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Androgen Receptor Stimulates Bone Sialoprotein (BSP) Gene Transcription via cAMP Response Element and Activator Protein 1/Glucocorticoid Response Elements

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Abstract Bone sialoprotein (BSP) is an early marker of osteoblast differentiation. Androgens are steroid hormones that are essential for skeletal development. The androgen receptor (AR) is a transcription factor and a member of the steroid receptor superfamily that plays an important role in male sexual differentiation and prostate cell proliferation. To determine the molecular mechanism involved in the stimulation of bone formation, we have analyzed the effects of androgens and AR effects on BSP gene transcription. AR protein levels were increased after AR overexpression in ROS17/2.8 cells. BSP mRNA levels were increased by AR overexpression. However, the endogenous and overexpressed BSP mRNA levels were not changed by DHT (10^{-8} M, 24 h). Whereas luciferase (LUC) activities in all constructs, including a short construct (nts −116 to +60), were increased by AR overexpression, the basal and LUC activities enhanced by AR overexpression were not induced by DHT (10^{-8}M, 24 h). The effect of AR overexpression was abrogated by 2 bp mutations in either the cAMP response element (CRE) or activator protein 1 (AP1)/glucocorticoid response element (GRE). Gel shift analyses showed that AR overexpression increased binding to the CRE and AP1/GRE elements. Notably, the CRE-protein complexes were supershifted by phospho-CREB antibody, and CREB, c-Fos, c-Jun, and AR antibodies disrupted the complexes formation. The AP1/GRE-protein complexes were supershifted by c-Fos antibody and c-Jun, and AR antibodies disrupted the complexes formation. These studies demonstrate that AR stimulates BSP gene transcription by targeting the CRE and AP1/GRE elements in the promoter of the rat BSP gene. J. Cell. Biochem. 102: 240–251, 2007.

Key words: androgen; androgen receptor; bone sialoprotein; cAMP response element; activator protein 1; osteoblasts; transcription

Androgens are important in male sexual differentiation and testicular function [Kang et al., 2001, 2004]. Thus, androgen deficiency results in various abnormalities of bone metabolism, including a tall eunuchoid stature due to unfused growth plates and thinner cortical bones as well as a lower peak bone mass and accelerated bone loss due to increased bone resorption, resulting in a higher fracture risk [Orwoll and Klein, 1995]. While the clinical and biochemical effects of androgens on bone metabolism are well appreciated [Baran et al., 1978; Finkelstein et al., 1992; Ongphiphadhanakul et al., 1995; Katznelson et al., 1996; Anderson

Abbreviations used: AR, Androgen receptor; BSP, bone sialoprotein; FCS, fetal calf serum; MEM, minimum essential medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FGF, fibroblast growth factor; FRE, FGF response element; bp, base pair(s); nts, nucleotides; LUC, luciferase; CRE, cAMP response element.

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et al., 1997; Behre et al., 1997; Sih et al., 1997], the cellular and molecular actions of androgens on bone cells have remained largely unclear.

Androgen receptor (AR) is a ligand-activated transcription factor that is a member of the steroid receptor superfamily [Mangelsdorf et al., 1995]. AR enhances myogenin expression in C2C12 myoblasts and accelerates differentiation [Lee, 2002]. Steady-state levels of osteoblastic and osteoclastic genes are reduced in tibia from both male and female AR-transgenic mice, with the exception of increased osteoprotegerin expression in male [Wiren et al., 2004]. Functional ARs are detected in pluripotent bone marrow stromal cells [Bellido et al., 1995], which represent osteoblast precursor cells, hypertrophic chondrocyes [Abu et al., 1997], osteocytes, and osteoclasts [Mizuno et al., 1994]. The detection of functional ARs in various bone cells has implicated bone as a target tissue for androgen action, and has fueled an increase in further investigations on the direct and indirect effects of androgens on bone cells, as well as the sequelae of clinical and experimental androgen deficiency and its correction on bone metabolism.

Bone sialoprotein (BSP) is a member of the small integrin binding ligand, N-linked glycoprotein (SIBLING) family that is characterized by its ability to mediate cell attachment through an RGD sequence and to bind hydroxyapatite through polyglutamic acid sequences [Oldberg et al., 1988; Ganss et al., 1999]. The restricted distribution and temporal changes in the expression of BSP mRNA and the ability of BSP to nucleate hydroxyapatite crystal indicate a potential role for this protein in the initial mineralization of bone [Chen et al., 1992; Hunter and Goldberg, 1993]. BSP is also expressed by breast and prostate cancers, which could metastasize to bone, suggesting that BSP may play a role in the pathogenesis of bone metastases [Waltregny et al., 2000]. BSP genes have been cloned [Li and Sodek, 1993; Kerr et al., 1993; Kim et al., 1994; Benson et al., 1999] and the promoters partially characterized. These promoters have an inverted TATA box (nt −24 to −19) [Li et al., 1995] overlapping with vitamin D response element [Kim et al., 1996], and an inverted CCAAT box (−50 to −46), which is required for basal transcription [Kim and Sodek, 1999; Shimizu and Ogata, 2002]. In addition, a fibroblast growth factor 2 (FGF2) response element (FRE; −92 to −85) [Shimizu-Sasaki et al., 2001; Samoto et al., 2003; Shimizu et al., 2004a, 2005, 2006; Nakayama et al., 2006], a cAMP response element (CRE; −75 to −68) [Samoto et al., 2002, 2003; Shimizu et al., 2006], a transformation growth factor-β activation element (TAE; −499 to −485) [Ogata et al., 1997; Shimizu et al., 2004b, 2005], a pituitary-specific transcription factor-1 (Pit-1) motif (−111 to −105) that mediates the stimulatory effects of parathyroid hormone [Ogata et al., 2000; Shimizu et al., 2004a], and a homeodomain protein-binding site (HOX; −199 to −192) [Benson et al., 2000; Shimizu et al., 2004b; Nakayama et al., 2006] have been characterized. Further upstream, an activator protein 1 (AP1) site overlapping with a glucocorticoid response element (AP1/GRE) [Ogata et al., 1995; Yamauchi et al., 1996] has also been identified.

To elucidate the molecular mechanism of the androgen and AR regulation of BSP transcription, in this study we have analyzed the effects of androgen and AR on the expression of BSP in ROS17/2.8 cells.

