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# Novel Regulation of 25-Hydroxyvitamin $D_3$ 24-Hydroxylase (24(OH)ase) Transcription by Glucocorticoids: Cooperative Effects of the Glucocorticoid Receptor, C/EBPβ, and the Vitamin D Receptor in 24(OH)ase Transcription

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## ABSTRACT

Glucocorticoid-induced bone loss has been proposed to involve direct effects on bone cells as well as alterations in calcium absorption and excretion. Since vitamin D is important for the maintenance of calcium homeostasis, in the present study the effects of glucocorticoids on vitamin D metabolism through the expression of 24(OH)ase, an enzyme involved in the catabolism of $1,25(OH)_2D_3$, were examined. Injection of vitamin D replete mice with dexamethasone (dex) resulted in a significant induction in 24(OH)ase mRNA in kidney, indicating a regulatory effect of glucocorticoids on vitamin D metabolism. Whether glucocorticoids can affect 24(OH)ase transcription is not known. Here we demonstrate for the first time a glucocorticoid receptor (GR) dependent enhancement of $1,25(OH)_2D_3$-induced 24(OH)ase transcription. Dex treatment of GR and vitamin D receptor (VDR) transfected COS-7 cells and dex treatment of osteoblastic cells (in which VDR and GR are present endogenously) potentiated $1,25(OH)_2D_3$-induced 24(OH)ase transcription. In addition, GR was found to cooperate with C/EBPβ to enhance VDR-mediated 24(OH)ase transcription. Using the rat 24(OH)ase promoter with the C/EBP site mutated, GR-mediated potentiation of $1,25(OH)_2D_3$-induced 24(OH)ase transcription was inhibited. Immunoprecipitation indicated that that GR can interact with C/EBPβ and ChIP/re-ChIP analysis showed that C/EBPβ and GR bind simultaneously to the 24(OH)ase promoter. These findings indicate a novel mechanism whereby glucocorticoids can alter VDR-mediated 24(OH)ase transcription through functional cooperation between C/EBPβ and GR that results in an enhanced ability of C/EBPβ to cooperate with VDR in the regulation of 24(OH)ase. J. Cell. Biochem. 110: 1314–1323, 2010. 

**KEY WORDS:** VITAMIN D; CCAAT ENHANCER BINDING PROTEIN BETA; GLUCOCORTICOID RECEPTOR; 25-HYDROXYVITAMIN D3 24-HYDROXYLASE

Vitamin D is a principal factor that maintains calcium homeostasis and is required for bone development and maintenance [Christakos et al., 2003]. The hormonally active form of vitamin D, 1,25-dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$), is produced by two sequential hydroxylations of vitamin D; by 25 hydroxylase in the liver and by 25 hydroxyvitamin $D_3$ 1α hydroxylase (1α(OH)ase) in the kidney [Christakos et al., 2003; DeLuca, 2004]. $1,25(OH)_2D_3$ regulates gene expression in target cells by binding to a high-affinity intracellular receptor protein (vitamin D receptor, VDR) which heterodimerizes with the retinoid X receptor and binds to vitamin D response elements within the promoter of target genes and, together with coactivators, affects target gene transcription [Christakos et al., 2003; DeLuca, 2004].

The maintenance of calcium homeostasis by vitamin D depends not only on VDR and coactivators needed for the expression of vitamin D-regulated genes but also on a balance between the production and degradation of $1,25(OH)_2D_3$. Parathyroid hormone (PTH) and low calcium stimulate 1α(OH)ase and $1,25(OH)_2D_3$ and the phosphaturic factor FGF23 reduce 1α(OH)ase [Omdahl et al., 2002; Shimada et al., 2004]. To provide additional control, $1,25(OH)_2D_3$ strongly induces the 25 hydroxyvitamin $D_3$ 24-hydroxylase (24(OH)ase) enzyme in kidney, in osteoblastic cells and in many

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other tissues [Omdahl et al., 2002]. 24(OH)ase plays an important role in the regulation of $1,25(OH)_2D_3$ since it catalyzes the first step in the metabolic inactivation of $1,25(OH)_2D_3$ [Reddy and Tserng, 1989]. Studies using a 24(OH)ase null mutant mouse provided the first in vivo evidence that 24(OH)ase initiates a major catabolic process that regulates $1,25(OH)_2D_3$ [St-Arnaud et al., 2000]. Thus, by inducing 24(OH)ase $1,25(OH)_2D_3$ regulates its own metabolism, protecting against hypercalcemia. 24(OH)ase is reciprocally regulated when compared to the regulation of 1α(OH)ase (stimulated by $1,25(OH)_2D_3$ and FGF23 and inhibited by PTH and low calcium) [Omdahl et al., 2002; Shimada et al., 2004]. Previous studies indicated that PTH inhibits renal 24(OH)ase by altering its stability [Zierold et al., 2001]. We recently reported that a member of the C/EBP family of transcription factors, C/EBPβ, is induced by $1,25(OH)_2D_3$ in kidney and osteoblastic cells and is a potent enhancer of VDR-mediated 24(OH)ase transcription [Dhawan et al., 2005]. We are only now beginning to understand the mechanisms involved in the regulation of 24(OH)ase and how various factors coordinately regulate 24(OH)ase gene expression and thus affect $1,25(OH)_2D_3$ metabolism and the maintenance of calcium homeostasis.

Glucocorticoids suppress the immune response and are used to treat inflammatory symptoms of various diseases [Franchimont, 2004]. However, glucocorticoid therapy is frequently associated with bone loss and pathological fractures. The mechanisms involved in glucocorticoid-induced bone loss are incompletely understood. Studies in mice and humans as well as in vitro studies have indicated that glucocorticoid-induced osteoporosis results, in part, from direct effects on bone [Weinstein et al., 1998, 2002; Canalis and Delany, 2002; Kim et al., 2006]. Glucocorticoids increases apoptosis of osteoblasts and osteocytes and decrease osteoblastogenesis, resulting in a decrease in bone formation [Weinstein et al., 1998; Canalis and Delany, 2002]. Glucocorticoids prolong longevity of osteoclasts but also disrupt actin ring formation resulting in dysfunctional osteoclasts [Weinstein et al., 2002; Kim et al., 2006]. It has been suggested that disruption of the cytoskeleton of the osteoclast by glucocorticoids suppresses the resorptive phase of remodeling, thus contributing to retarded bone formation [Kim et al., 2006]. In addition to direct effects on bone, glucocorticoids also induce bone loss by affecting calcium metabolism through alterations in intestinal calcium absorption and renal calcium excretion. Treatment with pharmacological doses of glucocorticoids results in impaired gastrointestinal absorption of calcium (which has been associated with a decrease in TRPV6, the $1,25(OH)_2D_3$-induced epithelial calcium channel, and a decrease in the intestinal calcium binding protein, calbindin-$D_{9k}$) [Reid, 1997; Huybers et al., 2007]. Evidence suggests the effectiveness of vitamin D and calcium in preventing glucocorticoid-induced bone loss [Amin et al., 1999]. Since vitamin D is critical for maintenance of calcium homeostasis, in the present study the effect of glucocorticoids on vitamin D metabolism through the regulation of 24(OH)ase, the enzyme involved in the catabolism of $1,25(OH)_2D_3$, was examined. Our findings indicate for the first time that glucocorticoids can directly affect 24(OH)ase transcription and define a novel mechanism through functional cooperation of glucocorticoid receptor (GR), C/EBPβ, and VDR in the regulation of 24(OH)ase.

