Identification of Multiple Glucocorticoid Receptor Binding Sites in the Rat Osteocalcin Gene Promoter†

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ABSTRACT: The biosynthesis of osteocalcin (OC), a bone-specific, noncollagenous protein, is stringently regulated during differentation of the osteoblast phenotype. Glucocorticoids, and also $1,25(OH)_2D_3$, mediate the developmental regulation of OC gene transcription. In this study, we established that the −1097 to +23 promoter (pOCZCat) of the rat OC gene confers glucocorticoid responsiveness to both basal and vitamin D-induced OC expression. The presence of multiple glucocorticoid receptor (GR) binding sites in the proximal rat OC gene promoter was determined by the combined use of DNase I footprinting, dimethyl sulfate fingerprinting, and gel mobility shift analysis with glucocorticoid receptor protein. One glucocorticoid receptor binding element (GRE) resides immediately downstream of the TATA box (−16 to −1). In vivo activity was established by cotransfection of ROS 17/2.8 osteosarcoma cells with an OC–CAT construct in the presence of cloned GRE sequences (wild type or mutant) as competitors. A putative second, less protected GR binding site is located further upstream in the OC gene basal promoter within the region overlapping the TATA box. This is in direct contrast to the organization of GREs in the human OC proximal promoter wherein GR binding at the upstream GRE overlapping the TATA is stronger than at the downstream GRE. In addition, we detected sequence-specific binding of GR protein to another basal promoter element, the OC box (−99 to −76), which contains a central CCAAT motif. The presence of multiple GR binding sites in the rat OC gene proximal promoter indicates that regulation of basal and vitamin D-enhanced transcription by glucocorticoids may involve the integrated activities of multiple, independent GREs.

Steroid and steroid-like hormones are potent modulators of transcription that bind to intracellular receptors. Steroid hormone receptors belong to a large superfamily of ligand-dependent transactivating zinc finger proteins. These receptors undergo a conformational change upon binding of their ligand and interact with specific DNA recognition sites, steroid responsive elements (Beato, 1989; Evans, 1988; Muller & Renkawitz, 1991). Glucocorticoids induce hepatic gluconeogenesis; they promote the development of various organs and also stimulate and/or inhibit the growth of many cell types in vivo [reviewed in Muller and Renkawitz (1991)].

Glucocorticoids have significant effects on bone and mineral metabolism (Gennari, 1985). In vivo, the skeletal effects of glucocorticoids, which include increased bone resorption (DeFranco et al., 1992) and decreased bone formation (Gennari, 1985), are associated with diminished osteoblastic activity and a decline in serum levels of osteocalcin (OC) (Ekenstamm et al., 1988; Lukert et al., 1986). In vitro, an inhibition of OC biosynthesis has been observed in bone organ culture (Eilam et al., 1980), in outgrowth cultures from trabecular explants (Wong et al., 1990), and in several osteosarcoma cell lines (Morrison et al., 1989; Rodan et al., 1984), but not in fetal rat-derived calvarial cells (Chen et al., 1977). Effects of the synthetic glucocorticoid dexamethasone on OC gene expression in normal diploid rat osteoblasts (Shalhoub et al., 1992), in human osteoblast-like cells (Wong et al., 1990; Subramanian et al., 1992), and in ROS 17/2.8 osteosarcoma cells (Schepmoes et al., 1991) at the mRNA, transcription, and protein levels have been observed.

The biosynthesis of osteocalcin, a bone-specific, noncollagenous protein, is stringently regulated during development and maintenance of the osteoblast phenotype (Owen et al., 1990). Selective developmental expression of the osteocalcin gene, both in normal rat osteoblasts in culture and in several transformed osteosarcoma cell lines, is transcriptionally regulated by a broad spectrum of hormones and other physiological mediators, as established by activities of the rat as well as the human OC gene promoters, in vivo and in vitro [reviewed in Lian et al. (1992) and Stein et al. (1992)]. The active metabolite of vitamin D, $1,25(OH)_2D_3$, enhances OC gene expression, and the vitamin D responsive elements (VDREs) have been identified in the proximal rat OC gene promoter (Demay et al., 1990; Terpening et al., 1991; Yoon et al., 1988; Markose et al., 1990) and the human promoter (Morrison et al., 1989; Kerner et al., 1989). Dexamethasone represses basal OC gene expression in Ros 17/2.8 cells and significantly reduces vitamin D-stimulated OC gene expression (Morrison et al., 1989; Schepmoes et al., 1991; Bortell et al., 1992; Terpening et al., 1991). The transcription inhibitory effects of glucocorticoids have been shown to be mediated by proximal promoter sequences (−196 to +34) in the human OC gene (Morrison et al., 1989). In vitro analysis identified a glucocorticoid receptor binding element (Karin et al., 1984)in the proximal human OC gene promoter that overlaps the TATA box (Stromstedt et al., 1991).

In this study, we identified multiple GR binding sites in the proximal rat OC gene promoter and demonstrated differences

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Table I: DNA Sequence of Gene Segments Used for Nuclear Factor Binding and Competition Studies[a]

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-141  -114        -98                          -76  -43  -31      -16              -1      +23
---------TGACC----TGACCCCCAATTAGTCCTGGCAG-----------TATA---AGAACA--AGTCCC-------------
PstI                                                 DdeI                          HindIII

Rat osteocalcin promoter:
                -26                                                 +9
  GRE wt        5' AAGGTATTGCAGAACAGACAAGTCCCACACAGCAA 3'

  GRE mu        5' AAGGTATTGCATACCAGACAAGGCGCACACAGCAA 3'

                -120                                          -89            -76
  rOCbox45      5' TGGGTTTGACCTATTGCGCACATGACCCCCAATTAGTCCTGGCAG 3'

  rOCbox32      5' TGGGTTTGACCTATTGCGCACATGACCCCCAA 3'

Human histone promoter:

  TM3           5' TCCCCTGATATACAAGAGTATCGGACCAGATTGAAAACCGAAAGCGGATC 3'
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[a] Steroid half-elements (TGACC) and glucocorticoid receptor binding half-elements (6 nt) are underlined, and GRE point mutations and CCAAT and TATA elements are in boldface.

in the location of GR binding sequences within 5′ regulatory domains between the rat and human OC genes (Stromstedt et al., 1991). One GRE (–16 to –1) resides immediately downstream of the TATA box. A second binding site is found further upstream in the basal promoter overlapping the TATA box, which corresponds with the sequence organization of the GRE/TATA domain in the human OC gene promoter. Additionally, we detected sequence-specific binding of the GR within another basal promoter element, the OC box (–99 to –76) containing a CCAAT motif as a central core. The presence of multiple GR binding sites indicates that regulation of basal and vitamin D-enhanced transcription by glucocorticoids may involve the integrated activities of (1) multiple, independent GREs within the modularly organized OC gene promoter and (2) functional interactions between the activities of several factors that exhibit occupancy of sequence-specific transcriptional regulatory domains.

MATERIALS AND METHODS

Cell Culture. Rat osteosarcoma-derived ROS 17/2.8 cells (gift of S. Rodan and G. Rodan; Merck Sharp & Dohme, West Point, PA) were maintained in Ham's F12 medium supplemented with 5% fetal bovine serum, calcium to 1.1 mM, and antibiotics (Majeska et al., 1980). ROS 17/2.8 cells were plated at a density of 5×10^5 per 100-mm dish and were passaged every 10 days. Cells were harvested between day 8 and day 10. Levels of secreted osteocalcin were quantitated by radioimmunoassay (Gundberg et al., 1984).