MATERIALS AND METHODS

Materials

α-Minimum essential medium (α-MEM), fetal calf serum (FCS), Lipofectamine, penicillin, streptomycin, and trypsin were obtained from Invitrogen (Carlsbad, CA). The pGL3-basic vector and pSV-β-galactosidase (β-Gal) control vector were purchased from Promega Co. (Madison, WI). 5α-dihydrotestosterone (DHT) and guanidium thiocyanate were purchased from Wako Pure Chemical (Tokyo, Japan). EXScript RT reagent Kit and SYBR Premix Ex Taq were purchased from Takara (Tokyo, Japan). AR expression vector was kindly gifted by Dr. Chawnshang Chang.

Cell Cultures

The rat clonal cell line, ROS17/2.8 cells, was used in these studies as osteoblast like cells [Ogata et al., 1995]. Cells were cultured in 60 mm tissue culture dishes in α-MEM medium containing 10% FCS. Twenty-four hours after plating, cells at 40–60% confluence were transfected using a Lipofectamine regent. The transfection mixture included 1 μg of an AR expression plasmid (PCMV-AR) [Yeh and Chang, 1996]. Thirty-six hours posttransfection, the cells were cultured in phenol red free

α-MEM without serum for 12 h and incubated with DHT (10^{-8}M) for 24 h. Total RNA was isolated from triplicate cultures and analyzed for the expression of BSP mRNA by Northern hybridization as described below.

Northern Hybridization

ROS17/2.8 cells were transfected with or without PCMV-AR for 48 h, followed by DHT (10^{-8}M) treatment for another 24 h. Total RNA was extracted with guanidium thiocyanate and, following purification, 20 μg aliquots of total RNA were fractionated on 1.2% agarose gel and transferred onto a Hybond-N^+ membrane, as described previously [Ogata et al., 1997]. Hybridizations were performed at 42°C with a ^{32}P-labeled rat BSP or rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes. Following hybridization, the membranes were washed four times for 5 min each at 21°C in 300 mM sodium chloride, 30 mM trisodium citrate pH 7.0, 0.1% SDS. The hybridized bands, representing the two polyadenylated forms (1.6 and 2.0 kb) of rat BSP mRNA, were scanned with a bio-imaging analyzer (BAS2000, Fuji BAS2000).

Real-Time PCR

Total RNA (1 μg) was used as a template for cDNA synthesis. cDNA was prepared using EXScript RT reagent Kit. Quantitative real-time PCR was performed using the alkaline phosphatase (ALP) primer sets: ALP forward, 5′-TTGAATCGGAACAACCTGACTGAC-3′; ALP reverse, 5′-GATGGCCTCATCCATCTCCAC-3′; GAPDH forward, 5′-GACAACTTTGGCATCGTGGA-3′; GAPDH reverse, 5′-ATGCA-GGGATGATGTTCTGG-3′ using the SYBR Premix Ex Taq in a TP800 thermal cycler dice real-time system (Takara). The amplification reactions were performed in 25 μl of final volume containing 2 × SYBR Premix EX Taq (12.5 μl), 0.2 μM forward and reverse primers (0.1 μl), and 25 ng cDNA (2.5 μl). To reduce variability between replicates, PCR premixes, which contain all reagents except for cDNA, were prepared and aliquoted into 0.2 ml Hi-8-tubes (Takara). The thermal cycling condition was 10 s at 95°C and 40 cycles of 5 s, 95°C and 30 s, 60°C. PostPCR melting curves confirmed the specificity of single-target amplification and -fold expressions of ALPase relative to GAPDH were determined in triplicate.

Transient Transfection Assays

Exponentially growing ROS17/2.8 cells were used for the transfection assays. Twenty-four hours after plating, cells at 40–60% confluence were transfected using a Lipofectamine regent. The transfection mixture included 1 μg of a luciferase (LUC) construct [Ogata et al., 1995], 1 μg of an AR expression plasmid (PCMV-AR) and 2 μg β-Gal plasmid as an internal transfection control. Thirty-six hours posttransfection, the cells were cultured in phenol red free α-MEM without serum for 12 h and incubated with DHT (10^{-8}M) for 24 h. The LUC assay was performed according to the supplier's protocol (PicaGene, Toyo Inki, Japan) using a luminescence reader (Acuu FLEX Lumi 400; Aloka) to measure the LUC activity.

Gel Mobility Shift Assays

Double-stranded oligonucleotides encompassing the inverted CCAAT (nts, −61 to −37, 5′-CCGTGACCGTG**ATTGG**CTGCTGAGA), CRE (nts, −84 to −59, 5′-CCCACAGCC**TGACGTCG**-CACCGGCCG), and AP1/GRE (nts, −925 to −898, 5′-TAGCT**GAGTCA**CTA**GGACCT**GGG-CAACA) in the rat BSP promoter, while consensus AP-1 (5′-CGCTTGA**TGAGTCA**GCCGG-AA) and consensus GRE (5′-TCGAC**TGTACA**G-GA**TGTTCT**AGCTACT) were purchased from Promega. For gel shift analysis, the double-stranded-oligonucleotides were end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Nuclear and cytosolic protein extracts (3 μg) were incubated for 20 min at room temperature with 0.1 pM radiolabeled double-stranded-oligonucleotides in buffer containing 50 mM KCl, 0.5mM EDTA, 10mM Tris-HCl, pH7.9, 1 mM dithiothreitol (DTT), 0.04% Nonidet P-40, 5% glycerol, and 1 μg of poly (dI-dC). For competition experiments, unlabeled oligonucleotides for the CRE, AP1/GRE, consensus AP-1 and consensus GRE were used with a 40-fold molar excess. Following incubation, the protein DNA complexes were resolved by electrophoresis on 5% nondenaturing acrylamide gels (38:2 acrylamide/bis acrylamide) run at 150 V at room temperature. Following electrophoresis, the gels were dried and autoradiograms prepared and analyzed using an image analyzer.

