Regulation of Human Osteocalcin Promoter in Hormone-
independent Human Prostate Cancer Cells*

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Osteocalcin (OC)1 is the major noncollagenous bone matrix
deposited by mature osteoblasts at the time of bone mineral-
the osteoblast lineage (2). OC is synthesized, secreted, and
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ase activity; RT, reverse transcriptase.

osteoblasts. The discovery of OC expression in prostate cancer
specimens led us to study the regulation of OC gene in
androgen-independent metastatic human prostate PC3
cells. An 800-bp human OC (hOC) promoter-luciferase
construct exhibited strong basal and vitamin D-induced
activity in OC-positive human prostate and osteosarcoma
cell lines. Through deletion analysis of the hOC promoter,
the functional hierarchy of the cis-acting elements, OSE1,
OSE2, and AP-1/VDRE, was established in PC3 cells
(OSE1 > AP-1/VDRE > OSE2). By juxtaposing dimers of
these 3 cis-elements, we produced a minimal hOC pro-
moter capable of displaying high tissue specific activity in
prostate cancer cells. Our study demonstrated three
groups of transcription factors, Runx2, JunD/Fra-2, and
Sp1, responsible for the high hOC promoter activity in
PC3 cells by binding to the OSE2, AP-1/VDRE, and OSE1
elements, respectively. Among the three groups of tran-
scription factors, the expression levels of Runx2 and Fra-2
are higher in the OC-positive PC3 cells and osteoblasts,
compared with the OC-negative LNCaP cells. Interest-
ingly, unlike the mouse OC promoter, the OSE1 site in
hOC promoter is regulated by members of Sp1 family
instead of the osteoblast-specific factor Osf1. The molec-
ular basis for androgen-independent prostate cancer cells
behaving like mature osteoblasts may be explained by the
interplay and coordination of these transcription factors
under the tight regulation of autocrine and paracrine mediators.

Osteocalcin (OC)1 is the major noncollagenous bone matrix
protein expressed in bone (1). OC expression is transcription-
ally regulated by vitamin D and limited exclusively to cells of
the osteoblast lineage (2). OC is synthesized, secreted, and
deposited by mature osteoblasts at the time of bone mineral-
ization. It serves as a phenotypic marker for mature osteoblasts

(3). Despite its well characterized specificity of expression in
transgenic mouse (4), the precise function of OC in bone remodeling
remains unclear. The location of OC at the bone-forming
surfaces (5) and the increased bone mineralization observed in
OC gene knockout mice (6) supports a role of OC in suppression
of bone mineralization.

Due to its tissue specificity, regulation of OC expression has
been studied extensively in bone cells. Many regulatory ele-
ments have been identified in the proximal 800-bp region of the
promoters. These include OSE1, OSE2 (7), AP-1/VDRE (8), and
GRE (9). OSE1 and OSE2 were identified in mouse OC (mOC)
and are responsible for its tissue specific activity in
osteoblasts. Both of these cis-elements are occupied by osteo-
blast-specific transcription factors, Osf1 and Runx2, respec-
tively (7, 10). Runx2 belongs to the Runt domain transcription
factor family (11) and it has an indispensable role in osteoblast
differentiation, maturation, and bone formation (12). Runx2
was shown to bind the OSE2 site and regulates the mOC
promoter in a tissue-specific manner (13).

In contrast to mOC promoter (14), hOC promoter is highly
inducible by vitamin D3 (15). As a result, studies have mostly
stressed its regulation by vitamin D3 in bone cells (16). Little is
known about the basal regulation of the gene. Vitamin D re-
ponse element (VDRE) has been mapped to the proximal pro-
moter and it is contiguous to an AP-1 site (8). Studies suggest
that various AP-1 factors occupy this site at different stages
during osteoblast development and they tightly regulate the
interactions between vitamin D receptor (VDR) and its cognate
binding sites. In proliferating osteoblasts, the binding of c-Fos
and c-Jun heterodimers to the AP-1 site suppresses the rat OC
(rOC) promoter activity, while the association of Fra-2 and
JunD in the post-proliferate osteoblasts facilitates VDR/reti-
noid X receptor binding to the rOC promoter and induces its
activity (17).