Gel Mobility Shift Assays. The following rat OC gene DNA probes (Table I) were used in gel mobility shift assays: a *Dde*I–*Hin*dIII fragment (–43 to +23) (containing the TATA box region); an oligonucleotide, designated rOCbox45, spanning the OC box domain (–120 to –76); and a *Pst*I–*Hin*dIII fragment which encompasses the OC box region and the TATA region (–141 to +23). All probes were labeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase (New England Biolabs, Berkeley, MA) and were purified by polyacrylamide gel electrophoresis.

The truncated human glucocorticoid receptor protein was overexpressed in insect cells using the baculovirus expression system as described previously (Alnemri et al., 1991). This GR contains the first 532 amino acids which encompass the entire N-terminal transcriptional activation domain (Hollenberg & Evans, 1988) and the DNA binding domain (Dalman et al., 1988; Freedman et al., 1988), but it lacks the C-terminal steroid binding domain (Denis & Gustafsson, 1989; Pratt et al., 1989).

The truncated glucocorticoid receptor was partially purified from nuclei by extraction with 50 mM $MgCl_2$ in 50 mM HEPES buffer, pH 7.4. The receptor preparation was more than 80% pure after dialysis against 50 mM HEPES buffer (pH 7.4)/150 mM KCl. ROS 17/2.8 nuclear extracts were prepared by the method of Dignam et al. (1983) as modified by Holthuis et al. (1990). DNA binding reactions were performed as described previously (Markose et al., 1990). One microgram of poly(dG-dC)·poly(dG-dC) (Pharmacia, Piscataway, NJ) was included in gel mobility shift assays for the TATA region probe (–43 to +23), and the 179 bp probe (–141 to +23) with ROS 17/2.8 nuclear extract and 0.1 μg of poly(dI-dC)·poly(dI-dC) was used in assays with purified GR protein. For ^{32}P-end-labeled rOCbox45 oligonucleotide (Table I), 2 μg of poly(dG-dC)·poly(dG-dC) and 1 μg of poly(dA-dT)·poly(dA-dT) or poly(dI-dC)·poly(dI-dC) were used. Antibodies (ab) were incubated with the binding reactions for 20 min prior to (preincubation) addition of probe. The GR antibody is the hybridoma supernatant of a monoclonal (mouse) anti-GR antibody (IgG_2) (clone BUGR2) (both reagents obtained from Affinity BioReagents, Neshantic Station, NJ) (Shea et al., 1991). Two migrograms of the GR antibody was used in gel mobility shift assays. An HSP90 monoclonal antibody which does not react with GR was also obtained from Affinity Bioreagents. For the competition experiments, double-stranded oligonucleotides were synthesized and are listed in Table I.

DNase I Footprinting and DMS Protection. DNA binding reactions were carried out in a volume of 50 μL with 10–20 pmol (0.5–1 μg) of purified GR protein, 0.1 μg of poly(dI-dC)·poly(dI-dC) DNA, 1 mM $MgCl_2$, and bovine serum albumin (BSA) to bring the binding reaction to 6 μg of protein and ^{32}P-end-labeled probe. After incubation for 15 min at room temperature, DNase I (Promega, Madison, WI) was added (0.25–2 units) and incubated for 30 s–2 min. The reaction was stopped by adding an equal volume of 0.2% SDS/40 mM EDTA. Following an ethanol precipitation and an ethanol wash, the dry pellet was resuspended in formamide buffer and loaded on an 8% denaturing polyacrylamide gel.

For dimethyl sulfate (DMS) protection analysis, a similar DNA binding reaction, but without the addition of $MgCl_2$, was incubated for 15 min at room temperature. After incubation, 1 μL of DMS and 150 μL of H_2O were added, and the methylation reaction was stopped after 30 s. Methylation of guanine residues and the subsequent piperidine cleavage were performed as described by Maxam and Gilbert (1977).

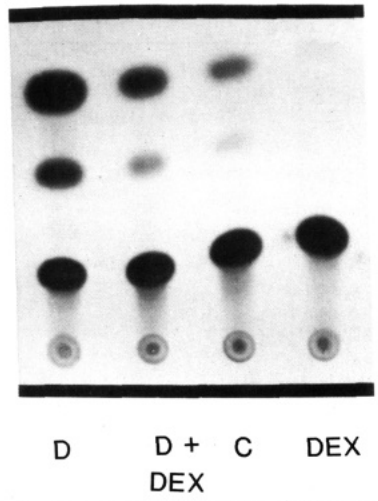

FIGURE 1: Influence of glucocorticoids on transcriptional activity of the rat OC gene promoter. ROS 17/2.8 osteosarcoma cells were transfected with pOCZCat (the −1097 to +23 rat OC promoter linked to the CAT reporter gene) and treated with complete media (C), 10^{-8} M $1,25(OH)_2D_3$ (D), 10^{-6} M dexamethasone (DEX), or 10^{-8} M $1,25(OH)_2D_3$ and 10^{-6} M dexamethasone (D+DEX). Four independent experiments with duplicate assays for each treatment were done. A representative autoradiogram of CAT assays is shown.

Gradient Gel Electrophoresis. The DNA binding reaction mixtures from gel mobility shift assays were electrophoretically fractionated in native [80:1 acrylamide:bis(acrylamide) ratio] 20–4% gradient polyacrylamide gels at 200 V for 24 h at 4 °C.

Transfection Assays. ROS 17/2.8 cells plated at a density of 5×10^5 cells/100-mm plate were used for transient transfection experiments by the DEAE-dextran method (Ausubel et al., 1989). Transfections for glucocorticoid responsiveness were done with 10 μg of pOCZCat (−1097 to +23) (Schepmoes et al., 1991) and 10 μg of salmon sperm DNA for a total of 20 μg of DNA/plate. Cells were subjected to glycerol-shock 3 h post-transfection and then incubated in F12 supplemented with 5% fetal calf serum (control), 10^{-8} M $1,25(OH)_2D_3$ (vit D), 10^{-6} M dexamethasone (dex), or 10^{-8} M $1,25(OH)_2D_3$ plus 10^{-6} M dexamethasone (vit D + dex). Cells were harvested at 60 h, and CAT activity was determined.

Cotransfections were performed with pOCZ-CAT (1 μg) as the target plasmid and the cloned oligonucleotides (10 μg) as the competitors. The competitors are constructs that consist of oligonucleotides that include the downstream GRE (−16 to −1) or a mutated GRE (Table I). For these experiments, the native and mutated GRE-containing sequences are cloned in pUC19, and 9 μg of pUC19 carrier DNA was transfected. In the control assays, 1 μg of pOCZCAT and 19 μg of pUC19 carrier DNA were transfected. Cells were glycerol-shocked as above and then cultured 48 h in complete culture media supplemented with 10^{-8} M $1,25(OH)_2D_3$ to promote maximal levels of OC gene transcription.

CAT Assays. Chloramphenicol acetyltransferase activity was determined as described (Ausubel, 1989). The samples were incubated with 0.25 μCi of [^{14}C]chloramphenicol (Dupont, Boston, MA) for 12 h. The samples were extracted with ethyl acetate and subjected to thin-layer chromatography (TLC) (Whatman Labsales, Hillsboro, OR). The TLC plates were exposed to KODAK X-OMAT.AR film, and CAT activity was quantified by photodensitometry.

RESULTS

Identification of OC Gene Glucocorticoid Responsiveness and GR Promoter Binding Sequences. We demonstrated in Figure 1 for these experiments that glucocorticoids (dexamethasone) inhibit both basal and vitamin D-stimulated expression of a rat OC promoter −1097 to +23–reporter construct transfected into ROS 17/2.8 cells, confirming previous reports (Schepmoes et al., 1991; Terpening et al., 1991; Shalhoub et al., 1992). To address the molecular mechanisms that mediate glucocorticoid regulation of OC gene transcription, we examined the proximal rat OC gene 5′ regulatory sequences for GR binding sites.