Protein Chemistry

ROS17/2.8 cells were cultured in 100 mm tissue culture dishes in α-MEM medium containing 10% FCS. Twenty-four hours after plating, cells at 40–60% confluence were transfected with 5 μg of AR (PCMV-AR) expression plasmids using Lipofectamine. Thirty-six hours posttransfection, the cells were cultured in phenol red free α-MEM without serum for 12 h and incubated with or without DHT (10^{-8}M) for 24 h. Nuclear and cytosolic proteins were extracted by the method of Dignam and Lebovitz [1983] with the addition of extra proteinase inhibitors (the extraction buffer was 0.42 M NaCl, 1.5 mM $MaCl_2$, 0.2 mM EDTA, 1 mM DTT, 25% (v/v) glycerol, 0.5 mM phenylmethyl-sulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 1 μg/ml aprotin, pH 7.9) [Shimizu-Sasaki et al., 2001]. For Western blot analyses, protein samples were separated on 10% SDS–PAGE and transferred onto a Hybond-P membrane. The membranes were then incubated for 3 h with antiAR, histone H4, tubulin, c-Fos, and CREB antibodies (diluted 1:1000, Santa Cruz Biotechnology). Antimouse and rabbit IgG conjugated with HRP (diluted 1:5,000, Amersham Biosciences) were used as the secondary antibodies. Immunoreactivity was determined by means of the ECL chemiluminescence reaction (Amersham Pharmacia Biotech). For immunoprecipitation, nuclear proteins were precipitated with CREB or c-Fos antibodies. Thereafter, Western blot analyses were performed using AR, c-Fos and CREB antibodies.

Statistical Analysis

Triplicate samples were analyzed for each experiment, and experiments replicated to ensure the consistency of the responses to androgens and AR. Significant differences between the control, androgen, and AR treatments were determined using Student's *t*-test.

RESULTS

Western Blot Analysis of Endogenous AR and AR Overexpression in Nuclear and Cytosolic Proteins

The expression of AR in the ROS17/2.8 cells with and without DHT was analyzed by Western blotting using an antiAR monoclonal antibody. AR protein levels were increased after AR overexpression in nuclear and cytosolic proteins (Fig. 1A). The endogenous and overexpressed AR protein levels did not changed by DHT (10^{-8}M, 24 h) treatment (Fig. 1A). Nuclear and cytosolic proteins reacted with antihistone H4 and antitubulin antibodies respectively (Fig. 1A).

Effects of AR Overexpression on BSP and ALP mRNA Levels

BSP mRNA levels were increased by AR overexpression. However, the endogenous and overexpressed BSP mRNA levels did not change following DHT (10^{-8}M, 24 h) treatment. The expression of GAPDH was examined as a control (Fig. 1B). ALP mRNA levels were increased by AR overexpression and almost completely abolished by DHT (10^{-8}M, 24 h) treatment (Fig. 1C).

Transient Transfection Analysis of Rat BSP Promoter Constructs

To determine AR overexpression and the DHT effects on the BSP transcription, we used LUC constructs that included various regions of the rat BSP gene promoter transfected into ROS17/2.8 cells (Fig. 2). Whereas the LUC activities of the BSP promoter were not influenced by DHT (10^{-8}M, 24 h), the LUC activities (pLUC6; −938 to +60, pLUC5; −801 to +60, pLUC4; −425 to +60 and pLUC3; −116 to +60) were increased by AR overexpression. When we deleted the −938/−801 and −938/−425 segments (pLUC5 and pLUC4; deletion of AP1/GRE element), LUC activities increased by AR overexpression were partially inhibited. In shorter constructs (pLUC2; −43 to +60, pLUC1; −18 to +60), LUC activities were not increased by AR overexpression (Fig. 2). Within the DNA sequence that is unique to pLUC3, an inverted CCAAT box (nts −50 to −46), a CRE (nts −75 to −68), a putative Cbfa1 (nts −84 to −79), a FRE (nts −92 to −85) and a Pit-1 motif (nts −111 to −105) are present (Fig. 3). By using a series of 5′ deletion constructs between nts −116 to −43, we found that the AR response region was mediated by a region between nts −84 and −60 of the promoter sequence (Fig. 4). Next, we used mutation constructs (2 bp mutations in the putative response elements) targeted by AR and androgen within the pLUC3 (M-CCAAT, M-CRE, and M-FRE) and pLUC6 (M-AP1/GRE) (Fig. 5). The basal activities and responses for AR overexpression and DHT treatments of wild type (pLUC3) and M-FRE were almost