Prostate cancer is the second leading cause of cancer death in
Northern American men. Despite its common occurrence, the
molecular mechanisms responsible for prostate cancer growth,
androgen-independent (AI) progression and acquisition of bone
metastatic potential are poorly characterized. Bone matrix pro-
teins such as OC, bone sialoproteins, and osteopontin are ex-
pressed at high levels in primary and metastatic prostate can-
cer specimens (18). We hypothesized previously that prostate
cancer acquires “bone-like properties” to thrive and grow in the
bone microenvironment (19). The goal of this study therefore is
to better understand the osteomimetic properties of prostate
cancer cells by studying the regulation of hOC gene expression
in an AI prostate cancer cell line, PC3. PC3 cells were estab-
lished from the bone metastatic lesions of a prostate cancer

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¶ The abbreviations used are: OC, osteocalcin; hOC, human osteocalcin;
mOC, mouse osteocalcin; rOC, rat osteocalcin; VDRE, vitamin D
response element; VDE, vitamin D response; AI, androgen-indepen-
dent; EMSA, electrophoretic mobility shift assay; RLA, relative lucifer-
ase activity; RT, reverse transcriptase.
patient. It is considered a highly aggressive AI cell line which expresses neither AR nor PSA, and it does not require androgen for growth or survival. When injected intracardially in athymic mouse, PC3 cells have high propensity to metastasize to bone.

In this study, we established the functional hierarchy of three regulatory elements, OSE1, OSE2, and AP-1/VDRE, in the regulation of the hOC promoter activity in PC3 cells (OSE1 > AP-1/VDRE > OSE2). Furthermore, through EMSA we identified Runx2, JunD/Fra-2, and Sp1 as the transcription factors which regulate OSE2, AP-1/VDRE, and OSE1, respectively. By expressing osteoblast-specific factor-like Runx2 and selectively activating Ap1 factor Fra-2, PC3 is able to behave like a mature osteoblast in activating OC expression. Based on our results, a model was constructed to explain how prostate cancer cells might become osteomimetic. This biochemical switch may contribute to the osteolytic/osteoblastic phenotype of prostate cancer cells frequently observed in metastatic skeletal lesions.

MATERIALS AND METHODS

Cell Culture and Transfection—Prostate cancer cells (PC3, DU145, and LNCaP) and MG63 (human osteosarcoma cells) were cultured in T-medium (20) supplemented with 5% fetal bovine serum. Rat osteosarcoma cells (rat osteosarcoma cells) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum. For transfections, cells were plated at a density of 1.0 × 10^5 (PC3, DU145, and rat osteosarcoma cells)/well in 12-well plates 24 h or 3 × 10^6 cells/well (LNCaP) in 6-well plates 48 h before transfection. Plasmid DNAs were introduced into cells either by complexing with DOTAP (Roche Molecular Biochemicals) or Confectin (CLONTECH, Palo Alto, CA). Briefly 1–3.5 μg of tested DNA constructs were used in the experiments. DNA-lipid complexes were allowed to form for 5–15 min at room temperature prior to their addition to each well containing 0.5 or 1 ml of serum-free and phenol red-free RPMI 1640 medium or serum-free Dulbecco's modified Eagle's medium. The cells were incubated with the complexes for 4–5 h at 5% CO_2, 37 °C. DNA-lipid containing medium was then replaced with fresh medium with fetal bovine serum. Cells were collected after 36–48 h of additional incubation.

Luciferase Assay—Cells were washed with 0.5 ml/well of phosphate-buffered saline and lysed in 100–300 μl of 1 × lysis buffer (Promega, Madison, WI). Cell lysates were vortexed for a few seconds and spun for 2 min. For luciferase activity detection, 20 μl of the supernatant was mixed with 100 μl of luciferase substrate (Promega) and measured by a luminometer (Monolight 2010, Analytical Luminescence Laboratory, Sparks, MD). For β-galactosidase activity detection, 50 or 100 μl of the supernatant was mixed with an equal volume of 2 × β-galactosidase substrate (Promega) and incubated at 37 °C for 15–90 min. The β-galactosidase activity was determined by a reader at 405 nm wavelength. For protein assays, 10 μl of cell extracts were mixed with 200 μl of Coomassie plus protein reagent (Pierce, Rockford, IL) and measured at 590 nm. Data are expressed as relative luciferase activity (RLA), of Coomassie plus protein reagent (Pierce, Rockford, IL) and measured at 590 nm. Data are expressed as relative luciferase activity (RLA), of Coomassie plus protein reagent (Pierce, Rockford, IL) and measured at 590 nm.

