Nurr1, an NGFI-B nuclear orphan receptor, which transactivates promoters through an NGFI-B response element (NBRE), is strongly induced by parathyroid hormone through the cAMP-protein kinase A signaling pathway in osteoblasts. Here, we demonstrate that multiple agents activating diverse signaling pathways in osteoblasts induce Nurr1. The strongest Nurr1 inducers were activators of cAMP-protein kinase A-coupled signaling, followed by protein kinase C- and calcium-coupled signaling activators. Receptor tyrosine kinase activators had minimal effect, whereas serine/threonine kinase activators had no effect on basal Nurr1 mRNA levels. Computer analysis of osteoblastic promoters indicated two potential NBREs in the rat osteocalcin (Ocn) promoter. Intriguingly, the proximal site maps to the cAMP-responsive cis-element. We tested whether Nurr1 induces Ocn expression through the NBRE-like site. Recombinant and endogenous Nurr1 protein from primary mouse osteoblasts bound to a consensus NBRE in EMSAs. Nurr1 induced a consensus 3×NBRE-luciferase reporter construct in mouse osteoblasts. Recombinant and endogenous Nurr1 protein bound to the proximal NBRE-like site in the Ocn promoter in EMSAs. Endogenous Nurr1 protein bound to this site as a monomer, because neither retinoid X receptor β nor retinoid X receptor β antibody supershifted the protein-DNA complex. Ocn promoter-luciferase constructs lacking or containing a mutated proximal NBRE-like site had markedly blunted responses to Nurr1 overexpression. Finally, adenovirally expressed Nurr1 protein bound to the proximal NBRE-like site in chromatin immunoprecipitation assays and induced Ocn mRNA in primary rat osteoblasts. We conclude that Ocn is a Nurr1 target gene, which positions Nurr1 in the core of transcriptional factors regulating osteoblastic gene expression.

Osteoblasts are bone-forming cells derived from a multipotent mesenchymal bone marrow precursor (1, 2). In addition to osteoblasts, the precursor cell gives rise to chondroblasts, adipocytes, myoblasts, and marrow stromal cells. Differentiation along any of these lineages is determined by expression of cell type- and stage-specific markers. Precursors that differentiate along the osteoblast lineage express Runx1/cbfa1 and osteopontin at an early stage, whereas osteocalcin (Ocn) is a late stage marker for mature osteoblasts (3). Identifying the regulators of osteoblastic gene expression is critical to understanding the control of precursor differentiation along specific lineages.

Parathyroid hormone (PTH), a potent stimulator of cAMP-dependent signaling (4) and Ocn transcription (5–12), induces expression of the NGFI-B nuclear orphan receptor Nurr1 through the cAMP-protein kinase A (PKA) pathway in osteoblasts (13). Nurr1 gene expression is also induced in other culture systems (14–16) through calcium (14, 15) and protein kinase C (PKC) (16) signaling. As a primary response gene (13) induced through multiple signaling pathways, Nurr1 is ideally positioned to mediate late response gene expression in a variety of cell types. Thus, identification of Nurr1 target genes is critical to understanding its role in regulating cell differentiation and function.

Nurr1 transactivates target promoters by binding to cis-elements as a monomer (17, 18), homodimer (19, 20), or Nurr1–RXX heterodimer (17, 21, 22). As a monomer, Nurr1 binds to an extended hormone response element (5′-AAAGGTCA-3′) referred to as an NGFI-B response element (NBRE) (23–25). Computer analysis of the rat Ocn promoter revealed two NBRE-like sites at nt −934/−927 and nt −120/−103. Interestingly, the downstream NBRE-like site maps entirely within the previously identified cAMP-responsive region (26). These data prompted us to hypothesize that Nurr1 regulates Ocn gene expression in osteoblasts through direct binding and transactivation as a monomer.