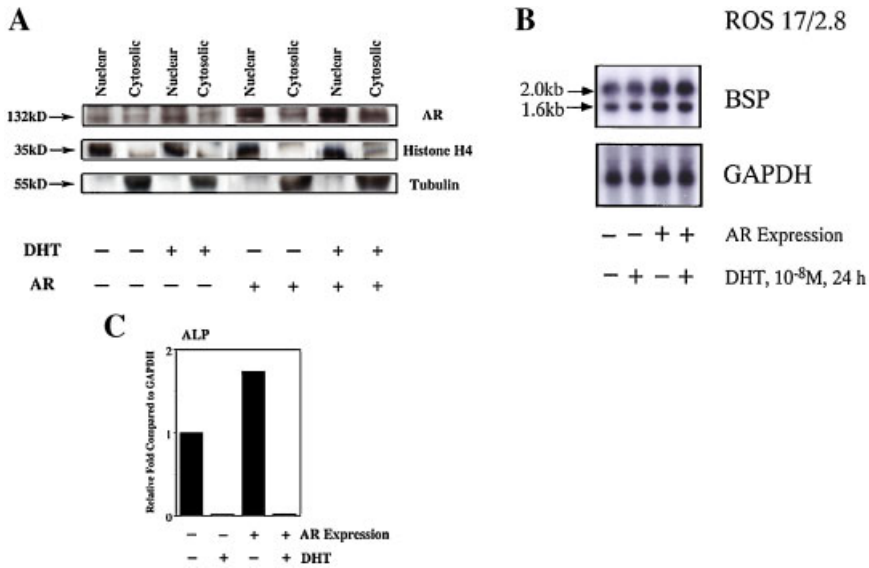

Fig. 1. Effects of AR overexpression and 5α-DHT on BSP proteins and mRNA levels in ROS 17/2.8 cells. **A**: Endogenous AR and AR overexpression in nuclear and cytosolic proteins in ROS 17/2.8 cells. Cells were transfected with or without AR expression plasmids (PCMV-AR). Thirty-six hours posttransfection, the cells were cultured in phenol red free α-MEM without serum for 12 h and treated with or without DHT (10^{-8} M) for 24 h and nuclear and cytosolic proteins were extracted. The expressions of AR, histone H4, and tubulin were analyzed by Western blotting using antiAR, histone H4, and tubulin antibodies. **B**: Effects of AR overexpression and 5α-DHT on BSP mRNA levels in ROS 17/2.8 cells. Cells were transfected with or without AR expression vector and treated with or without DHT (10^{-8} M) for 24 h. Then, total RNA was extracted, and the expressions of BSP and GAPDH mRNA in the cells were analyzed by Northern hybridization analysis. Two BSP mRNAs originate from alternative polyadenylation. **C**: Relative gene expression for ALPase generated from real-time PCR of ROS17/2.8 cells treated with DHT (10^{-8} M). The expression of GAPDH was also examined as control. The relative amounts of mRNA of ALPase to GAPDH were calculated. The experiments were performed in triplicate for each datapoint. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the same. The basal transcriptional activities of M-CCAAT and M-CRE were lower than the basal level of pLUC3. The transcriptional stimulations by AR overexpression were almost completely abrogated in the M-CCAAT, M-CRE, and M-AP1/GRE (Fig. 5). To confirm the functional elements, we performed double mutation analyses. When mutation was made in the CRE or AP1/GRE, the AR-induced LUC activities were partially abolished (Fig. 6). When mutations were made in the CRE and AP1/GRE sites, the effect of AR on the LUC activity was almost totally abrogated (Fig. 6). These results indicated that CRE and AP1/GRE act as functional response elements for AR regulation of BSP transcription.

Gel Mobility Shift Assays

To identify the binding proteins to the CRE and AP1/GRE elements, double-stranded oligonucleotides were end-labeled and incubated with equal amounts (3 μg) of nuclear proteins from ROS17/2.8 cells that were induced by DHT or AR overexpression. When the inverted CCAAT, CRE, and AP1/GRE were used as probes, DNA-protein complexes did not change after stimulation with DHT (10^{-8}M) for 24 h (Fig. 7A; lanes 2,5,8). The formation of CCAAT, CRE, and AP1/GRE-protein complexes were increased by AR overexpression (Fig. 7A; lanes 3,6,9). That those DNA-protein complexes represent specific interactions were demonstrated by competition experiments in 40-fold molar excess of unlabeled double-stranded oligonucleotides. CRE, AP1/GRE, and consensus AP1 reduced the amount of CRE-protein complex formation (Fig. 7B; lane 3,4,6). Whereas AP1/GRE reduced the formation of upper and lower AP1/GRE-protein complexes (Fig. 7B; lane 9), consensus GRE and AP1 reduced lower and upper bands respectively (Fig. 7B; lanes 10,11). In contrast, consensus GRE did not compete with CRE-protein complexes formation (Fig. 7B; lane 5), and CRE did not compete with AP1/GRE protein complexes formation (Fig. 7B; lane 12). To further characterize the proteins in the complexes formed with the CRE and AP1/GRE, we used antibodies for several transcription factors. The CRE-protein complexes showed CREB related doublet

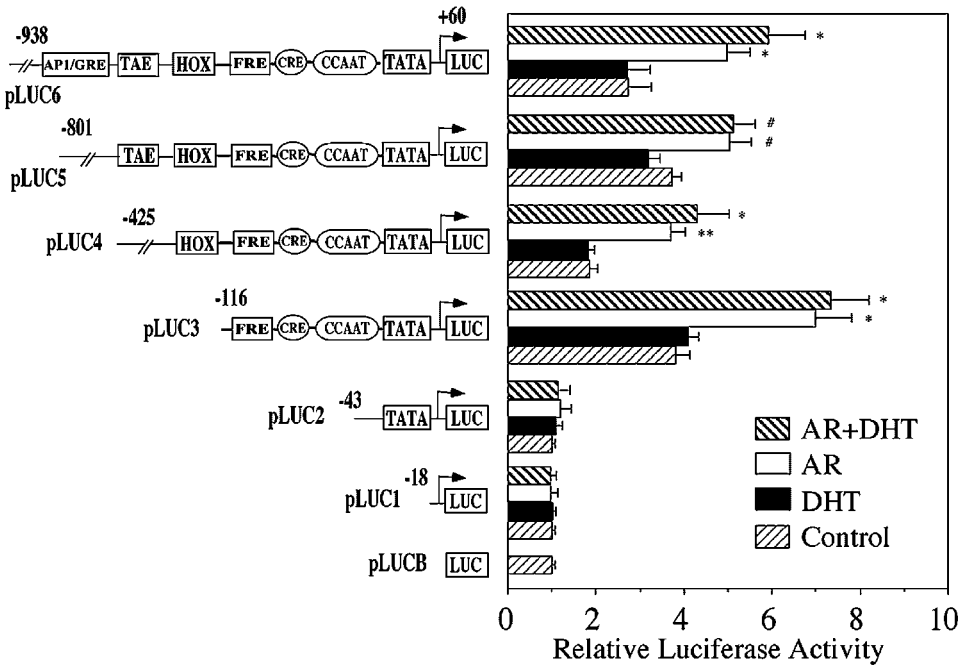

Fig. 2. AR overexpression up-regulates BSP promoter activity. LUC analyses were performed to determine transcriptional activities of chimeric constructs that included various regions of the rat BSP gene promoter ligated to a LUC reporter gene in ROS 17/2.8 cells, which were transfected with or without AR expression vector and treated with or without DHT (10^{-8} M, 24 h). The results of transcriptional activity obtained from three separate transfections with constructs; pLUC basic (pLUCB) and pLUC1 to pLUC6 have been combined and the values expressed with standard errors. Significant differences from the control: **($P < 0.05$); *($P < 0.1$); #($P < 0.2$).

bands, and three bands below the doublet (Fig. 8). Upper band of the CREB related doublet was supershifted by antiphospho-CREB antibody (Fig. 8; lane 5). AntiCREB, c-Fos, c-Jun, and AR antibodies disrupted the CREB related doublet bands (Fig. 8; lanes 4,6–8). Three bands below the CREB related doublet disappeared by c-Fos and c-Jun antibodies (Fig. 8; lanes 6,7). Especially, antiAR antibody completely blocked CREB related doublet and two bands below the CREB (Fig. 8; lane 8). The AP1/GRE-protein complexes were supershifted by c-Fos antibody (Fig. 9; lane 4). Antic-Jun and AR antibodies disrupted the complexes formation (Fig. 9; lanes 4,6–8).