Plasmids—Genomic DNA was used in the PCR of the 800-bp human osteocalcin promoter (16), subsequently cloned into luciferase reporter vector-pGL3/Basic (Promega). The deletion constructs were generated by digesting with either BamHI and AatII or BamHI and NdeI and ligated to pGL1- and NdeI-digested pGL3/TATA vector to generate OSE1, mOSE2, OSE2, and AV/TATA. Ligating respective constructs digested with either BamHI and AatII or BamHI and NdeI generated different combinations of the OSE1, OSE2, and AV/TATA.

Western Blot Analysis—Immunoblotting was performed using the NOVEX (Invitrogen, Carlsbad, CA) system. Briefly, 20 μg of nuclear extracts were separated on 4–12% Tris glycine PAGE gels and transferred onto a 0.2-μm nitrocellulose membrane. Nonspecific binding was blocked with 5% nonfat milk in TBS-T for 1 h at 37 °C. Primary antibody was used at a 1:500-Roxun2 (22) or 1:200-Fra-2 and JunD (Santa Cruz Biotechnology, Santa Cruz, CA) dilution. Secondary antibody (horseradish peroxidase-anti-rabbit antibody) (Amersham Bioscience, Inc., Piscataway, NJ) was used in a 1:4000 dilution. The incubation of both primary and secondary antibodies was done at 37 °C for 1 h with 30 min washing (TBS-T) in between. ECL plus (Amersham Bioscience, Inc.) reagent was used for detection.

EMSA—PAGE purified oligos (Sigma Genosys, Woodlands, TX) were annealed by heating up to 95 °C and slowly cooled down to room temperature. The oligos sequences used as probes or competitors were as follows: ARE-III, 5′-tgcagggagcatgtcatgcgta-3′ (23); SP-1, 5′-atctggtagctggctggcaggg-3′; mSP-1, 5′-atctggtagctggctggcaggg-3′; AP-1, 5′-gggtgtagaactcagccgaag-3′; AV-3, 5′-atctggtagctggctggcaggg-3′; VDRE, 5′-atctggtagctggctggcaggg-3′; and mVDRE, 5′-atctggtagctggctggcaggg-3′. The double-stranded probes were end-labeled with [γ-32P]ATP by using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Nuclear extracts were prepared as described in Current Protocols (24). 40,000 cpm of labeled probe and 5–10 μg of nuclear extracts were incubated with binding buffer containing 10 μl Tris-HCl (pH 7.5), 50 μM NaCl, 0.5 μM EDTA, 0.5 μM diethrio-itol, 4% glycerol, 1 μg of poly(dI-dC) (Amersham Bioscience, Inc.), and 1 μl KCl at room temperature for 30 min. The samples were subjected to electrophoresis at room temperature on a 4% nondenaturing polyacryl-amide gel in 0.5 × TBE at 35 mA for 2 h. For experiments using Runx2 antibody, 2 μg of antibody was added to the reaction mixture for 30 min after the incubation period of the probe and nuclear extracts. For experiments using Sp1, Sp2, Sp3, VDR, Fra-2, JunD and AR antibodies (Santa Cruz Biotechnology), nuclear extracts and 2 μg of antibody were preincubated at room temperature for 30 min before the addition of probe. In competition experiments, competitor oligos were incubated with nuclear extracts for 30 min at room temperature before the addition of the probe.

RT-PCR—RNA was extracted using RNeasy B (Tetstel, Friendswood, TX). Reverse transcription was performed using Superscript II reverse transcriptase (Invitrogen), according to the manufacturer's protocol. Each RT reaction contained 5 μg of total RNA, 0.5 μg of oligo(dT) (Amersham Bioscience, Inc.), and 0.5 μg of random hexamer (Amersham Bioscience, Inc.) in a total volume of 20 μl which were then incubated at 42 °C for 1 h. Subsequently, 3 μl of the fresh RT reaction was used as template for PCR. The primers used for hOC PCR were catttggtg (23); ocatactgagatcagcgg (GOC) and gcagaactgcatgcg (OCR). The primers used for human runx2 PCR were: acctatggctgcatcga and cattaagcttgtgctgct. The cycle for PCR was 94 °C 30 s, 60 °C 30 s, and 72 °C 30 s for 35 cycles. The 1.25-(OH)_2D_3 (vitamin D_3) used in the experiment was a generous gift from Hoffmann-LaRoche, Inc. (Nutley, NJ).

