possess a mechanism enabling interactions with other GR dimers or with other trans-acting factors to form macromolecular complexes involved in the transcriptional process (Ankenbauer et al., 1988; Kakidani and Ptashne, 1988; Yang-Yen et al., 1990). Synergistic actions of multiple responsive elements have been demonstrated using reporter genes in mammalian cells; however, using these assays it was not possible to determine whether or not the increase in activity was due to interactions between receptor dimers and/or to the involvement of other transcription proteins (Ankenbauer et al., 1988). The fact that purified components were employed in the present study supports the former explanation (see also Schmid et al., 1989; Tsai et al., 1989; Perlmann et al., 1990; Tully and Cidlowski, 1990).

Although the glucocorticoid-responsive consensus element has been defined as a sequence of 15 base pairs, the present experiments show that longer sequences optimize GR binding. Fragments containing 15 bp consensus elements 25 to 40 bp in length were ineffective in competing for MMTV(−644:−103) and the minimum sequence found to bind to GR was a 45 bp fragment from the 1.3 region of MMTV. These results indicate that flanking DNA sequences interact with GR, an observation consistent with footprinting and gel retardation analysis (Payvar et al., 1983; DeFranco et al., 1985; Schmid et al., 1989; Chalepakis et al., 1990). Comparisons of the sequences surrounding MMTV sites 1.2 and 1.3 show them to be similar, each containing the sequence TAAGTTTTT adjacent to the consensus sequence and suggesting that these nucleotides may impart specific structural information. In addition to specific binding at glucocorticoid responsive elements, GR binds in a nonspecific manner to DNA, albeit at lower affinity, as reported here and by other groups (Jantzen et al., 1987; Tully and Cidlowski, 1989, 1990; Hard et al., 1990b; Chalepakis et al., 1989, 1990; Perlmann et al., 1990).

The emerging picture derived from in vitro binding experiments and from cellular transcriptional assays is that GR acts as a catalyst for the macromolecular assembly of competent transcription complexes. The potential to identify other factors critical to complex formation render the use of reconstitution binding assays as an effective tool to complement other studies in understanding gene regulation by glucocorticoids and related nuclear receptors.

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