Here we demonstrate that multiple signaling activators important in osteoblast differentiation and function induced Nurr1 expression. Using chimeric and endogenous sequences, we show that monomeric Nurr1 bound to and transactivated NBRE-containing promoters. Importantly, Nurr1 induced Ocn mRNA levels in osteoblasts via direct binding to and transac-

The abbreviations used are: Ocn, osteocalcin; mut, mutant; PTH, parathyroid hormone; PKA, protein kinase A; PKC, protein kinase C; NBRE, NGFI-B response element; nt, nucleotide(s); b, bovine; h, human; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor; IGF, insulin-like growth factor; EGF, epidermal growth factor; MOB, mouse osteoblast; ROB, rat calvarial osteoblast; PGE2, prostaglandin E2; EMSA, electrophoretic mobility shift assay; PBS, phosphate-buffered saline; ChIP, chromatin immunoprecipitation; FSK, forskolin; PMA, phorbol 12-myristate 13-acetate; WT, wild type; MAPK, mitogen-activate protein kinase; BMP, bone morphogenetic protein; PTHrP, parathyroid hormone-related protein.
tivation of the NBRE-like site in the proximal promoter. Taken together with previous data, we conclude that Ocn is a Nurr1 target gene in osteoblasts.

EXPERIMENTAL PROCEDURES

Reagents—Bovine PTH (bPTH), hPTHrP, insulin, human FGFα (hFGFa), hPDGF, hTGFβ, hBMP-2, hBMP-7, hIGF-1, and murine EGF were purchased from Caymen Chemical (Ann Arbor, MI). Fluprostenol was purchased from Calbiochem (San Diego, CA). Recombinant mouse EGF were purchased from Sigma-Aldrich. Fluprostenol was purchased from Fluka (Buchs, Switzerland).

Cell Culture—Primary mouse osteoblasts (MOBs) were collected from 6- to 8-day-old CD-1 mice (Charles River Laboratories, Wilmington, MA) and were maintained as previously described (13). Primary rat calvarial osteoblasts (ROBs) were isolated from E18–E19 Sprague-Dawley fetuses (Charles River Laboratories). Briefly, calvariae were minced, digested five times in collagenase/trypsin-EDTA solution (1 mg of collagenase/5 ml of 0.125% trypsin-EDTA) for 20 min (min) on a rocker platform at 37 °C/5% CO2. Digest 1 was discarded, and digests 2–5 were subjected to reverse transcription and real-time PCR was used to determine fold induction over vehicle-treated MOBs (Con). Data shown are means ± S.E.

MOBs were plated at 100,000 cells/ml in 24-well tissue culture plates (Costar, Corning Life Sciences, Acton, MA) and were transfected when 80–90% confluent using LipofectAMINE and Plus Reagent (Invitrogen). For each reaction, a total of 0.36 g of DNA (promoter-luc reporter with or without Nurr1 expression with or without empty pcDNA3.1(−) vectors) was transfected per well according to the manufacturer’s protocol. Luciferase activity was assayed after 24–48 h using the Luciferase Assay System (Promega, Madison, WI), normalized, and reported as percent control. Each experiment was repeated 5–15 times.

Electrophoretic Mobility Shift Assays—Recombinant proteins and nuclear extracts from MOBs were as previously described (27). For EMSAs, 5 μg of nuclear proteins was incubated with 2–5 × 106 cpm of [32P]dCTP-labeled oligonucleotides. The consensus NBRE oligonucleotides probe sequence was 5'-AAAGTCA-3'. The mutNBRE probe sequence was 5'-AAGGTCA-3'. The rOcn (199–138) probe sequence was 5'-GCGCCAGCCTCTGATGTGCTCCTCCTCCCTCCAGCTTGTTCCGGGCAGCTCCAGTGA-3', and the rOcn (142–83) probe sequence was 5'-GGTCCACAACACGACACCTCTTGGTGTGACCTTTGACGATGCACGACATGCCACCTCGCCAGACGAGATGATGCCG-3', (accession #J054500). The rOcn (120–103) probe sequence was 5'-GGTTGTGAACATTGG-3'. The mutated rOcn (120–103) probe sequence was 5'-GGTTGTTACCAATTGG-3'. Supershifts were performed using Nurr1 (sc-990x), RXRα (sc-553x), RXRβ (sc-831x), and E4bp4 (sc-9549x) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA). E4bp4 antibody was used as a nonspecific antibody, and it failed to supershift protein complexes.