RESULTS

Androgen-independent Metastatic Prostate Cancer Cell Lines Have High and Inducible HOC mRNA and Promoter Activity—We demonstrated previously that OC protein was not detectable in normal human prostate tissue, yet it was prevalently expressed in primary prostate cancer (85%) and in prostate cancer lymph node (100%) and bone metastasis specimens (100%) (26). To understand the molecular mechanism of hOC expression in prostate cancer cells, we examined the expression pattern of hOC mRNA in different cancer cell lines. RNA from four cell lines in two different conditions were extracted and used as templates for RT-PCR. Ethanol (the control groups) or 5 nm 1,25-(OH)_2D_3 (vitamin D_3) was added to the medium when the cells reached 60% confluence. Cells were collected after an additional 48 h incubation. A human osteosarcoma cell line (MG63) known to express endogenous OC (27) was used as a positive control. HOc mRNA was detected in both PC3 bone metastatic AI prostate cancer cells and the positive control MG63. Upon addition of vitamin D_3, all the AI prostate cancer cell lines (DU145 and PC3) and MG63 showed an elevated level of hOC mRNA. However, the androgen-dependent/sensitive nonmetastatic prostate cancer cell LNCaP cells did not show any hOC expression in either the presence or absence of vitamin D_3.

To investigate whether the expression of hOC mRNA is controlled at the transcriptional level, the 800-bp hOC promoter was cloned and inserted upstream to a luciferase reporter gene (hOC/luc) for transient expression analysis (Fig. 1B). In agreement with the RT-PCR data, PC3 exhibited the highest basal hOC/luc activity (34,850 ± 7,434 RLA) among the
three independent experiments containing duplicates.

**Fig. 1. Basal and vitamin D₃-induced hOC mRNA expression and promoter activity in human prostate cancer cell lines (LNCaP, PC3 and DU145) and osteosarcoma cell lines (MG63 and Ros).** A, mRNA level of hOC in cancerous cell lines. RT-PCR was performed using total RNA prepared from 4 different human cell lines with or without 5 nM vitamin D₃ treatment. Human osteosarcoma MG63 is the positive control in this experiment. The size of hOC cDNA fragment is 275 bp. B, activity of hOC promoter. The 800-bp hOC promoter was inserted upstream to a luciferase reporter gene (hOC/luc) and transiently co-transfected with CMV/β-galactosidase into the cell lines. In this experiment Ros cells served as the positive control. The RLA was obtained by normalizing the luciferase activity with internal control β-galactosidase activity. RLA is expressed as the mean ± S.D. of three independent experiments containing duplicates.

Three prostate cancer cell lines, whereas the activity (2,311 ± 312 RLA) observed in LNCaP cells was similar to the empty vector. Rat osteosarcoma cells (16) served as positive control in the transient transfection experiments because high transfection efficiency can be obtained with rat osteosarcoma cells, but not with MG63. In accordance with previous studies, the hOC promoter was inducible by the addition of vitamin D₃ (Fig. 1B) in all the cell lines tested. Thus the promoter is tissue-specific and regulated by vitamin D₃. In this study, PC3 (which has the highest basal hOC promoter activity) was the target cell line for investigating basal hOC promoter regulation in prostate cancer cells and LNCaP was used as the negative control cell line.

**OSE 1 and AP-1/VDRE Are Critical for Regulating Basal hOC Promoter Activity in PC3 Cells**—OSE1 and OSE2 of mOC promoter were reported to be responsible for its restrictive activity in bone cells (7), while AP-1/VDRE (AV) is required for the vitamin D₃ inductive response in hOC promoter (8). However, the roles of OSE1, OSE2, and AP-1/VDRE have never been examined in the regulation of basal hOC promoter activity in prostate cancer cells. To define the functional hierarchy of these cis-elements in the control of hOC promoter activity in PC3 cells, we used the recombinant PCR method to generate single, double, or triple deletions of these elements (Fig. 2A). Among the single deletion mutants, ΔOSE1 suffered the greatest activity drop, followed by ΔAV which contains deletion of half of the VDRE and the contiguous AP-1 site. Deletion of OSE2 did not seem to affect promoter activity to a great extent. Moreover, when OSE2 was removed together with either OSE1 or AV in double deletion, no additional decrease of activity was observed compared with the single deletion mutants (ΔAV or ΔOSE1). Thus, the OSE2 element may not be required for the maintenance of basal hOC promoter activity. On the other hand, OSE1 and AV single or double deletions have caused dramatic loss of activity in the hOC promoter indicating that these two cis-elements are crucial in conferring basal activity to the promoter in prostate cancer cells. It is conceivable that the OSE1 element exerts its effect by being in close proximity to the TATA box, which would allow the OSE1-binding factor(s) to act as a mediator between the binding factors of the other two cis-elements and the TATA binding complex.