## MATERIALS AND METHODS

### MATERIALS

Nylon membranes for the Northern blot and radiochemicals [$^{14}$C]chloramphenicol (50 mCi/mmol) and [α-$^{32}$P]ATP (3,000 Ci [111 TBq]/mmol) were purchased from NEN Life Science Products (Boston, MA).The PVDF membranes and prestained molecular weight markers were obtained from Bio-Rad Laboratories, Inc. (Hercules, CA). Dexamethasone was obtained from Sigma (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), random-primer DNA labeling kit and T4 polynucleotide kinase, were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) and charcoal-stripped FBS were from Gemini Biological Products (Calabasas, CA), C/EBPβ antiserum, GR antibody (p-20; sc1002) and antirabbit secondary antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). $1,25(OH)_2D_3$ was a generous gift from Milan Uskokovic (Hoffmann-LaRoche, Nutley, NJ).

### CELL CULTURES

COS-7 African green monkey kidney cells and UMR 106 rat osteosarcoma cells, obtained from the American Type Culture Collection (Manassas, VA), were cultured in DMEM and DMEM-F12, respectively. MC3T3-E1 mouse osteoblastic cells (Riken Cell Bank, Tsukuba, Japan) were cultured in α-MEM. Media were supplemented with 5–10% heat-inactivated FBS and 1% antibiotic mixture (penicillin, streptomycin, and neomycin). Osteoblast enriched bone cells were isolated from neonatal murine calvaria by serial collagenase digestion and were cultured in DMEM supplemented with 10% FBS [Dhawan et al., 2005]. Cells were grown in a humidified incubator with atmosphere of 95% air–5% $CO_2$ at 37°C. For treatments, cells were grown to 60–70% confluence and changed to medium supplemented with 2% charcoal–dextran-treated FBS before treatment. Cells were treated with vehicle, $1,25(OH)_2D_3$, dexamethasone (dex) or $1,25(OH)_2D_3$ + dex as described in the figure legends.

### PLASMIDS, TRANSFECTIONS, AND ASSAY OF CAT ACTIVITY

Chloramphenicol acetyltransferase (CAT) reporter construct of rat 24(OH)ase promoter (positions −1,367 to +74 [−1,367/+74], containing VDREs at −258/−244 and −151/−137), deletion constructs −671/+74, −291/+74 [Kerry et al., 1996], and construct −671/+74 with C/EBP site mutated [Dhawan et al., 2005] were used for transfection in COS-7 cells. The pMEX-C/EBPβ expression vectors and GST-C/EBPβ 22–297 were gifts of Simon Williams, Texas Tech University, Lubbock, Tex. The dominant negative C/EBP construct (C/EBP DN) was a gift from Dr. C. Vinson, NCI. The GR expression vector was provided by Dr. Keith Yamamoto (University of California, San Francisco). The hVDR expression plasmid pAVhVDR was from J.W. Pike, University of Wisconsin, Madison. Empty vectors were transfected to keep the total DNA concentration equal. Cells ($1 \times 10^6$ cells/plate) were transfected using the lipofectamine 2000 (Invitrogen) or using calcium phosphate DNA precipitation method. For calcium phosphate method, 12–16 h after transfection, cells were shocked for 1 min with phosphate-buffered saline (PBS) containing 10% dimethyl sulfoxide, washed with PBS, and treated as described in the Results Section in the appropriate

medium supplemented with 2% charcoal–dextran-treated FBS. After treatment with vehicle or test compound(s), cells were harvested and cell extracts were prepared by freezing and thawing five times. The CAT assay was performed using equivalent amounts of protein and/or with constant β-galactosidase activity. CAT activity was quantitated by scanning thin-layer chromatography plates with the Packard Instant Imager system (Packard Instrument Co., Meriden, CN).

### NORTHERN BLOT ANALYSIS, RT-PCR, AND WESTERN BLOT ANALYSIS

RNA-Bee reagent was used to isolate total RNA from kidney or cultured cells. Northern blots were prepared, probed, and analyzed as described previously [Varghese et al., 1988]. $^{32}$P-labeled cDNA probes were prepared using random primer DNA labeling systems Invitrogen according to the random primer method [Ausubel et al., 2002]. The 3.2-kb rat 24(OH)ase cDNA was obtained by *Eco*RI digestion and was a gift from K. Okuda (Hiroshima University School of Dentistry, Hiroshima, Japan. The β-actin cDNA was purchased from Clontech Laboratories, Inc. (Palo Alto, CA). Autoradiograms were analyzed by densitometric scanning using the dual-wavelength flying spot scanner. The relative optical density obtained using the 24(OH)ase probe was divided by the relative optical density obtained after probing with β-actin to normalize for sample variation.

For RT-PCR analysis of 24(OH)ase mRNA and C/EBPβ mRNA in osteoblastic cells, RT-PCR was performed using 1 μg total RNA and Superscript™ One Step RT-PCR System with Platinum Taq DNA polymerase (Invitrogen). Primers used were as follows: 24(OH)ase forward 5′-GCCGAGCCTGCTGGAA-3′ and reverse, 5′-CCCCA-TAAAATCAGCCAAGAC-3′ (35 cycles); C/EBPβ forward 5′-ATG-CAATCCGGATCAAAC-3′ and reverse, 5′-AACATCAACAGCAACA-ACC-3′ (35 cycles); actin forward 5′-CCTGTGGATCTGACAGCT-GAA-3′ and reverse 5′-TCCCAAATCGGTTGGAGATA-3′. The cycles were chosen so that the amplification was conducted in the linear range of amplification efficiency. The resulting PCR products were subjected to electrophoresis on a 1% agarose gel containing ethidium bromide and bands were visualized under UV light. Gel data were recorded using the Gene Genius bioImaging System (Syngene, Frederick, MD) and relative densities of the bands were determined using Gene Tool Software (Syngene). Data were normalized for the expression of β actin mRNA within the sample.