Nurr1 and LacZ Adenoviruses—The AdEasy™ Adenoviral Vector System (Invitrogen) was used to construct adenoviruses expressing Nurr1 or LacZ proteins according to the manufacturer’s protocol. ROBs were plated at 4 × 106 cells/well in 6-well tissue culture plates (Costar) in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 200 units/ml penicillin, and 100 μg/ml streptomycin. The following day, the cells were transduced with 100 Nurr1-expressing virions/cell in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 200 units/ml penicillin, and 100 μg/ml streptomycin. Experiments utilizing LacZ adenovirus and the same protocol demonstrated more than 90% transduction of the cells (data not shown). Two days after transduction, total RNA and nuclear extracts were collected for use in real-time PCR and EMSAs.

Real-time PCR—One microliter (1.5 ng) of reverse transcribed product was amplified with iQ SYBR Green master mix (Bio-Rad) and the primers used were: mouse Ocn forward primer 5'-GTGGCCCTCTTCAGGACATC-3' and reverse primer 5'-ATGGACTGTGGTCATGAGCC-3' (accession #M23627) forward primer 5'-GCACCACCGTTAGGGCAT-3' and reverse primer 5'-GATGAGTGGAGATGATGGG-3'. Mouse glyceraldehyde-3-phosphate dehydrogenase (accession number U86783) forward primer 5'-GCACCACCGTTAGGGCAT-3' and reverse primer 5'-GATGAGTGGAGATGATGGG-3'. Mouse glyceraldehyde-3-phosphate dehydrogenase (accession number U86783) forward primer 5'-AACTTGGGTTTGACCTATTGC-3' and reverse primer 5'-AAAGTTAACCCAATTTGG-3'. The amplification program was set for 1 cycle of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min.

FIG. 1.  Nurr1 mRNA levels are induced through multiple signaling pathways in osteoblasts. MOBs were treated for 1 h with selective signaling agonists. A, cAMP-PKA activators: 10 μM FSK, 10 nm PTH, 10 nm PTHrP, 1 μM PGE2. B, PKC-calcium activators: 1 μM PMA, 1 μM ionomycin (iono), 1 μM PFGα, 1 μM fluprostenol (flup). C, receptor tyrosine kinase activators: 10 ng/ml FGF, 1 ng/ml PDGF, 10 nm IGF-1, 1 ng/ml TGFα, 10 ng/ml EGF. D, serine/threonine kinase activators: 10 ng/ml TGFβ, 100 ng/ml BMP-2, 100 ng/ml BMP-7. Total RNA from MOBs was subjected to reverse transcription and real-time PCR was used to determine fold induction over vehicle-treated MOBs (Con). Data shown are means ± S.E.
Nurr1 Transactivates Osteocalcin in Osteoblasts

Fig. 2. Nurr1 mRNA levels are dose-dependent. MOBs were treated for 1 h with 0.01–100 nM PTHrP (A), 0.01–100 μM FSK (B), 0.01–100 μM PGE₂ (C), 0.001–10 μM PMA (D), 0.001–10 μM PGF₂α (E), and 0.001–1 μM fluprostenol (F). EC₅₀ is indicated by dashed lines. Data shown are means ± S.E. *, p < 0.05 compared with control.

Chromatin Immunoprecipitation Assays—Confluent ROB cells were transduced with Nurr1-expressing adenovirus. Two days after transduction, medium was aspirated, and cells were washed with PBS. Cells were fixed in cold 2% paraformaldehyde for 30 min at room temperature. After fixation, cells were washed in PBS and blocked for 1 h at room temperature with 3% normal goat serum in PBS containing 0.1% Triton. Cells were then washed in PBS and incubated for 2 h at room temperature with secondary antibody (Santa Cruz, sc-2004) diluted 1:200 in 1.5% normal goat serum in PBS containing 0.1% Triton. Cells were then washed in PBS and developed using 0.005% 3,3′-diaminobenzidine/0.02% H₂O₂ solution in PBS, pH 7.4. Cells were washed in PBS, rinsed through an increasing ethanol gradient, and immersed in xylene for 1 min.