The effect of deletions on vitamin D induction is depicted in Fig. 2B. Constructs that contain AV deletion become unresponsive to vitamin D stimulation, this data agrees with the current understanding that the binding of VDR and AP-1 factors is critical for vitamin D induction. Even though OSE1 and/or OSE2 deletions did not affect fold induction of vitamin D, the overall induced activities of the constructs are significantly less than those of the wild type promoter due to lower basal activities (Fig. 2A). Therefore, OSE1 and OSE2 may function independently of AV and they are required to confer maximum vitamin D-induced activity to hOC promoter.

To determine whether these cis-elements can function independently in gene transcription, we generated constructs with two copies of each of the cis-elements inserted upstream to an artificial TATA box. As shown in Fig. 2C, both AV and OSE1 could function independently in PC3 cells, with OSE1/TATA having the highest fold increase above the empty vector activity (28.1-fold) and OSE2/TATA the least activity (2-fold). These results are in agreement with the deletion data, indicating that OSE1 and AV are strong regulatory elements in the hOC promoter in PC3 cells. Interactions among the three elements were investigated by juxtaposing dimers of the elements in the pGL3/TATA vector. The results suggested that OSE1 could interact with AV and activate the simple TATA promoter in a synergistic manner (181.3-fold). Addition of OSE2 did not further increase the activity of either OSE1/TATA or AV/TATA. However, when OSE2 was inserted between AV and OSE1 in the AV₂-OSE₂/OSE₁/TATA construct, increased activity (299-fold) was observed. These results imply that the OSE2-binding factor is not sufficient to induce transcriptional activation, but it can cooperate with OSE1 and AV-binding factors and collectively activate hOC promoter.

Next, we generated a chimeric promoter, AV₂-OSE₂-OSE₁/TATA, which not only retained the tissue-specific character of hOC promoter and was also 8.1-fold more active than wild type hOC promoter in the OC-positive PC3 cells, but not in the OC-negative LNCaP cells (Fig. 2D). Tissue-specific transcription factor(s), which are only present in OC positive cells, may be involved in regulating the chimeric AV₂-OSE₂-OSE₁/TATA.
TATA promoter. Furthermore, the addition of vitamin D did not affect the activity of any of the constructs shown in Fig. 2C (data not shown). The activity of the 800-bp wild type hOC promoter (hOC/luc) was set to 100% in PC3 cells. The activities of various deletion constructs were then presented as % of the wild type promoter activity. Note AV and OSE1 but not OSE2 were required for the maintenance of the basal hOC promoter activity. AV deletion abolished vitamin D induction. 5 nM of 1,24,25-(OH)D3 was added to PC3 cells at the end of the 5-h transfection incubation period. Fold induction was obtained by dividing vitamin D-induced activities with the activities of the respective constructs in the presence of ethanol. RLA of the constructs in the presence of vitamin D is indicated to the right of each bar. C, interaction exists between OSE1 and AV sites. To evaluate the activities and interactions of the cis-elements, dimers of the cis-elements were inserted upstream to an artificial TATA box in different combinations. The normalized activities (RLA) of various constructs were divided by the normalized activity of the empty vector (pGL3/TATA) in PC3 cells and expressed as fold of control. D, juxtaposing OSE1, OSE2, and AP-1/VDRE reconstituted hOC promoter activity. The activities of the control (hOC/luc) in PC3 (34,850 ± 7,434 RLA) and LNCaP (2,311 ± 311.7 RLA) were set to be 1, respectively, and the activities of AV2-OSE22-2-OS12/TATA were expressed as fold of control.