For Western blot analysis, total cellular protein was prepared using a buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1.0% NP-40, and protease inhibitors. Fifty micrograms of protein was separated by SDS–10% polyacrylamide gel electrophoresis (PAGE) and analyzed by Western blot analysis as previously described [Barletta et al., 2002] by the enhanced-chemiluminescence Western blotting detection system (NEN Life Science Products). Protein concentration was assayed by the method of Bradford [1976].

### PROTEIN–PROTEIN INTERACTION ASSAYS

For immunoprecipitation assays nuclear extracts were prepared from COS-7 cells co-transfected with C/EBPβ and GR expression vectors or vector alone as well as from osteoblastic cells as previously described [Dhawan et al., 2005; Shen and Christakos, 2005]. Protein concentration was detected by the Bradford method [1976]. Five hundred micrograms of each preparation was used for immunoprecipitation with the addition of 4 μg of C/EBPβ antiserum or 4 μg or GR antiserum in the presence of dex (200 nM) for 24 h at 4°C. Then 30 μl of protein A-Sepharose 4 Fast Flow Beads (Amersham Biosciences) were added to each sample and after incubation by rotating at 4°C for 1 h the immunoprecipitated complex was collected by centrifugation at 3,000 rpm for 5 min. The complex was separated by 12% SDS–PAGE and probed with GR antibody or C/EBPβ antibody.

For glutathione *S*-transferase (GST) fusion protein pull down assays, nuclear extracts were prepared from COS-7 cells transfected with GR expression vector. Fusion proteins containing pGEX-C/EBPβ$_{22–297}$ created by S.C. Williams using PCR-generated fragments encoding amino acids 22–297 of C/EBPβ inserted into pGEX-4T3 (Pharmacia), was expressed in *E. coli* DH5α cells by isopropyl-β-D-thiogalactopyranoside (IPTG) induction and immobilized on glutathione–Sepharose beads as previously described [Dhawan et al., 2005]. Nuclear extracts of cells transfected with GR were added to 50% slurry and brought to a volume of 1 ml in GST binding buffer (20 mM Tris–HCl, pH 7.4, 180 mM KCl, 0.05% Nonidet P-40, and protease inhibitors). Binding reactions were incubated at 4°C, rotating for 1 h. The beads were washed four times in GST binding buffer containing 0.1% Nonidet P-40 and 0.15% Sarkosyl (Sigma) at 4°C for 20 min. The eluted proteins were analyzed by SDS–PAGE followed by Western blotting with GR antibody. GST antibody [GST (B14): sc-138] was used to indicate the presence of fusion protein in each binding assay.

### CHROMATIN IMMUNOPRECIPITATION (ChIP) ASSAY

Treated cells (UMR cells and primary osteoblastic cells) were used for the ChIP assay as previously described [Liu et al., 2005; Shen and Christakos, 2005]. Briefly, cells were first washed with PBS and subjected to a cross-link reaction with 1% formaldehyde for 15 min. The cross-link reaction was stopped by adding glycine to a final concentration of 0.125 M. Cells were washed with ice-cold PBS twice. The cells were collected by scraping and lysed sequentially in 5 mM Pipes, pH 8.0, 85 mM KCl, 0.5% Nonidet P-40 and then in 1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.1, for 20 min individually. The chromatin pellets were sonicated to an average DNA size of 500 bp DNA (assessed by 1% agarose gel electrophoresis) using a Fisher model 100 sonic dismembranator at a power setting of 1. The sonicated extract was centrifuged for 10 min at maximum speed and then diluted into ChIP dilution buffer (16.7 mM Tris–HCl, pH 8.1, 150 mM NaCl, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA). Immunoprecipitations were performed at 4°C overnight with the indicated antibody overnight. After a 1-h incubation with salmon sperm DNA and bovine serum albumin-pretreated Zysorbin (Zymed Laboratories, Inc., San Francisco, CA), the precipitates were collected by centrifugation. Precipitates were washed sequentially in buffer I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 150 mM NaCl), buffer II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 500 mM NaCl), buffer III (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris–HCl, pH 8.1), and TE buffer (10 mM Tris, 1 mM EDTA) twice. The

protein–DNA was then eluted by using 1% SDS and 0.1 M $NaHCO_3$ for 15 min twice. Cross-links were reversed by incubating at 65°C overnight in elution buffer with 0.2 M NaCl. DNA fragments were purified using Qiagen QIAquick PCR purification kits (Valencia, CA) and subjected to PCR using the primers designed to amplify the C/EBP motif at −395/−388 in the 24(OH)ase promoter [Dhawan et al., 2005]. PCR analysis was carried out in the linear range of DNA amplification. PCR products were resolved in 5% TBE acrylamide gel and visualized using ethidium bromide staining. DNA acquired prior to precipitation was collected and used as the input. Ten percent of input was used for PCR evaluation.

In re-ChIP experiments, complexes were eluted by incubation for 30 min at 37°C in 60 µl of elution buffer containing 10 mM dithiothreitol. The eluted samples were diluted 50 times with ChIP dilution buffer and subjected again to the ChIP procedure with GR antibody.

### STATISTICAL ANALYSIS

Results are expressed as means ± standard errors (SE), and significance was determined by analysis by Student's *t*-test for two-group comparison or by analysis of variance for multiple-group comparison.

## RESULTS

### INDUCTION OF RENAL 24(OH)ASE mRNA AND ENHANCEMENT OF VDR-MEDIATED 24(OH)ASE TRANSCRIPTION BY GLUCOCORTICOIDS

To examine a role of glucocorticoids in the regulation of 24(OH)ase, dexmethasone (2 mg/kg body weight) was administered to normal vitamin D replete mice for 5 days. Northern blot analysis revealed that 24(OH)ase mRNA expression was induced after dex treatment (Fig. 1A) [similar to previous findings by Akeno et al., 2000; Van Cromphaut et al., 2007]. To examine the mechanism of activation of