Gel mobility shift assays using 2–20 pmol (0.1–1.0 μg) of partially purified GR and a ^{32}P-end-labeled rat OC gene promoter fragment (−43 to +23) containing the TATA box revealed a slowly migrating protein–DNA complex and some minor, faster migrating complexes (Figure 2A). Progressively increasing amounts of protein resulted in an abrupt transition at 0.4 μg of GR from unbound ^{32}P-end-labeled DNA to the formation of a protein–DNA complex with reduced electrophoretic mobility. These nonlinear protein–DNA binding kinetics strongly suggest cooperative binding. The major, slowly migrating complex could specifically be competed by preincubating with a GR antibody (Figure 2B).

An estimation of the size of the slowly migrating GR–DNA complex formed with the partially purified GR was made by electrophoretic fractionation of a binding reaction mixture on a native [80:1 acrylamide:bis(acrylamide) ratio] 20–4% gradient gel with molecular mass markers (Figure 2C). The approximate size corresponds to a GR tetramer, although we cannot exclude the possibility that other GR-associated proteins, e.g., HSP90, are components of the protein–DNA complex [reviewed in Muller and Renkawitz (1991)]. A monocional HSP90 antibody causes a supershift of some minor GR–DNA complexes and competes with the major, high molecular mass protein–DNA complex (data not shown).

To identify the target sequences of the GR protein, we carried out DNase I footprint and DMS protection experiments using 10–20 pmol (0.5–1 μg) of GR protein and an asymmetrically ^{32}P-end-labeled OC gene promoter fragment (−43 to +23). The protected region in the upper strand spans the sequence from −18 to +7 and in the lower strand from −22 to +1 (Figure 3A). Increasing amounts of protein do not change the boundaries of the footprint (data not shown), and protection could be partially prevented by competing with a GRE-containing oligonucleotide, (−26 to +9; shown in Table I) but not by a TATA box oligonucleotide, designated TM3 (from another gene, H4 histone; Table I) (Figure 3B). A weak footprint upstream of the strongly protected region is present in both the upper and the lower strand, starting at nt −28 and −32, respectively (Figure 3A). The other boundary of the footprint is difficult to determine.

DMS protection analysis (Figure 4A) revealed several protected guanine residues in both the upper and the lower strands in the same region of the DNase I protection (Figure 4B). Additionally, weakly protected guanines were found immediately upstream of the GRE, in a region overlapping the TATA box and corresponding with the weak footprint (Figure 4B). The region of strong protection immediately downstream of the TATA box has a sequence motif which shares 10 nucleotides out of 12 with a positive GRE (Jantzen et al., 1987). However, the spacing between the two half-elements is 4 bp instead of 3 bp. The region of weak protection in the TATA box shows moderate homology with the consensus GRE and coincides with a GR binding site in the human OC promoter.

Contribution of OC Gene GREs to Transcriptional Control. We examined the contribution of the downstream GR binding site (−16 to −1) to regulation of transcriptional activity by performing in vivo competition cotransfections into ROS 17/

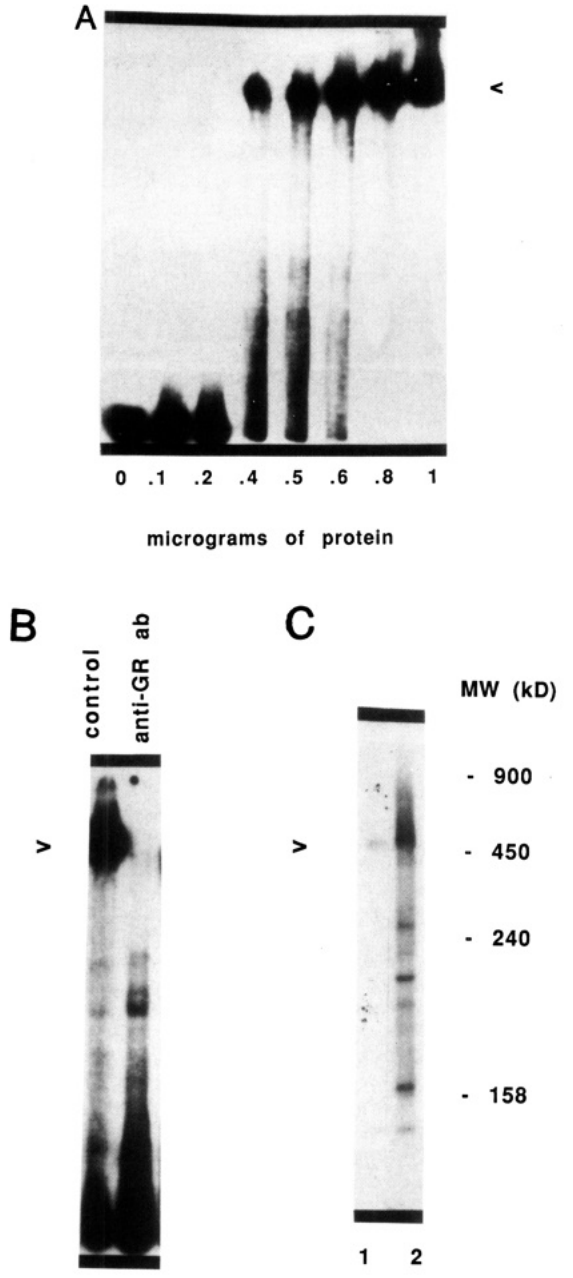

FIGURE 2: Glucocorticoid receptor binding at the proximal region of the rat OC gene. Gel mobility shift assays were performed with GR protein and a ^{32}P-end-labeled *Dde*I–*Hin*dIII OC gene promoter fragment (–43 to +23). (A) The abrupt formation of a strong protein–DNA complex (arrow) with increasing amounts of GR protein suggests cooperative binding. (B) Using 10 pmol (0.5 μg) of GR protein, the slowly migrating protein–DNA complex (arrow) (control) could specifically be competed by incubation with 2 μg of GR antibody for 20 min prior to addition of probe (anti-GR ab). (C) A molecular mass estimate of the slowly migrating purified GR protein–DNA complex (arrow) was made by electrophoretic fractionation of a 10-μL binding reaction (lane 1) in a native polyacrylamide (80:1) 20–4% gradient gel with molecular mass markers (lane 2).