Results

Multiple Osteoblastic Regulators Induce Nurr1 Gene Expression—We have previously shown that PTH induces Nurr1 mRNA and protein expression in MOBs (13). To investigate whether other regulators of osteoblastic function control Nurr1 gene expression, MOBs were treated with 10 nM PTHrP or agonists that activate cAMP-PKA signaling (Fig. 1A), PKC and/or calcium signaling (Fig. 1B), ligands of tyrosine kinase surface receptors (Fig. 1C), or members of the TGFβ-BMP family (Fig. 1D). cAMP-PKA pathway agonists (10 μM forskolin (FSK), 10 nM PTH, 10 nM PTHrP, 1 μM PGE₂) caused the strongest induction of Nurr1 gene expression followed by PKC and/or calcium signaling activators (1 μM PMA, 1 μM ionomycin, 1 μM PGF₂α, 1 μM fluprostenol) and receptor tyrosine kinase activators (10 ng/ml FGF, 1 ng/ml PDGF, 1 ng/ml TGFβ, 10 ng/ml EGF). Serum/threonine agonists (10 ng/ml TGFβ, 100 ng/ml BMP-2, 100 ng/ml BMP-7) and the receptor tyrosine kinase activators IGF-I (10 nM) and insulin (1 μM, data not shown) did not affect Nurr1 gene expression.

To further characterize Nurr1 induction in osteoblasts, we generated dose-response curves for the strongest agonists. Maximum Nurr1 mRNA levels were induced in MOBs following a 1-h treatment with 10 nM PTHrP (34.6-fold induction), 10 μM FSK (31.2-fold induction), 100 μM PGE₂ (15.3-fold induction), 10 μM PMA (8.71-fold induction), 10 μM PGF₂α (5.1-fold induction), and 1 μM fluprostenol (2.75-fold induction). The EC₅₀ for Nurr1 induction by these agonists was 0.109 nM PTHrP (Fig. 2A), 116 nM FSK (Fig. 2B), 115 nM PGE₂ (Fig. 2C), 7 nM PMA (Fig. 2D), and 11.9 nM fluprostenol (Fig. 2F). Interestingly, Nurr1 induction by PGF₂α never reached a plateau but continued to increase at higher doses (Fig. 2E). PGF₂α at high doses can cross-react and bind EP receptors to activate the cAMP-PKA pathway (29–31). Thus, the PGF₂α induction of Nurr1 mRNA levels is probably not mediated through FP receptor but rather through EP receptor signaling.

PTH-induced Nurr1 Protein Binds to and Transactivates a Consensus NBRE—After demonstrating the induction of Nurr1 gene expression through multiple signaling pathways in osteoblasts, we tested potential function of Nurr1 protein. Because
cAMP-PKA agonists yielded the strongest Nurr1 expression and PTH is an endogenous hormone, we utilized PTH to induce endogenous Nurr1 protein synthesis in subsequent studies. We have previously shown that 10 nM PTH causes maximum induction of Nurr1 gene expression (13). To determine if PTH-induced Nurr1 protein translocates to the nucleus and binds DNA target sequences, nuclear proteins from MOBs treated with 10 nM PTH for 0–6 h were utilized in EMSAs. PTH-inducible binding to a consensus WT NBRE probe peaked between 2 and 4 h (Fig. 3A). No binding to a mutated NBRE was detected. This binding was competed by a 50× WT unlabeled competitor. To determine if Nurr1 is part of this PTH-induced protein complex, supershift assay was performed on the same proteins. Nurr1-specific antibody supershifted the majority of the PTH-induced complex (Fig. 3B), whereas nonspecific antibody had no effect (data not shown).

Having determined that Nurr1 is part of the PTH-inducible complex that binds to the WT NBRE, we next investigated if Nurr1 protein induces NBRE-containing promoters. MOBs were co-transfected with an empty vector or the Nurr1 expression vector and a chimeric WT 3×NBRE-luc construct. Nurr1 overexpression induced luciferase activity through the chimeric promoter (Fig. 3C).

Nurr1-transactivated Rat Ocn Promoter Activity Requires the Proximal 199 bp—Having determined that PTH-induced Nurr1 protein bound to and transactivated an NBRE-containing promoter, we used computer analysis to identify osteoblastic genes with NBRE-containing promoters. The –1050 bp rat Ocn promoter has two sites with close homology (7/8 bp) to the consensus NBRE at nt –934/–927 and nt –115/–108.