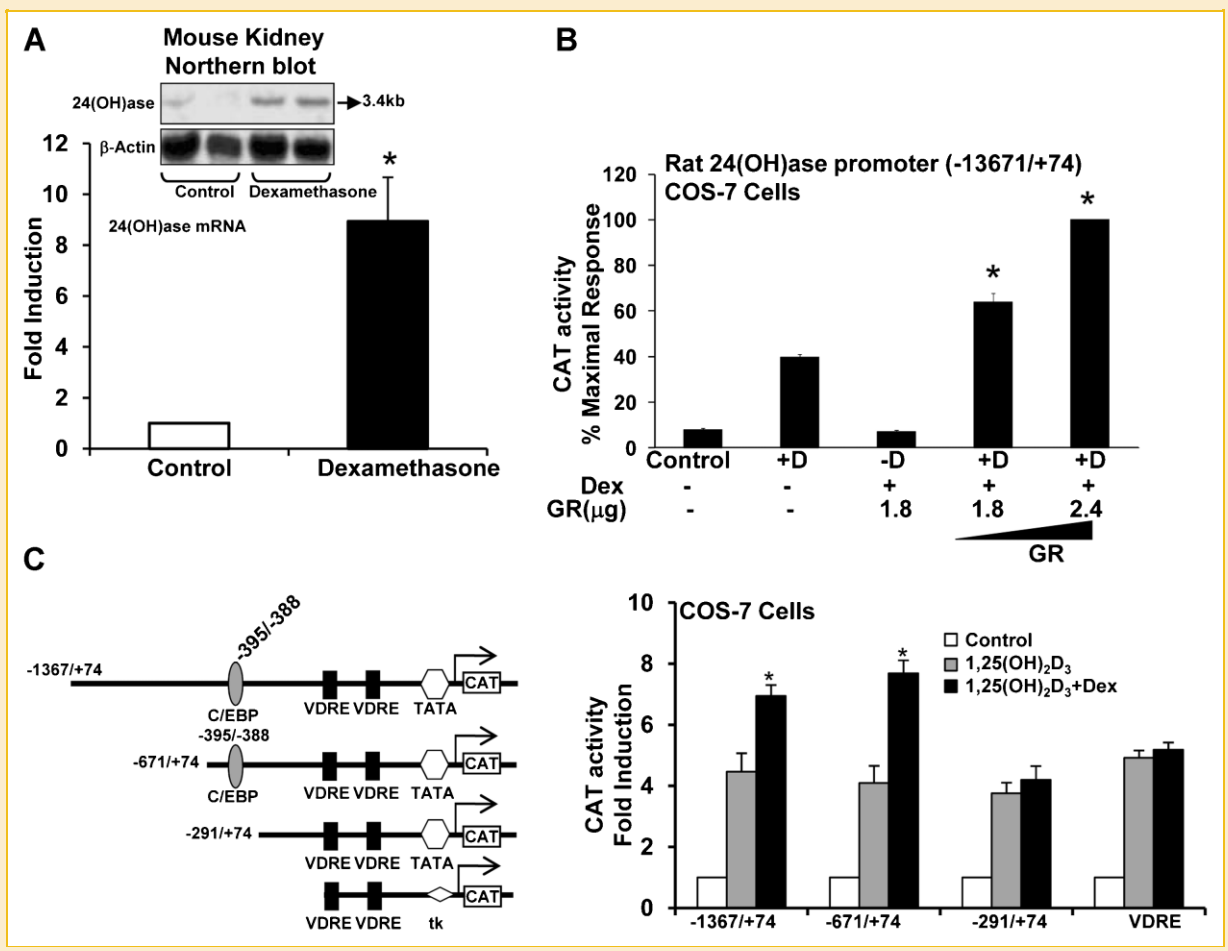


Fig. 1. Induction of renal 24(OH)ase mRNA and enhancement of VDR-mediated 24(OH)ase transcription by glucocorticoids. A: Northern blot analysis of 24(OH)ase mRNA in kidneys of mice injected with dex (2 mg/kg bw for 5 days) (dexamethasone) or vehicle (control). The results represent the mean ± SE of three independent experiments. (n = 3–5 mice per condition (control or dex-treated/experiment; $^*P < 0.05$ compared to control). Inset: Representative autoradiogram (longer autoradiographic exposure was needed to observe the band in lane 2, 24(OH)ase mRNA control). B: Enhancement of $1,25(OH)_2D_3$-induced 24(OH)ase transcription by dex. COS-7 cells were co-transfected with the rat 24(OH)ase promoter (−1,367/+74) and hVDR expression plasmid in the absence or presence of GR. Cells were treated with vehicle (control), $1,25(OH)_2D_3$ alone ($10^{-8}$ M), dex alone (200 nM), or with $1,25(OH)_2D_3$ + dex for 24 h. CAT activity is expressed as percentage of the maximal response (mean ± SE) and is representative of three or more separate experiments. C: Left panel: Schematic of CAT constructs. Right panel: CAT activity of the constructs (shown on the left) in extracts of COS-7 cells transfected with hVDR and GR and treated with $1,25(OH)_2D_3$ ($10^{-8}$ M) alone or with $1,25(OH)_2D_3$ and dex (200 nM) for 24 h. ($^*P < 0.05$ compared to cells treated with $1,25(OH)_2D_3$ alone.)

24(OH)ase by glucocorticoids, COS-7 cells were transfected with the rat 24(OH)ase promoter (−1,367/+74 as well as deletion constructs) and VDR expression vector in presence and in absence of GR and dex (Fig. 1B,C). In the presence of GR, dex was found to enhance $1,25(OH)_2D_3$-induced 24(OH)ase transcription. In the absence of $1,25(OH)_2D_3$, dex, and GR failed to induce 24(OH)ase transcription (Fig. 1B; 3rd bar). The enhancement of $1,25(OH)_2D_3$-induced transcription by dex/GR was not observed using the −291/+74 construct or the VDRE tk CAT construct, indicating that the 24(OH)ase VDRE is not sufficient to mediate the enhancement of $1,25(OH)_2D_3$-induced transcription through GR and that the dex/GR responsive region is between −671 and −291.

## GR FUNCTIONALLY COOPERATES WITH C/EBPβ AT THE C/EBP BINDING MOTIF PRESENT IN THE 24(OH)ASE PROMOTER FOR GLUCOCORTICOID POTENTIATION OF $1,25(OH)_2D_3$-INDUCED TRANSCRIPTION

We previously identified a C/EBP binding site at −395/−388 in the rat 24(OH)ase promoter [Dhawan et al., 2005]. Since cooperation between GR and C/EBPβ has previously been reported for the regulation of a number of genes, we tested the possibility that C/EBPβ may play a role in the enhancement of 24(OH)ase transcription by glucocorticoids. Transfection of COS-7 cells with the 24(OH)ase promoter and VDR in presence of C/EBPβ resulted in an enhanced induction of VDR-induced 24(OH)ase transcription [as we previously reported; Dhawan et al., 2005] (Fig. 2A). We found that dex, in presence of GR, potentiated the C/EBPβ-mediated enhancement of VDR-mediated 24(OH)ase transcription (Fig. 2A). To further assess a possible role of C/EBPβ in GR potentiation of $1,25(OH)_2D_3$-induced 24(OH)ase transcription, studies were done using dominant negative C/EBP (C/EBP DN). COS-7 cells, which contain endogenous C/EBPβ, were transfected with the rat 24(OH)ase promoter (−671/+74), VDR and GR in the presence or absence of DN C/EBP. DN C/EBP resulted in a dose-dependent suppression of the dex/GR-mediated enhancement of the 24(OH)ase transcription (Fig. 2B). In addition, dex treatment failed to enhance the $1,25(OH)_2D_3$-induced transcription of the 24(OH)ase promoter construct containing a mutated C/EBP binding site (−674/+74 MT) (Fig. 3A). Co-immunoprecipitation and GST pull down assays indicated that C/EBPβ binds to GR (Fig. 3B). These findings suggest that the glucocorticoid-mediated enhancement of 24(OH)ase transcription involves functional cooperation and possible interaction of GR with C/EBPβ.