Fig. 2. OSE1 and AP-1/VDRE (AV) are critical regulatory elements for basal hOC promoter activity in PC3 cells. A, deletion analysis of hOC promoter. Single, double, and triple deletions of OSE1, OSE2, and AV were generated in hOC promoter by the recombinant PCR method. The partial sequence of the hOC promoter is shown, with the location of the studied cis-elements underlined and the TATA box double-underlined. The activity of the 800-bp wild type hOC promoter (hOC/luc) was set to 100% in PC3 cells. The activities of various deletion constructs were then presented as % of the wild type promoter activity. Note AV and OSE1 but not OSE2 were required for the maintenance of the basal hOC promoter activity. B, AV deletion abolished vitamin D induction. 5 nM of 1,24,25-(OH)D3 was added to PC3 cells at the end of the 5-h transfection incubation period. Fold induction was obtained by dividing vitamin D-induced activities with the activities of the respective constructs in the presence of ethanol. RLA of the constructs in the presence of vitamin D is indicated to the right of each bar. C, interaction exists between OSE1 and AV sites. To evaluate the activities and interactions of the cis-elements, dimers of the cis-elements were inserted upstream to an artificial TATA box in different combinations. The normalized activities (RLA) of various constructs were divided by the normalized activity of the empty vector (pGL3/TATA) in PC3 cells and expressed as fold of control. D, juxtaposing OSE1, OSE2, and AP-1/VDRE reconstituted hOC promoter activity. The activities of the control (hOC/luc) in PC3 (34,850 ± 7,434 RLA) and LNCaP (2,311 ± 311.7 RLA) were set to be 1, respectively, and the activities of AV2-OSE22-2-OS12/TATA were expressed as fold of control.

TATA promoter. Furthermore, the addition of vitamin D did not affect the activity of any of the constructs shown in Fig. 2C (data not shown). The half VDRE in the AV element appears insufficient to confer vitamin D response to the constructs that contain AV2 (AV2/TATA, AV2-OS12/TATA, AV2-OS22/TATA, and AV2-OS22-2-OS12/TATA). Vitamin D may not be involved in the regulation of the cis-elements. Vitamin D is implicated as a protective factor against prostate cancer development and progression. Studies have indicated that there may be a local deficiency of the growth inhibitory vitamin D in prostate cancer patients (28, 29), resulting in increased proliferation, de-differentiation, and invasion of the cancer cells. Since the physiological level of vitamin D is uncertain in prostate cancer patients, the following sections focus on identifying transcription factors that regulate basal hOC promoter.

Runx2 is expressed in PC3 cells and active in associating

with OSE2—Even though OSE2 in mOC promoter was shown to associate with the osteoblast-specific factor, Runx2 (13), it is not clear whether the same transcription factor binds to the OSE2 on hOC promoter in PC3 cells. To address this question, we first determined the expression of Runx2 in PC3 cells by both RT-PCR and immunoblot analysis. Runx2 mRNA was expressed in all the OC-positive cell lines tested both in the presence and absence of vitamin D3, with PC3 having the highest level of expression (Fig. 3A). Vitamin D3 does not seem to regulate the mRNA level of runx2. On the protein level, Runx2 is highly expressed only in the OC-positive PC3 cells, but was not detected in the OC-negative LNCaP cells (Fig. 3B).

Although the OSE2 site was not necessary for transcriptional activation of hOC promoter, its binding factor Runx2 was expressed at a high level in PC3 cells. To determine whether Runx2 in PC3 cells was capable of binding DNA, we compared
EMSA profiles between PC3 and LNCaP nuclear extracts (Fig. 3C). PC3 nuclear extracts gave a specific DNA-protein complex (lane 7) that could only be competed away by a specific competitor, OSE2 (lane 8), but not by a nonspecific competitor, ARE-III (lane 9). With LNCaP nuclear extracts, no specific DNA-protein complex was observed (lanes 2–4). By adding Runx2 antibody or control AR antibody to the EMSA reactions, we observed that, as expected, the protein factor that associates with OSE2 in PC3 cells is indeed Runx2. In lanes 10 and 11, Runx2, but not AR antibody, could supershift the DNA-protein complex, suggesting that transcriptionally active Runx2 may play a significant role in the regulation of hOC promoter activity in PC3 cells as these cells acquire AI and skeletal metastatic potentials.