Immunoprecipitation

AntiCREB antibody precipitated AR and c-Fos in ROS 17/2.8 nuclear extract, and these protein complexes increased after AR overexpression. Whereas antic-Fos antibody precipitated AR and CREB, the protein complexes did not change after AR overexpression, thus suggests that AR may interact both Fos and CREB (Fig. 10).

DISCUSSION

Sex steroid hormones have major beneficial effects on the development and maintenance of the skeleton [Orwoll and Klein, 1995]. These include control of growth plate maturation and closure during longitudinal bone growth, differential regulation of cortical and cancellous bone metabolism, stimulation of the acquisition of peak bone mass and the inhibition of bone loss [Orwoll and Klein, 1995; Vanderschueren and Bouillon, 1995; Vanderschueren, 1996]. Steroid hormone receptors interact with steroid hormone response elements in the target gene promoters, and regulate their transcription directly and indirectly. Androgen and AR may play important roles in skeletal metabolism. Therefore, we wish to study the androgen and AR effects on BSP transcription, because BSP has an important role in bone formation and mineral crystalization. In this study, we have identified AR response elements in the rat BSP gene promoter as CRE and AP1/GRE, which mediate AR actions on BSP transcription. AP1/GRE was identified by our group [Ogata et al., 1995], through which the effects of

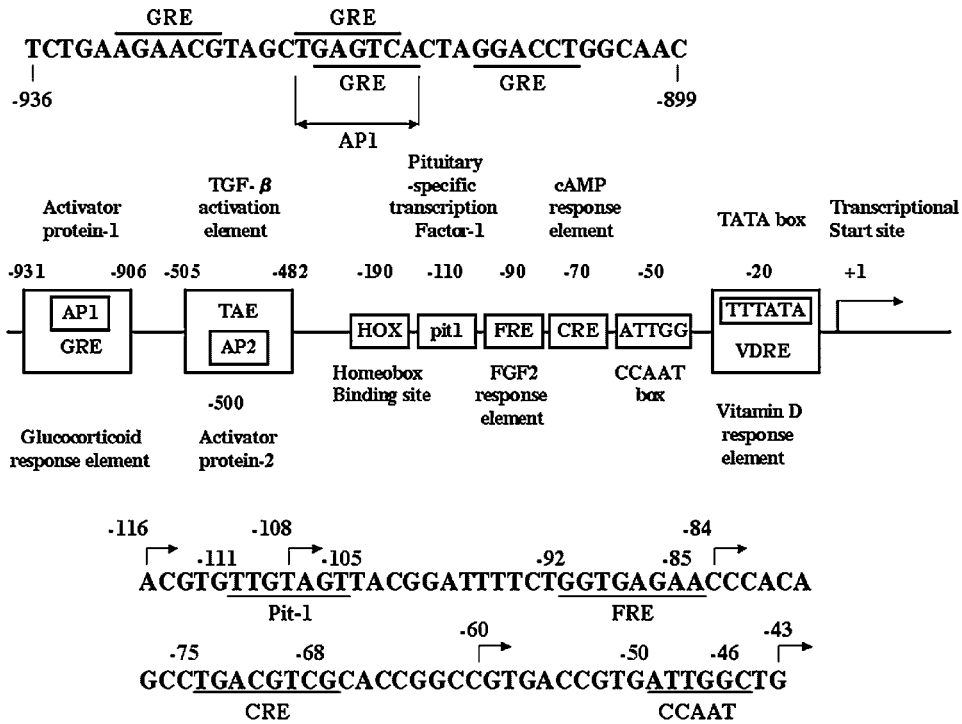

Fig. 3. Regulatory elements in the proximal rat BSP promoter. The positions of the inverted TATA and CCAAT boxes, a CRE, a FRE, a pituitary-specific transcription factor-1 binding site (Pit-1), a HOX, and a vitamin D response element (VDRE) that overlaps the inverted TATA box are shown in the proximal promoter region of the rat BSP gene, and a TAE overlapping an AP2 element and a GRE overlapping the AP1 in the distal promoter. The numbering of nucleotides is relative to the transcription start site (+1). **Upper panel**; the nucleotide sequences of the rat BSP gene promoter encompassing a GRE and AP1 are shown from −936 to −899. **Lower panel**; the nucleotide sequences of the rat BSP gene proximal promoter, encompassing an inverted CCAAT box, CRE, FRE, and Pit-1 are shown from −116 to −43.