## DEX ENHANCES THE EXPRESSION OF C/EBPβ AND 24(OH)ASE mRNA AS WELL AS $1,25(OH)_2D_3$-INDUCED 24(OH)ASE TRANSCRIPTION IN OSTEOBLASTIC CELLS

Glucocortiocoids enhance 24(OH)ase gene expression in osteoblastic cells as well as in kidney [Akeno et al., 2000; Kurahashi et al., 2002; Van Cromphaut et al., 2007]. In order to examine a possible role of C/EBPβ in glucocorticoid regulation of 24(OH)ase in osteoblastic cells, studies were done in UMR cells and in primary osteoblasts. We found that treatment of UMR cells and primary osteoblasts with $1,25(OH)_2D_3$ and dex resulted in an enhancement of 24(OH)ase mRNA and C/EBPβ mRNA over the levels observed with $1,25(OH)_2D_3$ alone (Fig. 4A,B). In addition, dex alone (200 nM for 12 h) resulted in an induction of C/EBPβ (but not C/EBPα) mRNA and protein in both UMR and primary osteoblasts (not shown). Dex

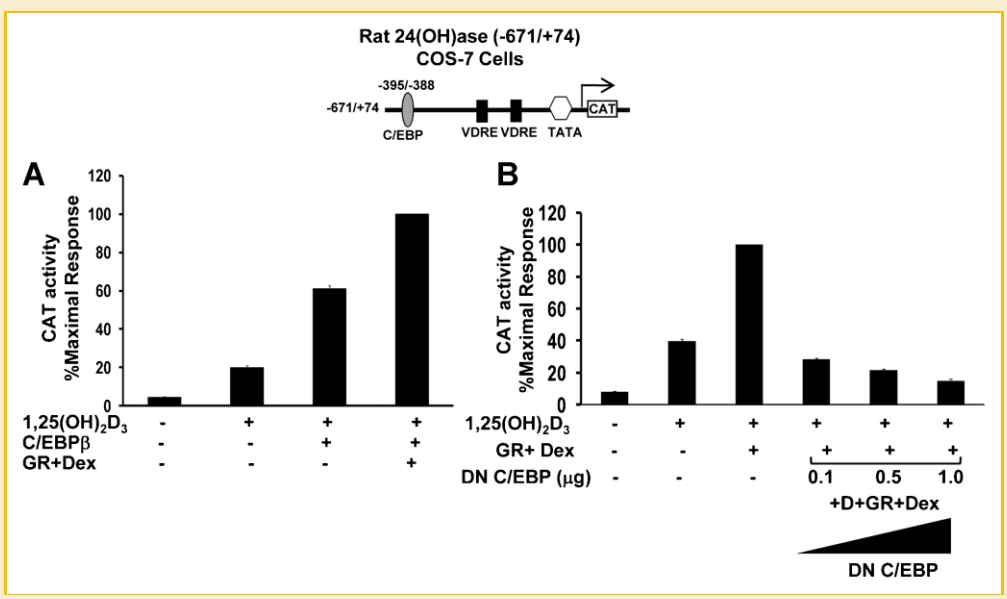


Fig. 2. Cooperative effects between GR and C/EBPβ in the enhancement of VDR-mediated 24(OH)ase transcription by glucocorticoids. A: COS-7 cells were transfected with the rat 24(OH)ase promoter construct (−671/+74) and hVDR expression plasmid in the presence or absence of C/EBPβ and GR as indicated. Cells were treated with $1,25(OH)_2D_3$ alone ($10^{-8}$ M), or $1,25(OH)_2D_3$ + dex (200 nM) for 24 h. B: COS-7 cells were transfected with the 24(OH)ase promoter (−671/+74) and hVDR or hVDR + GR in the absence or presence of increasing concentrations of C/EBP DN. Cells were treated with $1,25(OH)_2D_3$ ($10^{-8}$ M) or $1,25(OH)_2D_3$ + dex (200 nM). For both A and B, empty vectors were used to keep the total DNA concentration the same. Graphic representations are the results of three or more separate experiments and are expressed as mean percentages of the maximal response ± SE.

