Regulation of Osteocalcin Gene Expression by a Novel Ku Antigen Transcription Factor Complex*

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We previously described an osteocalcin (OC) fibroblast growth factor (FGF) response element (FRE) DNA binding activity as a target of Msx2 transcriptional regulation. We now identify Ku70, Ku80, and Tbdn100, a variant of Tubedown-1, as constituents of the purified OCFRE-binding complex. Northern and Western blot analyses demonstrate expression of Ku and Tbdn100 in MC3T3E1 osteoblasts. FGF2 treatment regulates Ku, but not Tbdn100, protein accumulation. Gel supershift studies confirm sequence-specific DNA binding of Ku in the OCFRE complex; chromatin immunoprecipitation assays confirm association of Ku and Tbdn100 with the endogenous OC promoter. In the promoter region −154 to −113, the OCFRE is juxtaposed to OSE2, an osteoblast-specific element that binds Runx2 (Osf2, Cbfa1). Expression of the Ku-Tbdn100 complex up-regulates both the basal and Runx2-dependent transcription driven by this 42-bp OC promoter element, reconstituted in CV-1 cells. Synergistic transactivation occurs in the presence of activated FGF receptor 2 signaling. Msx2 suppresses Ku- and Runx2-dependent transcription; suppression is dependent upon the Msx2 homeodomain NH2-terminal arm and extension. Pull-down assays confirm physical interactions between Ku and these regulatory transcription factors, consistent with the functional interactions identified. Finally, cultured Ku70−/− calvarial cells exhibit a profound, selective deficiency in OC expression as compared with wild-type calvarial cells, confirming the biochemical data showing a role for Ku in OC transcription. In toto, these data indicate that a novel Ku antigen complex assembles on the OC promoter, functioning in concert with Msx2 and Runx2 to regulate OC gene expression.

Expression of the osteoblast phenotype is regulated by morphogenetic, metabolic, endocrine, inflammatory, and mechanical signals that convey cognate physiological demands upon the skeleton (1). The osteocalcin (OC) promoter has emerged as a powerful tool for identifying and characterizing nuclear DNA-protein interactions mediating transcriptional responses to many of these signals in the osteoblast (2, 3). To date, much of what we know of the osteoblast transcriptional responses to the vitamin D receptor, Runx2 (also known as Osf2, PEBP2Aα, and Cbfa1), Msx2, Dlx5, the glucocorticoid receptor, and AP1 family members derive from studies of OC promoters cloned from human, rat, and mouse (OG2) genomes (4–13). Except for the absence of the vitamin D receptor-regulated enhancer in the mouse OG2 gene (14), key features of OC promoter regulation are remarkably well conserved across mammalian species (3).

The osteoblast homeoprotein Msx2 plays a crucial role in the regulation of osteoblast proliferation and differentiation (15). Msx2 and Msx1 are absolutely required for craniofacial bone formation (15). Msx2 controls the timing of osteoblast differentiation, including the temporospatial patterns of OC expression in the calvarium and teeth (12, 16). Msx2 plays a global role in determination of skeletal mass, elegantly demonstrated in murine genetic models; moreover, as highlighted by Maas and colleagues (15), osteogenic effects during development are dependent upon Msx gene dosage, suggesting that stoichiometry is an important feature of Msx2 action. Consistent with this, we (11, 12, 16–18) and others (2, 19) have shown that Msx2 and Msx1 participate in specific protein-protein interactions that control gene transcription. In the rat OC gene, transcriptional regulation by Msx2 converges on a 42-bp region at nucleotides −154 to −113 relative to the transcription initiation site, encompassing an FGF responsive element at nucleotides −142 to −136 (17). The OCFRE (OC FGF response element) is a GCAGTCA motif that confers both basal and FGF2-regulated expression of the OC gene in MC3T3E1 calvarial osteoblasts (17, 20, 21). A DNA binding activity up-regulated by FGF2 in MC3T3E1 cells recognizes this element and is constitutively expressed by MG63 osteosarcoma cells (17, 20). Msx2 exerts suppressive actions on the OC promoter in part by inhibiting the OCFRE DNA binding activity present in either MC3T3E1 cells or purified from MG63 cells (17). By contrast, Msx2 does not alter vitamin D receptor-dependent transcription or vitamin D receptor binding to its DNA cognate (17). Inhibition of OCFRE activity is dependent upon key regulatory domains encoded in the Msx2 homeodomain NH2-terminal arm and...
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extension (17). Interestingly, Stein and co-workers (22, 23) identified that this same OC promoter region mediates transcriptional repression in response to transforming growth factor-β, and thus named this same element the transforming growth factor-β response element. They identified that an AP1 complex mediates transforming growth factor-β repression in ROS17/2.8 cells. We have confirmed these observations,2 however, applying immunological and competition studies, we determined that the Msx2-regulated DNA binding activity was not an AP1 family member (20) (see below). Thus, the OCFCRE mediates both positive and negative regulation of OC gene transcription in osteoblasts.