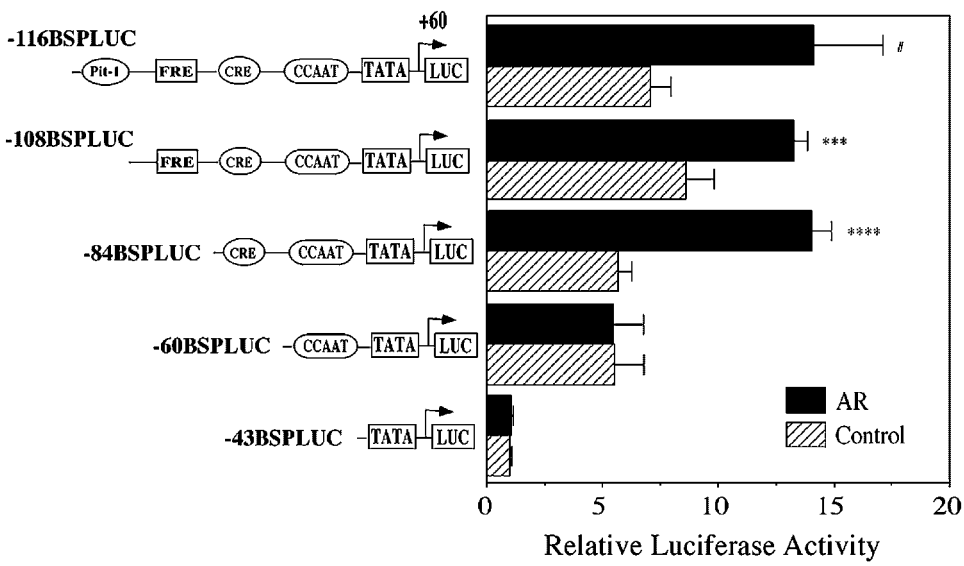

Fig. 4. Fine 5′-deletion mapping of the nts −116 to −43 element in the BSP promoter. A series of rat BSP promoter 5′ deletion constructs were analyzed for relative promoter activity after transfection into ROS17/2.8 cells and examined for induction by AR overexpression. The results of transcriptional activity obtained from three separate transfections with constructs; -43 BSPLUC (-43 to +60), −60 BSPLC (−60 to +60), −84 BSPLUC (−84 to +60), −108 BSPLUC (−108 to +60), and −116 BSPLUC (−116 to +60) have been combined and the values expressed with standard errors. Significant differences from the control: $^{\#}(P < 0.2)$; ***$(P < 0.02)$; ****$(P < 0.01)$.

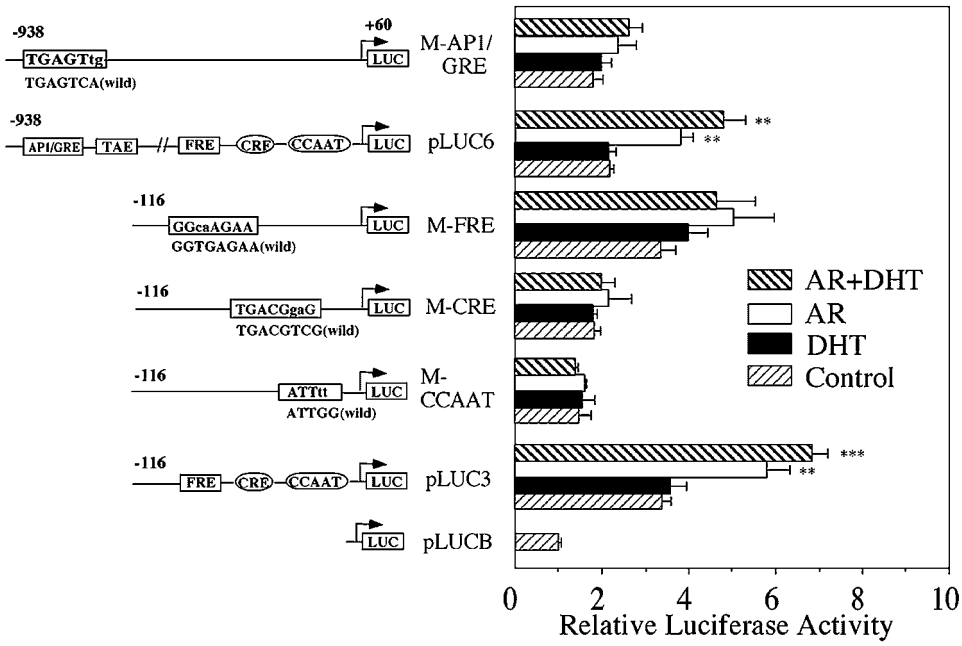

Fig. 5. Site mutation analysis of LUC activities. Dinucleotide substitutions were made within the context of the homologous −116 to +60 (pLUC3) and −938 to +60 (pLUC6) BSP promoter fragment. M-CCAAT (AATT**tt**), M-CRE (TGACG**ga**G), M-FRE (GG**ca**AGAA) and M-AP1/GRE (TGAGT**tg**) constructs were analyzed for relative promoter activity after transfection into ROS17/2.8 cells and examined for induction with AR overexpression and DHT (10^{-8} M, 24 h). The results of transcriptional activity obtained from three separate transfections with constructs were combined and the values expressed with standard errors. Significant differences from the control: ***($P < 0.02$); **($P < 0.05$).

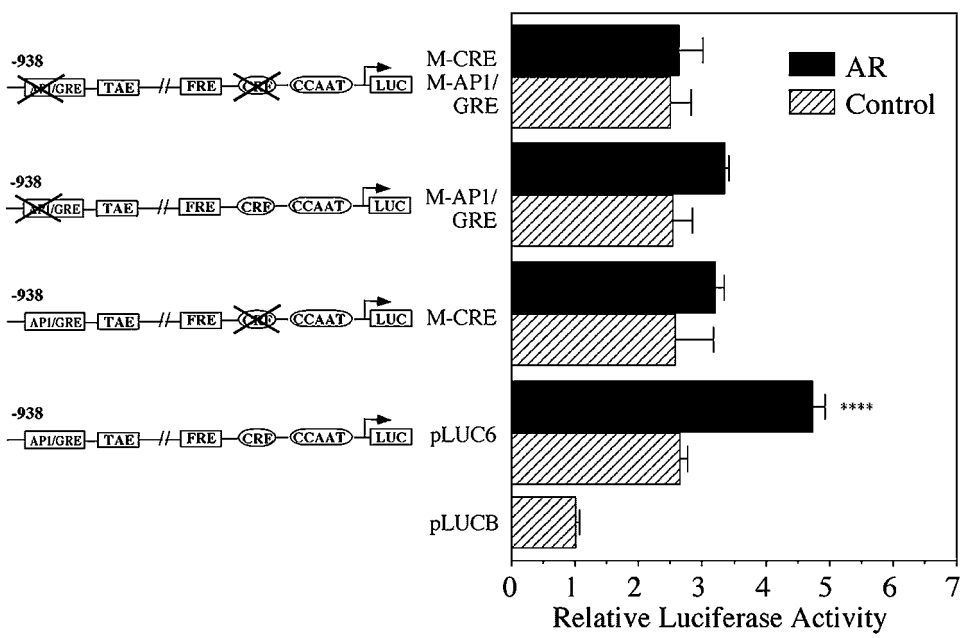

Fig. 6. LUC analyses of single- and double-mutated BSP promoter constructs (pLUC6). Dinucleotide substitutions in M-CRE and M-AP1/GRE described in this figure were analyzed for relative promoter activity after transfection into ROS 17/2.8 cells. Transcription was examined for induction by AR overexpression. The results of transcriptional activity obtained from three separate transfections with constructs were combined and the values expressed with standard errors. Significant differences from the control: ****($P < 0.01$).