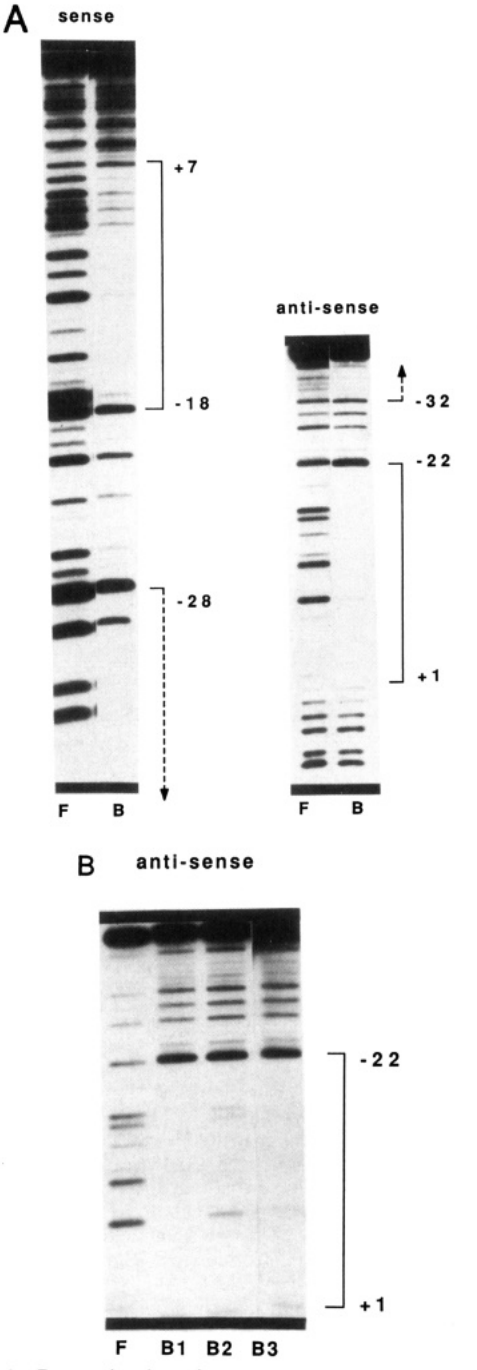

FIGURE 3: Determination of DNase I footprint boundaries with a proximal OC gene promoter fragment (–43 to +23). (A) 10–20 pmol (0.5–1 μg) of GR protein and an asymmetrically ^{32}P-end-labeled OC gene promoter fragment (–43 to +23) were incubated for 20 min in a 50-μL binding reaction with 1 mM $MgCl_2$ and BSA to a total amount of 6 μg of protein. Incubation with 0.5 unit of DNase I was for 1 min at room temperature. F, free probe; B, probe incubated with GR protein. (B) Addition of specific oligonucleotides to the binding reaction indicated that the nuclease protection was not due to TATA box binding factors, but by binding to a putative GRE. F, free probe; B1, probe incubated with GR protein; B2, probe incubated with protein and 100-fold molar excess of the GRE oligonucleotide; B3, probe incubated with protein and 100-fold molar excess of TATA-containing TM3 oligonucleotide. Brackets indicate regions of protection.

2.8 cells (Figure 5A). An OC gene promoter (–1097 to +23)–reporter construct, designated pOCZCAT, was transfected with an oligonucleotide spanning either the downstream GRE (–16 to –1) or a mutated GRE (see Table I); both competitor oligonucleotides were cloned in pUC19. Cotransfection of a 21-fold molar excess of the native GRE (–16 to –1) increased chloramphenicol acetyltransferase activity 3.6-fold compared to the control assay (Figure 5B). Cotransfection of the same molar excess of a cloned oligonucleotide, containing the mutated high-affinity GRE (–16 to –1), did not significantly alter transcriptional activity. This indicates that the GRE downstream of the TATA box (–16 to –1) competes for factors which regulate the transcriptional activity of the rat OC gene promoter. Moreover, increased transcriptional activity with cotransfected wild-type GRE suggests that the GRE acts as a negative cis regulatory element. This result is consistent with previous findings that dexamethasone decreased the transcriptional activity of the OC gene in ROS 17/2.8 cells,

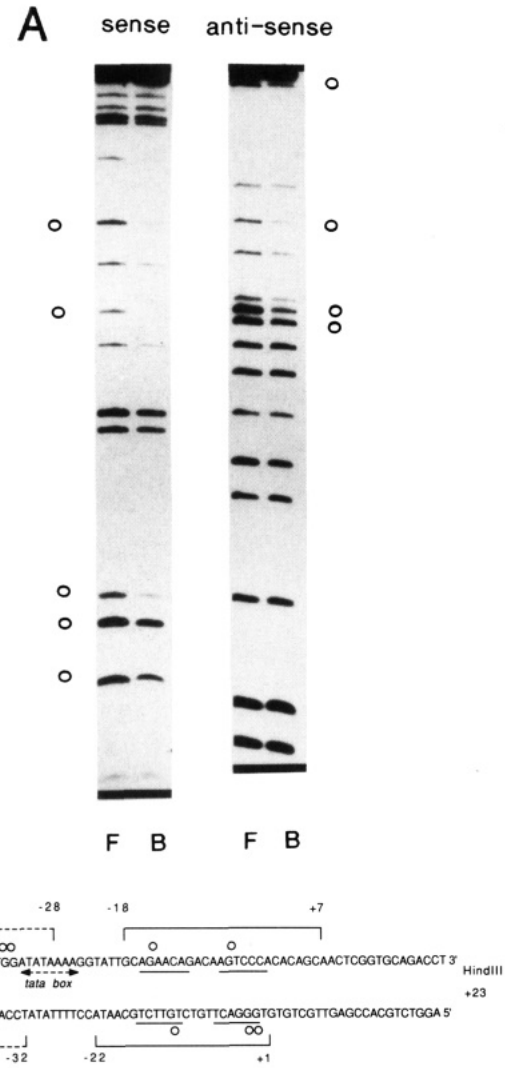

FIGURE 4: Dimethyl sulfate protection analysis at the TATA region (–43 to +23) of the OC gene promoter. (A) 10–20 pmol (0.5–1 μg) of GR protein and an asymmetrically ^{32}P-end-labeled TATA box containing OC gene promoter fragment (–43 to +23) were incubated for 20 min in a 50-μL binding reaction. 1 μL of DMS was added in a total volume of 200 μL and incubated for 1 min at room temperature. F, free probe; B, probe bound to protein. Circles indicate protected guanine nucleotides. (B) Summary of DNase I footprint regions and DMS-protected guanine residues for the –43 to +23 OC gene promoter fragment. The strong footprint is bracketed by a solid line, and the weaker footprint is indicated by a dashed line. G residue contacts are designated by open circles. Half steroid elements are underlined, and the TATA element is indicated by an arrow line.

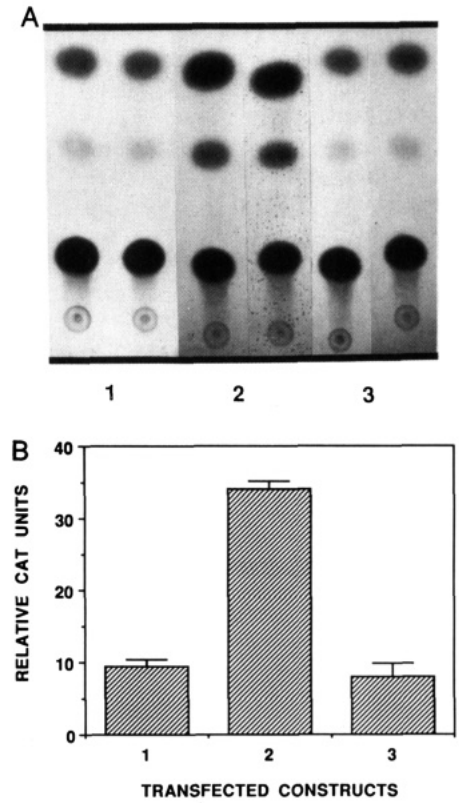

FIGURE 5: In vivo competition cotransfection into ROS 17/2.8 cells with pOCZCAT and wild-type or mutant GRE elements. (A) In panel 1, ROS 17/2.8 cells were cotransfected with 1 μg of pOCZCAT (–1097 to +23 segment of the OC gene fused to pGEMCAT) and 19 μg of pUC19 carrier DNA. In panel 2, cells were cotransfected with 1 μg of pOCZCAT, 9 μg of pUC19 carrier DNA, and 10 μg of wild-type GRE (–16 to –1) oligonucleotide cloned in pUC19 as a specific competitor. In panel 3, cells were cotransfected with 1 μg of pOCZCAT, 9 μg of pUC19 carrier DNA, and 10 μg of mutated GRE oligonucleotide cloned in pUC19. A representative autoradiogram of CAT assays done in duplicate is shown. See Table I for wild-type and mutated GRE sequences. (B) Quantitation of results presented in (A). The values represent mean ± SE of relative CAT assays from three independent experiments, each performed in triplicate.

independently or in combination with $1,25(OH)_2D_3$ (Schepmoes et al., 1991; Terpening et al., 1991).