AP-1 Proteins, Fra-2 and JunD, Bind to AP-1/VDRE Sites on the hOC Promoter—Different AP-1-binding proteins are known to operate at the AP-1/VDRE (AV) site in the rOC promoter during different stages of osteoblast development. In proliferating osteoblasts, c-Fos and c-Jun are involved in phenotypic suppression of the OC promoter while in the post-proliferate stage, JunD and Fra-2 are responsible for enhancing the expression of OC (30). Fig. 4A shows that the hOC AV was occupied by AP-1 protein factors and not by VDR, because the VDRE consensus site could not compete away the protein-DNA complex observed (lanes 5, 10, and 15) while AP-1 competitor could successfully compete away the complex (lanes 4, 9, and 14). Furthermore, we noticed that LNCaP nuclear extract yielded a lower level of the specific protein-DNA complex than the OC-positive PC3 and MG63. This result is further confirmed at the transcriptional level, where AV2/TATA showed lower activity in LNCaP than in PC3 cells (Fig. 4B).

We then defined the AP-1 protein factors that regulate AP-1/VDRE in hOC promoter by using various AP-1 antibodies in EMSA (Fig. 4C). Neither c-Fos nor c-Jun antibodies could supershift the DNA-protein complex observed (data not shown); however, with JunD antibody, the DNA-protein complex was clearly supershifted, while Fra-2 antibody acted more like a blocking antibody which prevented the protein-DNA complex formation and diminished the intensity of the original band. Furthermore, neither the control AR antibody nor VDR antibody could affect the complex. This confirms our previous observation that VDR does not associate with the AV site at the hOC promoter in the absence of vitamin D3 stimulation. We also demonstrated that there was less Fra-2 protein, but not less JunD (Fig. 4D) in the nuclear extracts of LNCaP compared with MG63 or PC3 cells. Therefore, PC3 cells behave like mature, post-proliferated osteoblasts, in which Fra-2 and JunD regulate the AV site and enhance the expression of hOC in cells.

OSE1 Is a Weak SP-1-binding Site—Osf1 was shown previously to be the osteoblast-specific transcription factor that associates with OSE1 in the mOC promoter (10). By sequence alignment, OSE1 is mapped to a similar location in hOC promoter with about 40% identity (7). Because no study has been done on the OSE1 in hOC promoter, little is known about the regulation of OSE1 in this promoter. In Fig. 5A, the mobility pattern of the protein factors that bind OSE1 was very similar to the Sp1 site except OSE1-protein complexes were weaker in intensity. Both the OSE1 and Sp1 sites gave three distinctive bands and their levels were similar among the three different nuclear extracts used. Based on published Sp1 studies, these bands were presumed to represent Sp1, -2, and -3 protein complexes. These specific protein-DNA complexes could be easily competed away with either unlabeled specific OSE1 competitor (lanes 3, 8, and 13) or SP-1 consensus competitor (lanes 4, 9, and 14). Moreover, antibody supershift experiments (Fig. 5B, lanes 16–19) showed that the OSE1-binding factors could
be supershifted by Sp2 and Sp3 antibodies, whereas Sp1 antibody blocked the Sp1 protein factor from binding to OSE1, hence the disappearance of the top band (lane 16). In the OSE1 mutation study, when the critical nucleotides for Sp1 binding were mutated within the OSE1 core sequence (cccctcc) (Fig. 5C), the mutated OSE1 (mOSE1) no longer bound the Sp1 factors (lane 5). Unlabeled mOSE1 and mSP-1 sites could not compete away the OSE1 complex (lanes 3 and 4). Furthermore, mutated OSE1 lost transcription activity tremendously (Fig. 5D) when compared with wild type OSE1. These results proved that OSE1 is a weak Sp1 site, which is regulated by the members of the Sp1 universal transcription factor family. Since Sp1

**FIG. 4.** Fra-2/JunD heterodimers regulate AP-1/VDRE (AV) element in hOC promoter. A, EMSA indicated AP-1 factors but not VDR bind the AV site. The OC-negative LNCaP cells show less specific DNA-protein complex (lane 2) than PC3 (lane 7) or MG63 cells (lane 12). The specific DNA-protein complex could not be competed off by a 100-fold excess of VDRE competitor (lanes 5, 10, and 15), while both AP-1 and AV competitors could effectively compete away the complex (lanes 3, 4, 8, 9, 13, and 14). B, comparison of AV/TATA activity in LNCaP and PC3 cells. On the transcriptional level, two copies of AV element also have higher activity in PC3 than in LNCaP cells. C, Fra-2/JunD heterodimers occupied the AP-1/VDRE site. Antibody of JunD could supershift (*) while Fra-2 antibody could specifically block the formation of protein-DNA complex in PC3 cells. D, Western blot analysis of Fra-2 and JunD protein expression. As shown, Fra-2 but not JunD was expressed at a higher level in the OC-positive PC3 and MG63 cells than the OC-negative LNCaP cells.
transcription factors have been known for their association with the general transcription complex TFIID, the binding of Sp1 factors at the OSE1 site could facilitate and/or stabilize the interactions between the Runx2 and Ap-1 factors and the general transcription machinery.