To determine whether Ocn is a potential Nurr1 target gene, we transiently co-transfected MOBs with the Nurr1 expression vector and luc-reporter constructs driven by 5’ serial deletions of the rat (–1050) Ocn promoter. Nurr1 overexpression maximally induced Ocn promoter activity in constructs containing nt –1050/–199 (Fig. 4). However, deletion to nt –138 attenuated, whereas further deletion to nt –83 abolished, Nurr1-induced Ocn promoter activity.

Nurr1 Is Part of a PTH-induced Nuclear Protein Complex That Binds the Rat Ocn Proximal NBRE—Our transfection data suggested that both nt –199/–138 and nt –138/–83 are important for Nurr1-mediated Ocn promoter induction (Fig. 4). Interestingly, computer analysis revealed an NBRE-like sequence within nt –138/–83 (–115/–108, ATAGTCA in the antisense strand) but not within nt –199/–138. To determine potential direct Nurr1-DNA interaction accounting for the reduced Nurr1-mediated transcription seen in the (–199/Ocn-luc and (–138/Ocn-luc constructs, we performed EMSAs utilizing recombinant Nurr1 or luc proteins and nt –199/–138 or nt –142/–83 as probes. Nurr1 protein strongly bound to the nt –142/–83 probe but did not bind to the nt –199/–138 probe (Fig. 5A). Recombinant luc protein was used as control. Because computer analysis revealed an NBRE-like sequence in the distal Ocn promoter at nt –934/–927, we performed EMSAs utilizing recombinant Nurr1 or PTH nuclear extracts and a probe corresponding to nt –942/–920 of the Ocn promoter. Neither recombinant Nurr1 nor PTH treated extracts bound to that site (data not shown).

Because the nt –142/–83 probe contains the putative NBRE at nt –115/–108, we tested the ability of endogenous Nurr1 protein to bind to this site. Nuclear proteins from MOBs treated with 10 nM PTH for 0–6 h showed peak binding to the nt –120/–103 probe at 2 h. Furthermore, a 2-nt mutation in the –120/–103 probe eliminated PTH-induced Nurr1 protein binding (Fig. 5B). The PTH-induced binding completely supershifted with Nurr1 antibody, whereas a nonspecific antibody had no effect (Fig. 5C). To determine if Nurr1 binds to the NBRE-like sequence alone (17, 18) or as an RXR-heterodimer (17, 21, 22), we performed supershift experiments on nuclear proteins from MOBs treated with 10 nM PTH for 0–6 h utilizing the nt –130/–103 probe and Nurr1, RXRα or RXRβ antibodies. Similar to Fig. 3B, Nurr1 antibody completely supershifted PTH-induced binding. No supershift was seen when the RXR antibodies were used (Fig. 5D).
FIG. 4. Optimum Nurr1-transactivated rat Ocn promoter requires the proximal 199 nucleotides. MOBs were transiently co-transfected with empty pcDNA 3.1 or Nurr1 expression vector and 5’ serial deletions of Ocn promoter-luciferase constructs. Data are expressed as percent control (pcDNA).

FIG. 5. Nurr1 protein binds nt −120/−103 from the rat Ocn promoter. A, recombinant Nurr1 protein (N) bound to the nt −142/−83, but not to the −199/−138, fragment from the rat Ocn promoter. Recombinant luciferase protein (L) was used as control. P, probe alone. B, nuclear proteins from MOBs treated with 10 nM PTH for 0–6 h bound to the WT but not mut nt −120/−103 fragment from the rat Ocn promoter. C, nuclear proteins from MOBs treated with 10 nM PTH for 0–6 h show peak binding to nt −120/−103, which supershifts with Nurr1 antibody but not with nonspecific E4bp4 antibody (+N.S. Ab). D, nuclear proteins from MOBs treated with 10 nM PTH for 0–6 h show peak binding to nt −120/−103, which supershifts with Nurr1 but not RXRα or RXRβ antibody.
Nurr1 Transactivates Osteocalcin in Osteoblasts

**DISCUSSION**

We have previously reported the PTH-induced expression of the nuclear orphan receptor Nurr1 in osteoblasts (13). Here, we determined that multiple signaling pathways important in osteoblastic function regulate Nurr1 expression (Figs. 1 and 2). The strongest inducers were cAMP-PKA activators; PKC and calcium signaling, as well as selective agonists of tyrosine kinase receptors, caused a moderate increase of Nurr1 gene expression. Interestingly, TGFβ-BMP family members, which are potent osteoblast differentiation factors (32), had no effect on Nurr1 mRNA levels.