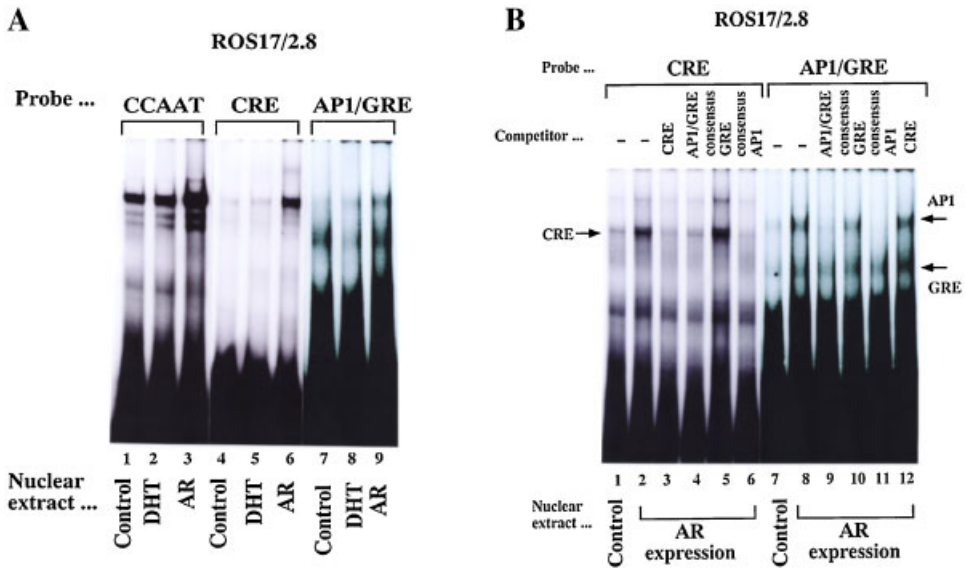

Fig. 7. Specific binding of nuclear proteins to the inverted CCAAT, CRE and AP1/GRE. **A**: Radiolabeled double-stranded CCAAT (−61 CCGTGACCGTG**ATTGG**CTGCTGAGA-37), CRE (−84 CCCACAGCC**TGACGTCG**CACCGGCCG-59) and AP1/GRE (−925 TAGCT**GAGTCA**CTA**GGACCT**GGGCAACA −898) were incubated with nuclear protein extracts (3 μg) obtained from ROS 17/2.8 cells stimulated without (**lanes 1**,**4**,**7**) or with DHT (10^{-8} M, 24 h; **lanes 2**,**5**,**8**) and AR expression vector (**lanes 3**,**6**,**9**). DNA-protein complexes were separated on 5% polyacrylamide gel in low-ionic-strength Tris-borate buffer, dried under vacuum and exposed to an imaging plate for quantitation using an image analyzer. **B**: Competition reactions were performed using a 40-fold molar excess of unlabeled CRE (CCCACAGCC**TGACGTCG**CACCGGCCG; **lanes 3**,**12**), AP1/GRE (TAGCT**GAGTCA**CTA**GGACCT**GGGCAACA; **lanes 4**,**9**), consensus GRE (TCGAC**TGTACA**GGA**TGTTCT**AGCTACT; **lanes 5**,**10**) and consensus AP1 (CGCTTGA**TGAGTCA**GCCG-GAA; **lanes 6**,**11**). DNA-protein complexes were separated on 5% polyacrylamide gel in low-ionic-strength Tris-borate buffer, dried under vacuum and exposed to an imaging plate for quantitation using an image analyzer. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

glucocorticoids on BSP gene transcription appear to be regulated. Interestingly, the AR effects are ligand-independent. These results suggest that AR might interact with CREB and AP1 transcription factors to activate BSP transcription in the absence of direct DNA binding. Estrogen receptor β (ERβ) have a ligand-independent effects on mouse gonadotropin-releasing hormone promoter activity [Pak et al., 2006]. ERβ is a potent modulator of estrogen response element and AP1 mediated transcription independent of ligand binding, and might have a functional role in modulating gene expression that is independent of steroid hormones [Pak et al., 2005]. Further study is necessary to elucidate the mechanism of ligand-independent activation of AR.

LUC activities using the rat BSP promoter constructs were not influenced by DHT (10^{-8}M, 24 h). However, the transcriptional activities (pLUC3–pLUC6) were increased by AR overexpression (Fig. 2). Moreover, the results of LUC analyses using 5′ deletion constructs between nts −116 to −43 in the BSP promoter indicated that the AR effects are targeted to a region encompassed by nts −84 and −60 (Fig. 4). The putative response element in this region is CRE (Fig. 3). Whereas the LUC activity of M-FRE is almost the same as that of the wild type, M-CCAAT resulted in the loss of basal transcriptional activity. In comparison, the luiferase activities of M-CRE and M-AP1/GRE indicate that these two elements are required for the induction of BSP transcription by AR overexpression (Figs. 5,6). The involvement CRE and AP1/GRE elements are further supported by gel shift assays. The formation of CRE and AP1/GRE-protein complexes were increased by AR overexpression (Fig. 7). The results of LUC assays using constructs with mutations in the CRE and AP1/GRE elements suggest that they are collectively required for optimal induction of BSP expression by AR (Fig. 6). There is no additional increase of transcriptional activity in pLUC6, which contains both CRE and AP1/GRE, in spite of the increasing transcriptional activity by AR overexpression to the same extent in pLUC3, which contains CRE