Specificity of Protein–DNA Interactions at the OC Gene GREs. To examine the specificity of GR binding to the basal promoter element of the OC gene, we performed gel mobility shift competition experiments with the ^{32}P-end-labeled basal promoter fragment (–43 to +23) and 5–10 pmol (0.25–0.5 μg) of GR protein (Figure 6). We used 100- and 200-fold molar excess of an oligonucleotide (–26 to +9) that included the GRE (–16 to –1), a GRE mutant oligonucleotide, and a TATA box oligonucleotide designated TM3 (Table I). The GRE-containing oligonucleotide competed for the slowly migrating protein–DNA complex, whereas the mutants and the unrelated TATA box-containing oligonucleotide, TM3, did not compete.

Unexpectedly, an oligonucleotide (rOCbox45) containing the OC box region (–120 to –76) competed equally as well as the GRE oligonucleotide for the GR–DNA complex (Figure 6). Upon examination of DNA sequences in the OC box region, we identified two identical steroid-responsive half-elements TGACC (Beato, 1989), separated by 11 nt. One steroid-responsive half-element resides immediately upstream of the CCAAT sequence in the OC box (–98 to –94). The second steroid half-element is located further upstream of the OC box (–114 to –110). Therefore, to examine the possibility of GR binding interactions in the OC box region, we performed gel mobility shift assays with the GR protein, using ^{32}P-end-labeled rOCbox45 oligonucleotide. A slowly migrating protein–DNA complex was formed which was similar to the protein–DNA complex found with the TATA region probe (–43 to +23) (data not shown). The same complex was found using ROS 17/2.8 cell nuclear extracts in combination with GR (Figure 7). In addition, the previously identified "steroid related" protein–DNA interactions that are competed by rOCbox32 were observed, which includes the two steroid half-elements TGACC (Heinrichs et al., unpublished results). The slowly migrating complex was not competed by rOCbox32 and is specifically competed by GR ab and by the osteocalcin GRE-containing oligonucleotide but not by a GRE mutant oligonucleotide (Table I).

Because of our observation that GR binds to a region of the OC gene proximal promoter which contains the CCAAT element (Figure 7), we further examined the location of GR binding sites by DNase I and DMS protection assays, using

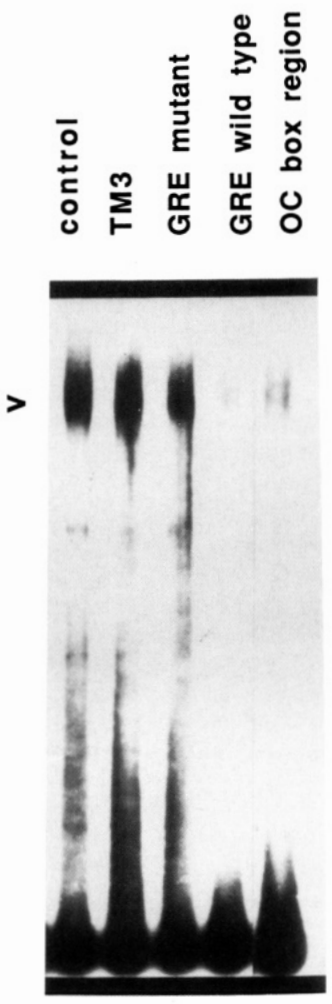

FIGURE 6: Binding specificity of the basal OC gene promoter fragment (–43 to +23) to the GR protein. Gel mobility shift assays with 10 pmol (0.5 μg) of GR protein and the –43 to +23 OC gene fragment as a probe were performed. Assays were performed in the absence of specific competitor DNA (control) or with a 200-fold molar excess of a TATA box oligonucleotide (TM3), a mutated GRE oligonucleotide (GRE mutant), an oligonucleotide containing the GR binding site (–16 to +1) downstream of the TATA (GRE wild type), or an oligonucleotide encoding the OC box region (–120 to –76). The sequences of the oligonucleotides are in Table I. The arrow indicates the GR protein–DNA interaction.

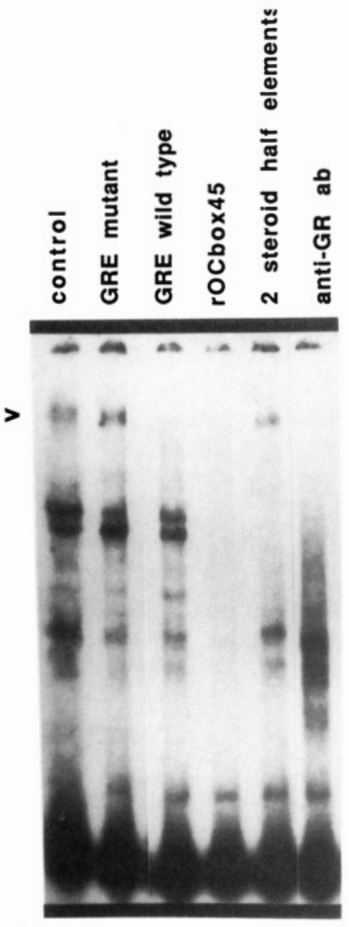

FIGURE 7: GR binding at the OC box region in the rat OC gene promoter (–120 to –76). Gel mobility shift assays with ^{32}P-end-labeled OC box45 (–120 to –76) oligonucleotide and a mixture of 1 μg of ROS 17/2.8 cell nuclear extract and 5 pmol (0.25 μg) of GR protein. Assays were performed in the absence of specific competitor DNA (control) or with a 200-fold molar excess of a mutated oligonucleotides (GRE mutant), an oligonucleotide containing the GR binding site (–16 to +1) downstream of the TATA (GRE wild type), an oligonucleotide containing the OC box domain (–120 to –76) (rOCbox45), an oligonucleotide containing only the distal OC box domain (–120 to –89) (2 steroid half elements), or 2 μg of GR antibody (anti-GR ab). The sequences of the oligonucleotides are in Table I. The arrow indicates the GR antibody-sensitive protein–DNA interaction which is not competed by the the two steroid half-elements oligonucleotide.

a probe spanning both the OC box region and the TATA region (–141 to +23). The assay showed two regions of DNase I protection (Figure 8): one downstream of the TATA region and the other which overlaps CCAAT-specific protein–DNA interaction sites (–96 to –76) (Heinrichs et al., unpublished results). This protected region contains a glucocorticoid-responsive half-element sequence immediately downstream of the CCAAT element. However, this putative GRE half-site, 5′AGTCCT3′, 3′TCAGGA5′, does not correspond to either of the two steroid-like half-elements upstream of the CCAAT element (see Table I, rOCbox45). These GR binding sites were corroborated by DMS protection assays, which showed protected nucleotides downstream of the TATA box, as well as two protected guanine residues immediately downstream of the CCAAT sequence in the OC box (Figure 9A). By using the OC box region as a competitor in DMS protection assays, protection of the nucleotides in the GRE downstream of the TATA box is lost in addition to that in the OC box. This confirms sequence-specific GR–DNA interactions and not a nonspecific DNA binding activity in the GR fraction. Unexpectedly, however, when we used this longer probe spanning both the OC box region and the TATA box domain, we no longer observed either DNase I or DMS protection in the GRE which overlaps the TATA box (–43 to –28). The DNase I footprint boundaries and the DMS-protected guanine residues are summarized in Figure 9B.