Induction of Nurr1 expression by various agonists and multiple signaling pathways has also been reported in other tissues. As in osteoblasts, cAMP signaling strongly induces Nurr1 expression in corticotrophs (33) and neuroblastoma and glial cells (34). Nurr1 is induced by luteinizing hormone in granulosa cells through PKCζ (16), vascular endothelial growth factor in human umbilical vein endothelial cells through the tyrosine kinase-coupled KDR receptor (15), and N-type calcium channel depolarization in primary sensory neurons (14).

The abundance of signaling regulators that induce Nurr1 raises the possibility that multiple signaling pathways may converge on Nurr1 to affect cell type specificity. Indeed, this seems to be the case for neuroblastoma and glial cells. Extracellular signal-regulated kinase, which mediates mitogen-activated protein kinase (MAPK) signaling, is required to mediate FSK-induced Nurr1 expression in N2A neuroblastoma cells but not in C6 glial cells (34). Likewise, MAPK signaling does not affect cAMP-induced Nurr1 expression in AtT-20 corticotrophs (33). In light of these data, the induction of Nurr1 by multiple agonists in osteoblasts stresses its potential importance in regulating target genes that affect osteoblast function.

To identify potential Nurr1 target genes in osteoblasts, we used a computer search to locate NBRE-containing promoters. NBRE-like elements were identified in the promoter/enhancer area of alkaline phosphatase, bone sialoprotein, osteopontin, type I(a)1 and type I(a)2 collagens, insulin-like growth factor I, collagenase, and osteocalcin genes. More specifically, we found two putative NBRE-like sites in the rat osteocalcin (Ocn) gene at nt −934/−927 and nt −120/−103. The downstream NBRE-like site maps entirely within a previously identified cAMP-responsive region (26). Indeed, we found that maximum Ocn promoter activity required at least the proximal −199 nt of the promoter. Deletion of nt −199/−139 greatly reduced, and deletion of nt −138/−83 eliminated, Nurr1-induced Ocn promoter activity (Fig. 4). The latter response is easily explained by the loss of the proximal NBRE-like site at nt −120/−103. However, we were surprised when Nurr1-mediated Ocn promoter activity declined when the nt −198/−139 fragment containing an E box, AP-1, and cbfa1 sites was deleted.

Nurr1-mediated transcription is highly dependent upon Nurr1 stability on DNA (35). Loss of the E box, AP-1, and cbfa1 sites and any proteins bound to them may disrupt Nurr1 stability, thereby reducing Nurr1-mediated transcription. Yet, no direct interactions between HLH, AP-1, or cbfa1 and Nurr1 are known. It could be argued that AP-1 proteins are candidates for affecting Nurr1-mediated transcription, because AP-1 proteins regulate nuclear receptors through competition for the co-activator CREB-binding protein (CBP) (36). Although nuclear receptors compete for CBP, Nurr1 is unusual in that it does not bind CBP or any other classic nuclear receptor cofactor (35).

The lack of evidence for interactions between Nurr1 and AP-1 proteins in the regulation of Ocn promoter activity does not rule out the possibility that both transcription factors coordinate regulation induced Ocn promoter activity. AP-1 proteins are heterodimers formed from members of the Jun and Fos family of basic leucine zipper transcription factors. During osteoblast differentiation the composition of AP-1 dimers changes from a mixture of all the Jun and Fos family members...
NURR1 Transactivates Osteocalcin in Osteoblasts