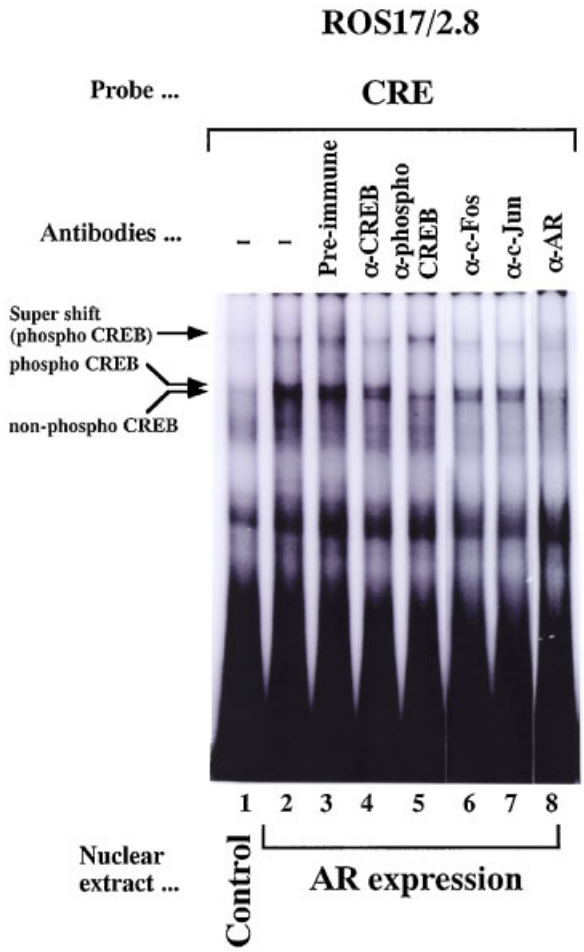

Fig. 8. Specific binding of nuclear proteins to the CRE. Radiolabeled double-stranded CRE (−84 CCCACAGCC**TGACGTCG**-CACCGGCCG-59) was incubated with nuclear protein extracts (3 μg) obtained from ROS 17/2.8 cells stimulated without (lane 1) or with AR expression vector (**lanes 2–8**). Supershift experiments were performed with 0.4 μg of antibodies against CREB, phospho-CREB, c-Fos, c-Jun, and AR added separately to each gel shift reaction. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

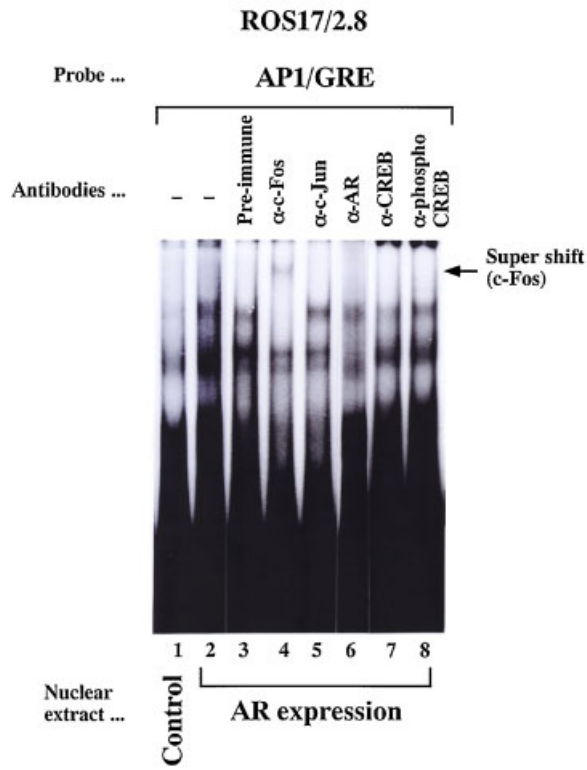

Fig. 9. Specific binding of nuclear proteins to the AP1/GRE. Radiolabeled double-stranded AP1/GRE (−925 TAGCT**GAGT-CA**CTA**GGACCT**GGGCAACA -898) was incubated with nuclear protein extracts (3 μg) obtained from ROS 17/2.8 cells stimulated without (**lane 1**) or with AR expression vector (**lanes 2–8**). Supershift experiments were performed with 0.4 μg of antibodies against c-Fos, c-Jun, AR, CREB and phospho-CREB added separately to each gel shift reaction. [Color figure can be viewed in the online issue, which is available at www.interscience. wiley.com.]

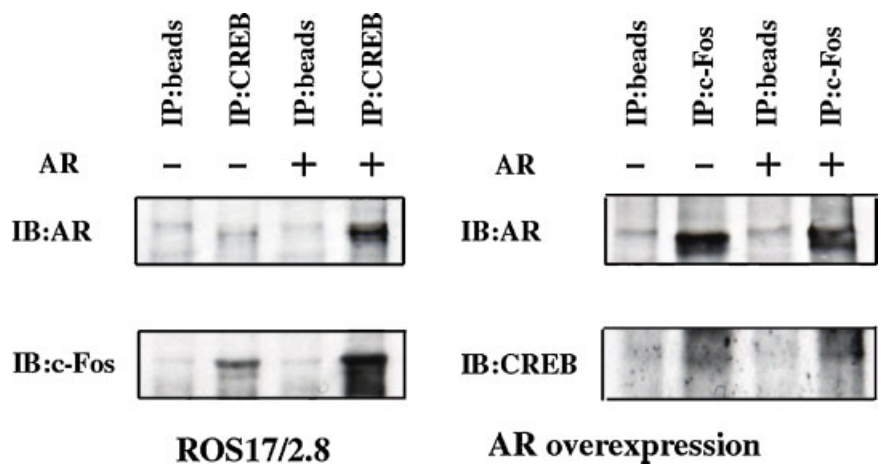

Fig. 10. Immunoprecipitation using antiCREB and antic-Fos antibodies in ROS 17/2.8 nuclear extract without or with AR overexpression. AntiCREB and c-Fos antibodies were used for immunoprecipitation. Thereafter, Western blot analyses were performed using antiAR, c-Fos, and CREB antibodies. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

alone. These suggest that the contribution of the AP1/GRE element to the activation of BSP transcription by AR overexpression might be weaker than that of the CRE element, or there might be repressor elements between pLUC4–pLUC6.

That the proteins bind specifically to CRE and AP1/GRE were demonstrated by the competition gel shift and supershift assays (Figs. 7B,8,9). CRE binding proteins could be identified as phospho-CREB, nonphospho-CREB, c-Fos, c-Jun, and AR. AP1/GRE binding proteins could be c-Fos, c-Jun, and AR by using specific antibodies (Figs. 8,9). The results of immunoprecipitation showed that CRE binding proteins were CREB, AR and c-Fos, and AP1/GRE binding proteins were AR and c-Fos. Further study is necessary to elucidate how these proteins interact with each other and bind to AR response elements.

In conclusion, our studies showed that AR increased BSP transcription in ROS17/2.8 cells, which targets CRE and AP1/GRE elements in the rat BSP gene promoter.

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