DISCUSSION

We have identified a strongly protected GR binding site in the rat OC gene promoter immediately downstream of the TATA box (–16 to –1) and a less protected binding site overlapping the TATA motif. This second GRE corresponds to a GRE in the human OC gene proximal promoter (–30 to –16), although the footprint borders and also the guanine residues exhibiting methylation interference are shifted several nucleotides upstream compared to the human OC gene promoter. Interestingly, the downstream GRE of the rat OC gene coincides with a region in the human OC promoter (–11 to –3) which showed DNase I protection in some, but not all, experiments reported by Stromstedt et al. (1991). Thus, the location of multiple GR binding sites in the human and rat OC promoter may be analogous, but the binding properties are apparently different; i.e., in the rat OC promoter, the more downstream GRE shows stronger protection, whereas in the human promoter the more upstream GRE shows greater

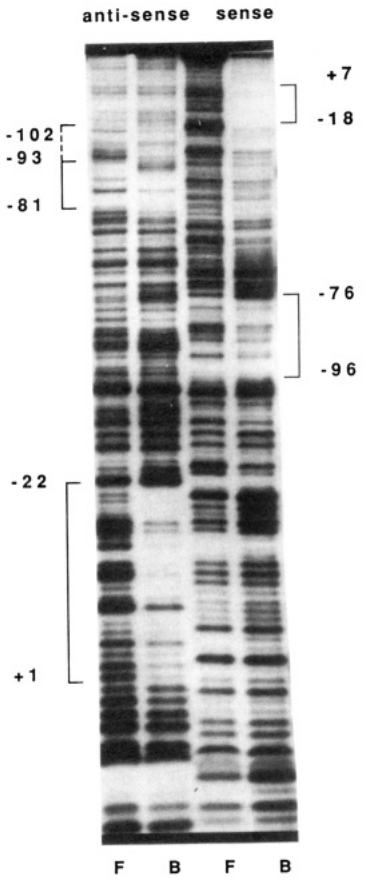

FIGURE 8: Determination of DNase I footprint boundaries with an OC gene promoter fragment containing both the OC box and the TATA region (–141 to +23). 10–20 pmol (0.5–1 μg) of GR protein and an asymmetrically ^{32}P-end-labeled OC gene promoter fragment (–141 to +23) were incubated for 20 min in a 20-μL binding reaction with 1 mM $MgCl_2$ and BSA to a total amount of 6 μg of protein. Incubation with 0.5 unit of DNase I was for 30 s at room temperature. F, free probe; B, probe bound to protein. Brackets indicate regions of nuclease protection.

protection. For each region of GR binding, the protected guanine residues are located within the corresponding DNase I footprint region, and also the extent of protection, as observed in both DMS protection and DNase I footprint assays, is similar.

The spacing of 4 bp in the downstream high-affinity GRE is different from the 3 bp consensus spacing which appears to be essential for optimal dimer formation (Luisi et al., 1991). However, several steroid response elements have been reported where a strict spacing between half-elements is not observed, and dimer binding occurs with similar efficiency (Mader et al., 1993). This also may explain the identification of GR binding sites which show homology with the consensus GRE in only one half-element.

The protein–DNA binding kinetics as observed in gel mobility shift assays suggest cooperative binding of GR protein to cognate steroid hormone regulatory promoter elements (Drouin et al., 1992). It has been shown that cooperative binding of the GR to the DNA is mediated by protein–protein interactions (Wright & Gustafsson, 1991). A recent report suggests that a novel GR complex which binds a negative GRE in the pro-opiomelanocortin gene appears to contain three GR molecules (Drouin et al., 1993). In our study, the estimated size of the slowly migrating GR protein–DNA complex (420 kDa), as established by electrophoretic fractionation in native gradient gels, may correspond to a GR tetramer; this supports cooperative binding of GR proteins and is consistent with the identification of multiple GREs.

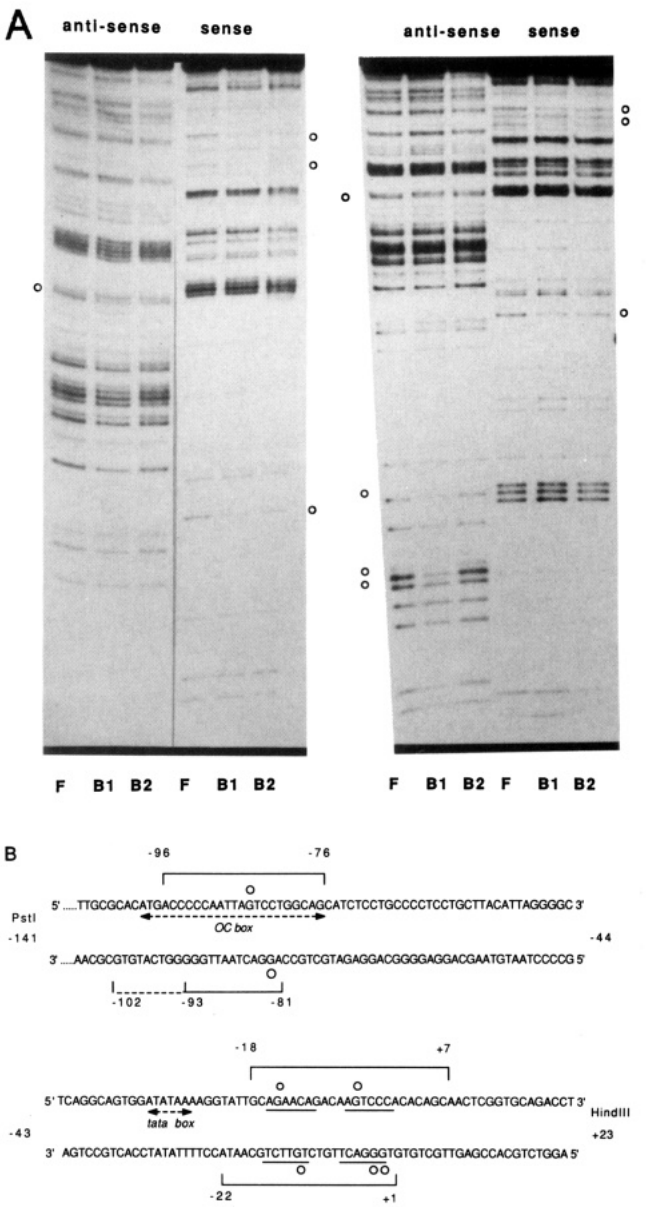

FIGURE 9: Dimethyl sulfate protection analysis of the basal OC promoter gene fragment spanning both the OC box and the TATA region. (A) 10–20 pmol (0.5–1 μg) of GR protein and an asymmetrically ^{32}P-end-labeled OC gene promoter fragment (–141 to +23) were incubated for 20 min in a 20-μL binding reaction. 1 μL of DMS was added in a total volume of 200 μL and incubated for 1 min at room temperature. F, free probe; B1, probe bound to protein; B2, free probe added to protein in the presence of a 200-fold molar excess of rOCbox45 oligonucleotide as competitor. Circles indicate protected guanine nucleotides. Left and right panels were analyzed under two different electrophoretic conditions. (B) Summary of DNase I footprint regions and DMS-protected guanine residues for the –141 to +23 OC gene promoter fragment. Strong footprinted sequences are bracketed by solid lines and weaker footprinted sequences by a dashed line. G residue contacts are designated by open circles. Half steroid elements are underlined, and the OC box and TATA elements are indicated by arrow lines.

In vivo data indicate a reduction of OC gene expression by glucocorticoids for both the human and the rat OC gene (Schepmoes et al., 1991; Morrison et al., 1989; Bortell et al., 1992; Shalhoub et al., 1992). Negative regulation by glucocorticoids has been observed in several genes. One mechanism of negative regulation involves steric hindrance by the GR to the binding of a positive factor at a cAMP responsive element in the human glycoprotein α-subunit gene promoter (Akerblom et al., 1988). For the bovine prolactin gene (Sakai et al., 1988), the rat α_1-fetoprotein (Guertin et al., 1988), and the rat pro-opiomelanocortin gene (Drouin et

al., 1987, 1989), it was shown that the GREs are localized close to or overlapping other transcription factor binding sites. Another mechanism for negative regulation of transcription by glucocorticoids involves direct interaction between c-Jun and GR in the presence of hormone as demonstrated in the collagenase gene (Jonat et al., 1990). The c-Jun–GR complex neither binds to DNA nor transactivates. The estrogen receptor has also been postulated to repress the prolactin gene in the absence of DNA binding, by inactivating an intermediate factor (Adler et al., 1988).