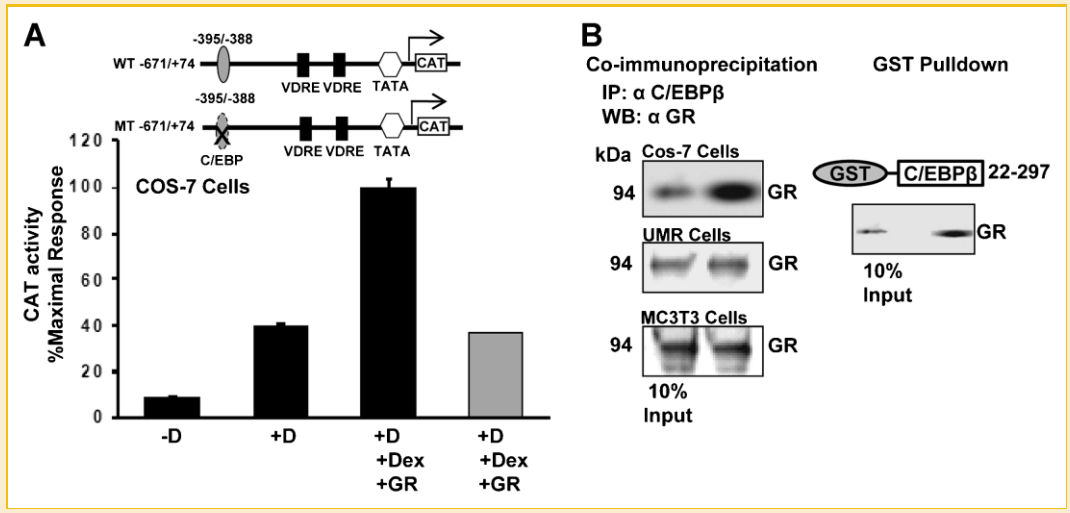


Fig. 3. Dex/GR potentiation of VDR-mediated 24(OH)ase transcription through the C/EBP binding site. Interaction between C/EBPβ and GR. A: Inset: Schematic of the rat 24(OH)ase promoter (−671/+74) and the C/EBP mutation introduced in the 24(OH)ase promoter. COS-7 cells were transfected with the rat 24(OH)ase promoter (−671/+24) or the −671/+24 24(OH)ase promoter with a mutated C/EBP motif (grey bar). Cells were co-transfected with hVDR or hVDR and GR and treated with $1,25(OH)_2D_3$ ($10^{-8}$ M) or $1,25(OH)_2D_3$ + dex (200 nM) for 24 h. CAT activity is represented as a percentage of the maximal response (mean ± SE; 3–4 observations/group; data are representative of at least three experiments). B: GR and C/EBPβ interact. Left panel: Co-immunoprecipitation. Interaction of GR and C/EBPβ using COS-7 cells (transfected with C/EBPβ and GR), UMR osteoblastic cells and MC3T3-E1 cells. The complexes, immunoprecipitated using C/EBP antibody and protein A beads, were separated by SDS–PAGE and probed with GR antibody. Similar results were observed when immunoprecipitation was done using GR antibody and Western blot was probed with C/EBPβ antibody (not shown). No band was observed when immunoprecipitations were done using IgG (not shown). Right panel: GST pull down assays done using GST fusion protein C/EBPβ 22–297 and nuclear extracts prepared from COS-7 cells transiently transfected with GR expression vector. GR antibody was used for the Western blot. Results are representative of three separate experiments.

treatment of vitamin D replete mice (as in Fig. 1A) also resulted in an induction of C/EBPβ (but not C/EBPα) in kidney (not shown). When UMR cells (which contain endogenous VDR and GR) were transfected with the 24(OH)ase promoter, treatment with dex enhanced $1,25(OH)_2D_3$-induced 24(OH)ase transcription. DN C/EBP suppressed the induction of 24(OH)ase transcription by $1,25(OH)_2D_3$ and dex (Fig. 5A). Similar results were observed using MC3T3-E1 osteoblastic cells (not shown). ChIP assays were done using UMR cells and primary osteoblastic cells treated with $1,25(OH)_2D_3$ or $1,25(OH)_2D_3$ + dex. The ChIP assay was performed using C/EBPβ antibody and primers designed to amplify the 300 bp region of the 24(OH)ase promoter containing the C/EBP site. ChIP assay results indicate enhanced recruitment of C/EBPβ to its motif in the 24(OH)ase promoter with co-treatment of dex + $1,25(OH)_2D_3$ compared to treatment with $1,25(OH)_2D_3$ alone (Fig. 5B,C; $P < 0.05$ D + Dex compared to D). ReChIP analysis after dex + $1,25(OH)_2D_3$ co-treatment shows that C/EBPβ and GR can bind simultaneously to the C/EBP motif in the 24(OH)ase promoter (Fig. 5B,C; re-ChIP).

## DISCUSSION

Vitamin D and glucocorticoids are both known to have effects on bone cells and on calcium transport. In this study we show for the first time that GR enhances $1,25(OH)_2D_3$-induced transcription of 24(OH)ase, the enzyme that initiates the catabolism of $1,25(OH)_2D_3$. Recently, Van Cromphaut et al. [2007] reported an induction of renal 24(OH)ase mRNA and a 50% decrease in free $1,25(OH)_2D_3$ serum levels in mice with dex treatment. A similar increase with dex treatment of renal 24(OH)ase mRNA as well as an increase in renal 24(OH)ase activity was noted in mice by Akeno et al. [2000]. However, in the study of Akeno et al. [2000], serum $1,25(OH)_2D_3$ levels were similar in control and dex-treated animals. It was suggested by Van Cromphaut et al. [2007] that an increase in 24(OH)ase without a decrease in $1,25(OH)_2D_3$ serum levels may be due to the lack of determination of free $1,25(OH)_2D_3$ serum levels in the study of Akeno et al. [2000]. Whether there is a direct stimulation by dex of 24(OH)ase or whether there is an indirect stimulation by glucocorticoids due to altered bone remodeling and effects on calcium homeostasis had not as yet been addressed. Our study shows a direct effect of glucocorticoids on 24(OH)ase transcription. It should be noted that results of studies in humans of $25(OH)D_3$ and $1,25(OH)_2D_3$ serum levels after glucocorticoid treatment are varied [Klein et al., 1977; Chesney et al., 1978; Prummel et al., 1991; Cosman et al., 1994]. Differences have been suggested to be due to different corticosteroid dose or different schedule of dosing [Klein et al., 1977]. In addition, it is possible that, similar to the studies in mice, differences may be due, in part, to lack of determination of free $1,25(OH)_2D_3$ serum levels. However, it has also been suggested that glucocorticoids affect vitamin D metabolism by inactivating $1,25(OH)_2D_3$ after tissue localization [Carre et al., 1974]. It is of interest that dex enhances 24(OH)ase mRNA and activity in osteoblastic and renal cells treated with $1,25(OH)_2D_3$ and that the effect of dex has been reported to be more marked in osteoblastic cells [Kurahashi et al., 2002]. These findings support an effect of glucocorticoids, that has been observed on induction of 24(OH)ase [Akeno et al., 2000; Kurahashi et al., 2002; Van Cromphaut et al.,