FIG. 7. Adenovirally expressed Nurr1 protein translocates to the nucleus where it binds to the proximal NBRE-like site in the Ocn promoter and induces Ocn mRNA levels. A, cells were transduced with 100:1 multiplicity of infection of Nurr1-expressing adenovirus. Non-transduced cells were used as controls (Cont). Immunocytochemistry utilizing a Nurr1 primary antibody indicates nuclear localization of Nurr1 protein 2 days post-transduction. B, ROBs were transduced with 100:1 multiplicity of infection of Nurr1- or LacZ-expressing adenovirus. Non-transduced cells were used as controls (Cont). Two days post-transduction nuclear proteins were extracted, and EMSAs were performed using the consensus NBRE as a probe. C, nurr1- or LacZ-transduced cells or non-transduced controls (Cont) were subjected to ChIP assays utilizing primary antibodies to Nurr1 or E4bp4 (N.S.) or no antibody (No Ab). Then DNA were amplified utilizing PCR primers that encompass the proximal NBRE in the rOcn promoter. D, reverse transcribed total RNA from Nurr1- or LacZ-transduced cells or non-transduced controls (Cont) was subjected to real-time PCR utilizing specific primers for Ocn and for β-actin. Data shown are mean ± S.E. *, p < 0.05 compared with control.

in proliferating preosteoblasts to only JunD-Fra-2 heterodimers in differentiated osteoblasts (37). Interestingly, PTH maximally induces Fra-2 binding to a consensus AP-1 sequence at 3 h of treatment (38). This coincides with peak PTH-induced Nurr1 binding to nt −120/−103 of the Ocn promoter (Fig. 3, A and B). This suggests that both AP-1 and Nurr1 proteins are present at the same time in PTH-treated osteoblasts and that they may coordinately induce Ocn promoter activity through the adjacent AP-1 and NBRE-like sites.

Our report here also adds Ocn to a short list of known Nurr1 target genes. CYP11B2 is induced through Nurr1 in angiotensin II-treated adenocortical cells (39). Three Nurr1 targets are critical for neuronal differentiation and function. Ret is a proto-oncogene encoding a receptor tyrosine kinase (40, 43), tyrosine hydroxylase, catalyzes the rate-limiting step in catecholamine synthesis (24, 44) and the dopamine transporter (DAT) regulates extracellular dopamine levels (25, 45). Similar to Nurr1−/− mice (46), Ret and tyrosine hydroxylase knockouts have severe neuronal agenesis that causes pre- or peri-natal death (47, 48). DAT−/− mice survive to adulthood and exhibit hyperlocomotion that mimics neurostimulant-induced activity (49). Interestingly, these mice are dwarfs due to anterior pituitary hypoplasia, which disrupts the growth hormone axis, and lactotroph hypoplasia, which decreases milk availability for nursing pups (50). The dwarfism correlates with decreased trabecular bone volume, cortical thickness, and cortical strength, although serum calcium, phosphorous, and PTH levels are unchanged compared with wild type littermates (51). Finally, very recently, osteopontin was shown to be a Nurr1 target in osteoblasts (22).

Interestingly, Nurr1 binds to the osteopontin promoter as a monomer, although it appears to be tethered to one of the RXR isoforms (22). In contrast, we found that Nurr1 bound to the Ocn promoter alone, because Nurr1 but neither RXRα nor RXRβ antibody successfully supershifted PTH-induced nuclear proteins (Fig. 5). Indeed, the NBRE-like site in the Ocn promoter is a true monomeric site, suggesting that although Nurr1 can either homodimerize (19, 20) or heterodimerize with RXR (17, 21, 22), there is no strict dimerization requirement for Nurr1 to transactivate osteoblastic gene expression.

Identification of both osteopontin and osteocalcin as Nurr1 target genes in osteoblasts is intriguing from a functional perspective. Neither osteopontin nor osteocalcin are required for normal skeletal development. Instead, both genes are needed for normal skeletal homoeostasis. Ocn−/− mice undergo progressive osteoporosis with age due to inappropriate skeletal remodeling (52), while osteopontin deletion impairs bone remodeling following ovariectomy (53) or PTH treatment (54). Interestingly, the two genes differ significantly in the timing of their expression during osteoblast development. Osteopontin is highly expressed in the earliest preosteoblast stage and remains high in fully mature osteoblasts (3). Conversely, Ocn is expressed only in fully mature osteoblasts (3). These data point to Nurr1 regulation of multiple genes along the osteoblastic lineage. Indeed, establishing Nurr1-induced Ocn gene expression points to Nurr1 as a potential transcriptional regulator influencing osteoblastic differentiation.

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