Interestingly, the region of the OC gene promoter protected by purified GR includes the TATA box, which is the binding site for the TFIID basal transcription factor. The location of GR binding sites close to or overlapping with the TATA box suggests competition or steric interference of the GR with TFIID binding as a mechanism for negative regulation by glucocorticoids. There are several examples of negative transcriptional control by regulatory factor competition for TFIID binding. Engrailed, a homeodomain protein, can repress in vitro transcription by competition with TFIID (Ohkuma et al., 1990a,b). Thyroid hormone binds to a responsive element adjacent to the TATA box in a negative hormone responsive promoter region (Chatterjee et al., 1989; Crone et al., 1990). In the ovalbumin gene promoter, a functional ERE half-palindromic sequence is located near the TATA box and confers estrogen inducibility (Kato et al., 1992).

In addition to the GR binding sites in the TATA box region, GR protein–DNA interactions were observed in the OC box, a highly conserved (22/24 nt) basal promoter element in the rat and human OC genes with a central CCAAT motif. Interestingly, the protected guanine residues in the OC box are adjacent to the CCAAT element, and the DNase I footprint overlaps the CCAAT sequence. In the pro-opiomelanocortin gene, a GRE overlaps a putative CCAAT motif (Drouin et al., 1987, 1989), and the proposed mechanism of negative regulation by glucocorticoids is displacement of a positive transcription factor by GR. However, the location of a GRE half-site close to the CCAAT sequence may indicate interaction between GR and CCAAT binding protein. Indeed, earlier reports show synergistic interactions of GR with a number of transcription factors that include NF1, CACCC box binding factor, CCAAT binding protein, and SP1. These synergistic promoter factor activities may involve direct or indirect protein–protein interactions (Strähle et al., 1988; Schüle et al., 1988). Conservation of the OC box in the rat and the human OC promoter raises the question whether there is a GR binding site in the human OC box, analogous to the GRE sequence in the rat OC box. The AGTCCT steroid half-element in the rat OC promoter differs by one nucleotide in the human, AGCCCT. Glucocorticoid responsive half-elements are able to bind GR monomer (Drouin et al., 1992), but the affinity is lower and is not hormone-responsive in vivo.

The presence of a GRE half-site in the OC box may also support interaction between the OC box and the TATA/GRE region and/or the VDRE (Bortell et al., 1992). These interactions are supported by in vivo data suggesting that vitamin D-enhanced OC gene expression is reduced by glucocorticoids in both rat (Schepmoes et al., 1991; Morrison et al., 1989) and human osteoblasts (Wong et al., 1990). The half-sites contributed by the OC box and the TATA/GRE domain could support formation of a functional GRE. Recent reports suggest that widely spaced estrogen response element (ERE) half-palindromic sequences are competent to bind estrogen receptor (ER) cooperatively and confer estrogen inducibility (Kato et al., 1992).

Two GR binding sites were observed using the TATA domain probe (–43 to +23) by DNase I footprint and DMS protection. However, only one of these sites was retained when a longer probe (–141 to +23) was used. These differences reflect the complexity of protein–DNA interactions in the OC gene proximal promoter (Figure 4B). This complexity is further indicated by the detection of the GR binding site in the OC box which may compete for binding of the GR to the weak, putative GRE overlapping the TATA box.

The presence of multiple GREs has been observed in several genes (Ray et al., 1990; Schmid et al., 1989; Jantzen et. al., 1987; Danesch et al., 1987; Drouin et al., 1987, 1989). Some of them are functionally independent, some are only functional in the presence of another GRE, and others are inactive (Jantzen et al., 1987; Drouin et al., 1992). Our in vivo competition cotransfection analysis indicated that the downstream GR binding site (–16 to –1) contributes to regulation of rat OC gene transcriptional activity. We are currently investigating the contribution of each of the other in vitro defined GREs to glucocorticoid responsiveness of the rat OC gene and their putative functional interrelationship.

In summary, we have demonstrated a strongly protected GR binding site downstream of the TATA element (–16 to –1) of the proximal rat OC promoter that differs from what was reported in the human OC promoter (Stromstedt et al., 1991). We have also shown a less protected GR binding site overlapping the TATA box domain at a site analogous to the human OC promoter. This suggests interference of GR with the binding activity of the TFIID positive transcription factor as a mechanism for negative regulation by glucocorticoids. However, since the more strongly protected GRE is downstream of the TATA in the rat promoter, this may allow for variations in the regulation of OC expression between these two species by glucocorticoids (Stromstedt et al., 1991; Schepmoes et al., 1991; Shalhoub, 1992; Morrison et al., 1989; Wong et al., 1990; Subramaniam et al., 1992). Additionally we have demonstrated a novel GR binding site not reported in the human OC promoter. This GR binding site is in the OC box contiguous to the CCAAT sequence, and may implicate cooperative binding of GR and CCAAT motif proteins. Taken together, these results suggest multiple mechanisms through which glucocorticoids can modulate basal and vitamin D-enhanced OC gene transcription. A basis is thereby provided for the integration of activities at several basal and steroid hormone-mediated enhancer sequences in the OC gene promoter and diversity of responsiveness to physiological regulatory signals that subsequently control OC gene expression during development of the osteoblast phenotype.

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REFERENCES

Adler, S., Waterman, M. L., He, X., & Rosenfeld, M. G. (1988) *Cell 52*, 685–695.

Akerblom, I. E., Slater, E. P., Beato, M., Baxter, J. D., & Mellon, P. L. (1988) *Science 241*, 350–353.

Alnemri, E. S., Maksymowych, A. B., Robertson, N. M., & Litwack, G. (1991) *J. Biol. Chem. 266*, 3925–3936.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., Struhl, K., Eds. (1989) *Current Protocols in Molecular Biology*, Section 9.2, Wiley and Sons, New York.

Beato, M. (1989) *Cell 56*, 335–344.

Bortell, R., Owen, T. A., Bidwell, J. P., Gavazzo, P., Breen, E., van Wijnen, A. J., DeLuca, H. F., Stein, J. L., Lian, J. B., & Stein, G. S. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6119–6123.

Chatterjee, V. K. K., Lee, J.-K., Rentoumis, A., & Jameson, J. L. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9114–9118.

Chen, T. L., Aronow, L., & Feldman, D. (1977) *Endocrinology 100*, 619–628.

Crone, D. E., Kim, H. S., & Spindler, S. R. (1990) *J. Biol. Chem. 265*, 10851–10856.

Dalman, F. C., Sanchez, E. R., Lin, A. L.-Y., Perini, F., & Pratt, W. B. (1988) *J. Biol. Chem. 263*, 12259–12267.

Danesch, V., Gloss, B., Schmid, W., Schutz, G., Schüle, R., & Renkawitz, R. (1987) *EMBO J. 6*, 625–630.

DeFRanco, D. J., Lian, J. B., & Glowacki, J. (1992) *Endocrinology 131*, 114–121.

Demay, M. B., Gerardi, J. M., DeLuca, H. F., & Kronenberg, H. M. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 369–373.

Denis, M., & Gustafsson, J.-A. (1989) *Cancer Res. (Suppl.) 49*, 2275s–2281s.

Dignam, J., Lebovitz, R., & Roeder, R. (1983) *Nucleic Acids Res. 11*, 1475–1489.

Drouin, J., Charron, J., Gagner, J.-P., Jeannotte, L., Nemer, M., Plante, R. K., & Wrange, O. (1987) *J. Cell. Biochem. 35*, 293–304.

Drouin, J., Trifiro, M. A., Plante, R., Nemer, M., Eriksson, P., & Wrange, O. (1989) *Mol. Cell. Biol. 9*, 5305–5314.