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the tissue-specific hOC promoter activity in cells. Therefore, the osteomimetic properties of prostate cancer cells in theory could allow them to metastasize, adhere, survive, and grow better in the bone microenvironment.

To better understand the osteomimetic properties of prostate cancer cells, we used PC3 cells as an in vitro model system to investigate the regulation of OC, a tissue-specific noncollagenous bone matrix protein. PC3 cells are AI bone metastatic prostate cancer cells that share with mature osteoblasts the unique feature of synthesizing and depositing a large amount of hOC. The 800-bp hOC promoter (16) was shown to have high and inducible activity in PC3 cells (Fig. 1B). By focusing on three well studied regulatory cis-elements (OSE1, OSE2, and AP-1/VDR) on the hOC promoter, we demonstrated that the molecular mechanism by which hOC promoter is turned on in PC3 cells is similar to that in osteoblasts. The evidence from the present report indicates that hOC promoter activity in both PC3 and bone cells are regulated by the interactions among three different sets of transcription factors: JunD/Fra-2 heterodimers, Runx2, and Sp-1. Unlike the OC-negative prostate cancer cell line LNCaP, PC3 has the ability to express the osteoblast-specific factor Runx2. Since overexpression of Runx2 in cells that normally express neither Runx2 nor osteoblast-specific genes leads to OC and α1-collagen expression (13), the turning on of runx2 expression in prostate cancer cells is one of the critical steps in making prostate cells bone-like.

The up-regulation of Fra-2 (Fig. 4D) is another feature shared by osteoblasts and PC3 cells. It has been established that in proliferating osteoblasts, c-Fos and c-Jun heterodimers are the predominant species at the AP-1 sites, while Fra-2 and JunD are the abundant AP-1 factors in differentiated osteoblasts (39, 40). The connection between Fra-2 translation and matrix mineralization in rat calvarial cells (30) has further suggested the functional significance of relatively high levels of Fra-2 compared with other AP-1 proteins for osteoblasts differentiation and maturation. With similar levels of JunD in OC-negative and OC-positive cells, the high level of Fra-2 in OC-positive cells allowed the cells to selectively and specifically push the equilibrium toward the formation of Fra-2/JunD heterodimer, activating hOC promoter in PC3 cells and mature osteoblasts.

A major finding of this study is the discovery of Sp1 universal transcription factors in the regulation of hOC promoter activity. Despite sharing homology (>40%) and similar location with mouse OSE1 (7), human OSE1 does not bind any bone-specific transcription factor. Our results are further supported by a recent Sp3-3 knockout study, in which the Sp3 null cells derived from the knockout animals showed normal levels of Runx2, but reduced OC expression and osteogenic differentiation capacity (41). Thus, Sp3 is an essential transcription factor involved in proper skeletal ossification. In light of the fact that OSE1 is indispensable for hOC promoter activity (Fig. 2A), Sp3 might be serving as a bridge to facilitate or stabilize the interaction between Runx2, Fra-2/JunD, and the general transcription machinery. Therefore, the delicate interplay and co-ordination of Sp3, Runx2, and Fra-2/JunD protein factors could specifically turn on hOC promoter in AI prostate cancer cells and confer bone-like phenotypes on them.

At present it is uncertain how prostate cancer cells acquire osteoblastic characteristics. It has been suggested that since prostate cancer cells and osteoblasts share a common growth
factor milieu (e.g. basic fibroblast growth factor, hepatocyte growth factor, bone morphogenetic protein, transforming growth factor-β, and insulin-like growth factor) (19), the reciprocal interactions between bone stroma and prostate cancer cells could regulate the expression of osteoblast-specific factors (Fig. 7). For example, basic fibroblast growth factor is abundantly present in the bone extracellular matrix. It is known as a potent angiogenic stimulus and a strong mitogen for the stimulation of both prostate cancer and osteoblast growth (42). It has been confirmed that basic fibroblast growth factor can induce hOOC promoter activity independent of vitamin D₃ and retinoid activation (43).

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