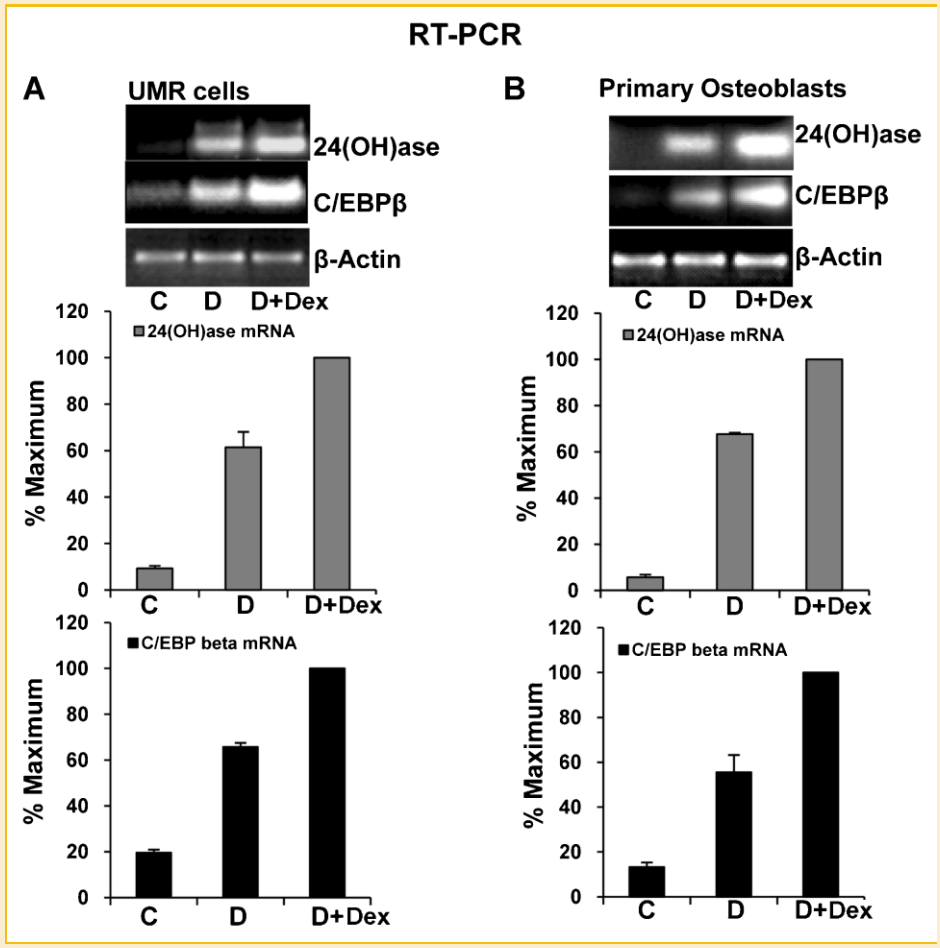


Fig. 4. Dex enhances the expression of $1,25(OH)_2D_3$-induced C/EBPβ mRNA and 24(OH)ase mRNA. A,B: Top panels: representative RT-PCR analyses of 24(OH)ase mRNA and C/EBPβ mRNA in UMR osteoblastic cells or primary osteoblastic cells treated with vehicle (C), $1,25(OH)_2D_3$ (D; $10^{-8}$ M for 36 h) or $1,25(OH)_2D_3$ + dex (200 nM) [D + Dex; $1,25(OH)_2D_3$ treatment ($10^{-8}$ M) was for 24 h followed by 200 nM dex treatment for 12 h in the presence of $10^{-8}$ M $1,25(OH)_2D_3$]. Lower panels: Quantitation of 24(OH)ase mRNA and C/EBPβ mRNA expression. Data represent the mean ± SE of three independent experiments. $1,25(OH)_2D_3$-induced C/EBPβ and 24(OH)ase mRNA levels are significantly enhanced by dex treatment (D + Dex, $P < 0.05$ compared to D treatment).

2007], to occur after tissue localization of $1,25(OH)_2D_3$. The need for controlled $1,25(OH)_2D_3$ homeostasis in target tissues was shown in studies in 24(OH)ase nullmutant mice [St-Arnaud et al., 2000]. Abnormalities in bone in these mice were suggested to be due to elevated $1,25(OH)_2D_3$ levels at specific sites in bone [St-Arnaud et al., 2000]. In transgenic mice, in which the VDR is over-expressed in mature cells of the osteoblastic lineage, bone structure is affected, providing additional evidence for the need to properly modulate tissue levels of $1,25(OH)_2D_3$ to prevent pathological consequences [Gardiner et al., 2000]. Thus, an additional mechanism of the adverse effect of glucocorticoids on bone may be by increasing catabolism of $1,25(OH)_2D_3$.

Glucocorticoids have been reported to affect the expression of other $1,25(OH)_2D_3$-regulated genes. In vivo studies have shown that treatment with glucocorticoids for 3–7 days reduces the expression of duodenal calbindin-$D_{9k}$ mRNA and TRPV6 mRNA [Li and Christakos, 1991; Lee et al., 2006; Huybers et al., 2007; Kim et al., 2009]. Renal TRPV5 mRNA and calbindin-$D_{28k}$ and calbindin-$D_{9k}$ mRNA were unaffected by glucocorticoid treatment [Li and Christakos, 1991; Huybers et al., 2007]. The reduction in intestinal TRPV6 mRNA and calbindin-$D_{9k}$ mRNA with glucocorticoid treatment was associated with diminished intestinal calcium absorption [Huybers et al., 2007]. Earlier studies in the chick also noted an inhibition of calbindin in intestine of rachitic or vitamin D-treated chicks that was accompanied by a decrease in intestinal calcium absorption [Feher and Wasserman, 1979; Tohmon et al., 1988]. It has been suggested that the effect of glucocorticoids on duodenal calcium transporters, unlike the effect of glucocorticoids on 24(OH)ase, can be independent of $1,25(OH)_2D_3$ [Feher and Wasserman, 1979; Tohmon et al., 1988; Huybers et al., 2007]. Whether the in vivo effects of glucocorticoids on intestinal calbindin and TRPV6 are due to a direct effect of glucocorticoids on the calbindin and TRPV6 genes remains to be determined. Glucocorticoids have also been reported to inhibit $1,25(OH)_2D_3$-stimulated osteocalcin (OC) expression in bone cells [Shalhoub et al., 1998]. The rat and human OC promoters contain multiple GREs and similar to the regulation of calbindin, the glucocorticoid effect on OC can be independent of $1,25(OH)_2D_3$ [Morrison et al., 1989;