Drouin, J., Sun, Y. L., Tremblay, S., Lavender, P., Schmidt, T. J., de Lean, A., & Nemer, M. (1992) *Mol. Endocrinol. 6*, 1299–1309.

Drouin, J., Sun, Y. L., Chamberland, M., Gauthier, Y., DeLean, A., Nemer, M., & Schmidt, T. J. (1993) *EMBO J. 12*, 145–156.

Eilam, Y., Silbermann, M., & Szydel, N. (1980) *Biochem. Biophys. Res. Commun. 96*, 299–305.

Ekenstamm, E., Stalenheim, G., & Hallgren, R. (1988) *Metabolism 37*, 141–144.

Evans, R. M. (1988) *Science 240*, 889–895.

Freedman, L. P., Luisi, B. F., Korszun, Z. R., Basavappa, R., Sigler, P. B., & Yamamoto, K. R. (1988) *Nature 334*, 543–546.

Gennari, C. (1985) in *Bone and Mineral Research* (Peck, W. A., Ed.) Vol. 3, pp 213–231, Elsevier Science Publishers, New York.

Guertin, M., Larue, H., Bernier, D., Wrange, O., Chevrette, M., Gingras, M.-C., & Belanger, L. (1988) *Mol. Cell. Biol. 8*, 1398–1407.

Gundberg, C. M., Hauschka, P. V., Lian, J. B., & Gallop, P. M. (1984) *Methods Enzymol 107*, 516–544.

Hollenberg, S. M., & Evans, R. M. (1988) *Cell 55*, 899–906.

Holthuis, J., Owen, T. A., van Wijnen, A. J., Wright, K. L., Ramsey-Ewing, A., Kennedy, M., Carter, R., Cosenza, S. L., Soprano, K. J., Lian, J. B., & Stein, G. S. (1990) *Science 247*, 1454–1457.

Jantzen, H.-M., Strähle, U., Gloss, B., Stewart, F., Schmid, W., Boshart, M., Miksicek, R., & Schutz, G. (1987) *Cell 49*, 29–38.

Jonat, C., Rahmsdorf, H. J., Park, K. K., Cato, A. C. B., Gebel, S., Ponta, H., & Herrlich, P. (1990) *Cell 62*, 1189–1204.

Karin, M., Haslinger, A., Holtgreve, H., Richards, R. I., Krauter, P., Westphal, H. M., & Beato, M. (1984) *Nature 308*, 513–519.

Kato, S., Tora, L., Yamauchi, J., Masushige, S., Bellard, M., & Chambon, P. (1992) *Cell 68*, 731–742.

Kerner, S. A., Scott, R. A., & Pike, J. W. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4455–4459.

Lian, J. B., Stein, G. S., Owen, T. A., Tassinari, M. S., Aronow, M., Collart, D., Shalhoub, V., Peura, S., Dworetzky, S., & Pockwinse, S. (1992) in *Molecular and Cellular Approaches to the Control of Proliferation and Differentation* (Stein, G. S., & Lian, J. B., Eds.) pp 165–222, Academic Press, San Diego.

Luisi, B. F., Xu, W. X., Otwinowski, Z., Freedman, L. P., Yamamoto, K. B., & Sigler, P. B. (1991) *Nature 352*, 497–505.

Lukert, B. P., Higgins, J. C., & Stoskopf, M. M. (1986) *J. Clin. Endocrinol. Metab. 62*, 1056–1058.

Mader, S., Leroy, P., Chen, J., & Chambon, P. (1993) *J. Biol. Chem. 268*, 591–600.

Majeska, R. J., Rodan, S. B., & Rodan, G. A. (1980) *Endocrinology 107*, 1494–1503.

Markose, E. R., Stein, J. L., Stein, G. S., & Lian, J. B. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1701–1705.

Maxam, A. M., & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 560–564.

Morrison, N. A., Shine, J., Fragonas, J.-C., Verkest, V., McMenemy, M. L., & Eisman, J. A. (1989) *Science 246*, 1158–1161.

Muller, M., & Renkawitz, R. (1991) *Biochim. Biophys. Acta 1088*, 171–182.

Ohkuma, Y., Horikoshi, M., Roeder, R. G., & Desplan, C. (1990a) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2289–2293.

Ohkuma, Y., Horikoshi, M., Roeder, R. G., & Desplan, C. (1990b) *Cell 61*, 475–484.

Owen, T. A., Aronow, M., Shalhoub, V., Barone, L. M., Wilming, L., Tassinari, M., Kennedy, M. B., Pockwinse, S., Lian, J. B., & Stein, G. S. (1990) *J. Cell. Physiol. 143*, 420–430.

Pratt, W. B., Sanchez, E. R., Bresnick, E. H., Meskinchi, S., Scherrer, L. C., Dalman, F. C., & Welsh, M. J. (1989) *Cancer Res. (Suppl.) 49*, 2222s–2229s.

Ray, A., LaForge, K. S., & Sehgal, P. B. (1990) *Mol. Cell. Biol. 10*, 5736–5746.

Rodan, S. B., Fischer, M. K., Egan, J. J., Epstein, P. M., & Rodan, G. A. (1984) *Endocrinology 115*, 951–958.

Sakai, D., Helms, D. D., Helms, S., Carlstedt-Duke, J., Gustafsson, J. A., Rottman, F. M., & Yamamoto, K. R. (1988) *Genes Dev. 2*, 1144–1154.

Schepmoes, G., Breen, E., Owen, T. A., Aronow, M. A., Stein, G. S., & Lian, J. B. (1991) *J. Cell. Biochem. 47*, 184–196.

Schmid, W., Strahle, U., Schutz, G., Schmitt, J., & Stunnenberg, H. (1989) *EMBO J. 8*, 2257–2263.

Schüle, R., Muller, M., Otsuka-Murakami, H., & Renkawitz, R. (1988) *Nature 332*, 87–90.

Shalhoub, V., Conlon, D., Tassinari, M., Quinn, C., Partridge, N., Stein, G. S., & Lian, J. B. (1992) *J. Cell. Biochem. 50*, 425–440.

Shea, W. K., Cowens, J. W., & Ip, M. M. (1991) *J. Steroid Biochem. Mol. Biol. 39*, 433–447.

Stein, G. S., Lian, J. B., Owen, T. A., Holthuis, J., Bortell, R., & van Wijnen, A. J. (1992) in *Molecular and Cellular Approaches to the Control of Proliferation and Differentiation* (Stein, G. S., & Lian, J. B., Eds.) pp 299–341, Academic Press, San Diego.

Strähle, U., Schmid, W., & Schutz, G. (1988) *EMBO J. 7*, 3389–3395.

Stromstedt, P. E., Poellinger, L., Gustafsson, J.-A., & Carlstedt-Duke, J. (1991) *Mol. Cell. Biol. 11*, 3379–3383.

Subramaniam, M., Colvard, D., Keeting, P. E., Rasmussen, K., Riggs, B. L., & Spelsberg, T. C. (1992) *J. Cell. Biochem. 50*, 411–424.

Terpening, C. M., Haussler, C. A., Jurutka, P. W., Galligan, M. A., Komm, B. S., & Haussler, M. R. (1991) *Mol. Endocrinol. 5*, 373–385.

Wong, M.-M., Rao, L. G., Ly, H., Hamilton, L., Tong, J., Sturtbridge, W., McBroom, R., Aubin, J. E., & Murray, T. M. (1990) *J. Bone Miner. Res. 5*, 803–813.

Wright, A. P. H., & Gustafsson, J.-A. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8283–8287.

Yoon, K., Rutledge, S. J. C., Buenaga, R. F., & Rodan, G. A. (1988) *Biochemistry 27*, 8521–8526.