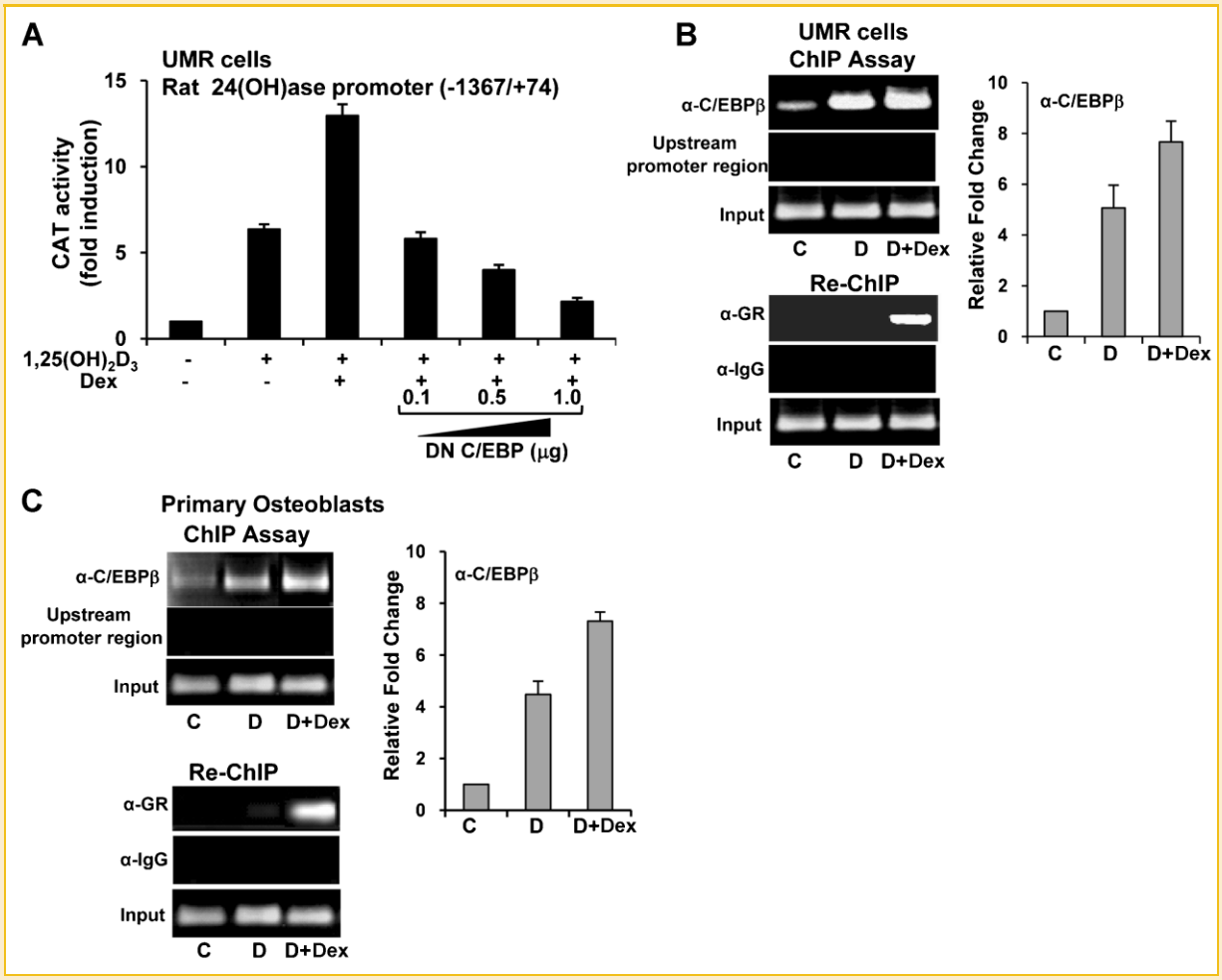


Fig. 5. Dex enhances $1,25(OH)_2D_3$-induced 24(OH)ase transcription in osteoblastic cells. A: Dex enhances $1,25(OH)_2D_3$-induced 24(OH)ase transcription in UMR osteoblastic cells. UMR osteoblastic cells were transfected with the 24(OH)ase promoter (−671/+74) and treated with $1,25(OH)_2D_3$ alone ($10^{-8}$ M) or $1,25(OH)_2D_3$ + dex (200 nM) in the absence or presence of increasing concentrations of C/EBP DN. CAT activity is represented as fold induction (mean ± SE; 3–4 observations/group). B,C. C/EBPβ and GR are recruited to the 24(OH)ase promoter in intact cells. UMR cells and mouse primary osteoblastic cells were treated as described in Figure 4. Cells were cross-linked and cell lysates were subjected to immunoprecipitation first with C/EBPβ antibody (ChIP) and then with GR antibody (re-ChIP). DNA was isolated and PCR (using specific primers designed against the C/EBP site on the 24(OH)ase promoter) was carried out in the linear range of DNA amplification. Immunoprecipitation with IgG was used as control (IgG panel). For ChIP assays three separate experiments were performed under the similar condition.

Stromstedt et al., 1991; Aslam et al., 1995]. Glucocorticoid responsive GREs have been identified in proximal and distal regions of the OC promoter [Aslam et al., 1995]. However, it was reported that these GREs are not the target for altered $1,25(OH)_2D_3$ regulation of OC. It was suggested, similar to our findings for the regulation of 24(OH)ase, that the glucocorticoid inhibition of $1,25(OH)_2D_3$ induction of OC may be mediated not by GR binding to a GRE, but rather by modification by dex/GR of protein–DNA interactions [Aslam et al., 1995]. The negative effect of glucocorticoids on $1,25(OH)_2D_3$ target genes in intestine and bone is consistent with the reported correlation between glucocorticoid treatment and decreased bone mineral density [Reid, 1997]. These findings, together with our findings of a enhanced induction of 24(OH)ase transcription, indicate differential regulation of $1,25(OH)_2D_3$ target genes by glucocorticoids that can be dependent or independent of $1,25(OH)_2D_3$.

In this study we found that GR cooperates with C/EBPβ to enhance VDR-mediated 24(OH)ase transcription. Cooperation between GR and C/EBPβ has been reported for the regulation of a number of genes. Cooperative effects have been reported to involve interaction of C/EBPβ and GR with the target gene promoter or the effect of GR, similar to our study, has been found to be mediated by C/EBPβ. The glucocorticoid induction of the phosphoenolpyruvate carboxykinase (PEPCK) gene is mediated through a complex glucocorticoid response unit, which includes GR binding sites [Cassuto et al., 2005]. Cooperative effects of STAT5 and C/EBPβ on β casein gene transcription, controlled by a composite response element, have been reported to be mediated by GR [Wyszomierski and Rosen, 2001]. Similar to our study, it was suggested that the cooperativity may be the result of a structural effect of GR, resulting in an activation complex recruited jointly by C/EBPβ and GR, rather than a transactivation effect of GR

[Wyszomierski and Rosen, 2001]. An interaction between GR and C/EBPβ was also suggested to be a mechanism involved in the glucocorticoid induction of the $\alpha_1$ acid glycoprotein gene [Nishio et al., 1993; Chang et al., 1998]. In addition to cooperative effects between GR and C/EBP in the regulation of gene transcription, glucocorticoids have an effect by inducing some of the C/EBP isoforms. Glucocorticoids have been shown to regulate C/EBPs in different tissues [McCarthy et al., 2000; Delany et al., 2001; Roesler, 2001; Balazs et al., 2008]. Similar to our findings, previous studies have also indicated an induction of C/EBPβ (but not C/EBPα) by glucocorticoids in osteoblasts and kidney [McCarthy et al., 2000; Delany et al., 2001; Balazs et al., 2008]. C/EBPβ (but not C/EBPα) is also induced by $1,25(OH)_2D_3$ in osteoblastic cells and in kidney[Gutierrez et al., 2002; Dhawan et al., 2005]. Since C/EBPβ is known to act as an enhancer of 24(OH)ase transcription [Dhawan et al., 2005], the induction of C/EBPβ by both $1,25(OH)_2D_3$ and glucocorticoids in kidney and osteoblasts as well as the cooperative effects between GR and C/EBPβ on transcription suggest both direct and indirect effects of these steroids that would result in enhanced 24(OH)ase transcription.

In summary, our results establish that liganded GR functionally cooperates with C/EBPβ at the C/EBP binding motif present in the 24(OH)ase promoter for glucocorticoid-mediated potentiation of $1,25(OH)_2D_3$-induced 24(OH)ase transcription. These results suggest that glucocorticoid mediated alterations in controlled $1,25(OH)_2D_3$ homeostasis in vitamin D target tissues may contribute to glucocorticoid-induced bone loss.

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