To fully characterize the DNA binding activities that recognize and regulate the OCFCRE, we have purified the major homeoprotein-regulated OCFCRE complex about 25,000-fold from MG63 human osteosarcoma cells, using DNA binding in gel shift as an assay. Ku70 and Ku80 (24–26) were identified as the Msx2-regulated DNA binding constituents (17) in the purified OCFCRE binding complex by nanobore LC-MS/MS “sequencing” of tryptic peptides (27). Additionally, we identify a novel 100-kDa variant of Tubedown-1, hence, named Tbdn100, as a constituent of this complex purified from this osteoblastic cell line. Tubedown is an N-acetylttransferase initially identified in immature endothelial cells (28); however, Northern blot analyses verify the expression of Tbdn in MC3T3E1 calvarial osteoblasts, a useful and relevant cell background for osteoblast gene expression studies, and in many other tissues. Western blot analyses with antibodies raised to the COOH-terminal domain of Tbdn100 confirm the accumulation of the 100-kDa Tbdn100 variant in nuclear fractions prepared from MC3T3E1 osteoblasts. Moreover, chromatin immunoprecipitation (ChIP) assays confirm the association of Ku and Tbdn100 with endogenous OC promoter, but not with the BSP promoter. Immunological supershift and competition studies confirm sequence-specific association of Ku in the nuclear complex assembled by the OCFCRE. In transient transfection assays, co-expression of the novel OC promoter region –154 to –113, the OCFCRE (20) is juxtaposed to OSE2 (29), an element that binds the osteoblast transactivator Runx2 (also known as Osf2 and Cbfa1) (3, 9). Runx2 and Ku:Tbdn100 synergistically activate transcription driven by the OC promoter region –154 to –113 when placed upstream of the un responsive RSV promoter. Synergistic activation of OC (–154/-113)-RSVLC is markedly augmented by FGFR2-ROS, a constitutively active FG receptor (30, 31). Importantly, Msx2 suppresses Ku- and Runx2-dependent transcription, consistent with our prior studies demonstrating that OCFCRE binding is inhibited by Msx2 (17).

Pull-down assays confirm physical interactions between Ku and Msx2, and Ku and Runx2. Finally, we show that calvarial cells lacking Ku70 exhibit profound, selective deficiencies in OC, but not OPN, gene expression. In toto, these data indicate that a novel Ku antigen complex assembles on the OC promoter, functioning in concert with Msx2 and Runx2 to regulate OC gene expression. Tbdn100 emerges as a nuclear transcriptional regulator that associates with the OC gene and promotes transactivation responses.

EXPERIMENTAL PROCEDURES

Reagents—Tissue culture supplies and custom synthetic oligodeoxyribonucleotides were obtained from Invitrogen. Reagents for protein purification were obtained from Pierce, Sigma, and Fisher. Radiolabeled DNA fragments were released by timed digestion with DNase I or fracture of nuclear matrix released by timed digestion with DNase I or fracture of nuclear matrix

by applying successive rounds of 5'-32P-end labeling of the radiolabeled duplex oligo used to follow OCFCRE binding activity is 5'-GGGACGTCCAGTCACCGGCCC-3' (the OC promoter sequence encompassing the OCFCRE is underlined). Total protein recovery was determined by the Pierce bichinchoninic acid assay after protein precipitation and resolubilization as described (33). High performance liquid chromatography (HPLC) was carried out using Waters (Milford, MA) ACC gradient controller with two model 515 pumps, fitted with a preparative scale 10-mL Rheodyne injector (Rohnert Park, CA), and low protein retention polyetheretherketone (PEEK) tubing. A Waters model 468 tunable ultraviolet detector set at 280 nm was used to follow protein elution. A 5-mL Amersham Bioscience Mono Q FPLC column (20 mM Tris buffered, pH 8.0) was used for anion exchange, and an Amersham Bioscience disposable 1-ml HiTrap heparin-Sepharose affinity column (20 mM Hepes buffered, pH 7.5) was used for final purification steps. For both HPLC applications, 1 mL/min flow rates with a 14 mM/min NaCl gradient were used to elute OCFCRE binding activity; 0.1 mM EDTA and 1 mM 2-mercaptoethanol were included in all HPLC buffers. Proteins were resolved by SDS-PAGE using an 11% 0.75-mm thick polyacrylamide gel poured on methanol-cleaned virgin glass plates (Mini-Protean II; Bio-Rad) and pre-run to clear oxyradicals. After electrophoresis, fractionated proteins were visualized by staining in Coomassie Brilliant Blue, briefly destained, and excised with a scalpel, reduced and alkylated with dithiothreitol and iodoacetamide, respectively, rinsed with 50% acetonitrile in deionized water with 0.1% acetic acid to remove SDS, and speed evaporated dry. After pre-swelling gel fragments in 50 mM NaHCO3, proteins were digested with L.20 (mass/mass) trypsin (Promega), and tryptic peptides were analyzed by nanobore LC-MS/MS at the Harvard Microchemistry Facility (B. Lane, director) using a Finnigan ion trap mass spectrometer (34). SEQUEST analysis (27) of the NCBI cDNA data base identified Ku70 (24 unique peptides) and Ku80 (17 unique peptides). Analysis of the translated EST data base in all 6 reading frames was required to identify peptides from the unknown 100-kDa subunit (8 unique peptides initially identified; 24 identified in final analysis, Fig. 3).

Cloning of Tbdn100—Because there were no matches for our uLC-MS/MS tryptic peptide data in the nonredundant cDNA data base for the 100-kDa protein, we searched the translated NCBI EST data base with BLAST (35). We identified several ESTs which assembled into a 900-base pair contig containing a putative stop codon and providing a contiguous open reading frame encoding 8 of the unique tryptic peptides we identified from mass spectroscopy analysis of the purified 100-kDa subunit. EST analyses revealed expression in the heart (and subsequently confirmed by Northern blot); therefore, the 900-bp fragment was amplified from commercially available human heart cDNA (Clontech Marathon Ready cDNA; Clontech) and sequenced to verify correct assembly. Groups of 5' rapid amplification of cDNA ends from this Marathon Ready cDNA and iterative EST data base searches, we assembled the complete sequences of both the mouse and human cDNAs (Fig. 3). After obtaining the complete sequence, reanalysis of our raw LC-MS/MS data identified a total of 24 peptides, including one at our predicted translation start site without Met (Fig. 3). During our studies, a variant of this 100-kDa protein was cloned by Gendron and colleagues (28) and named Tubedown-1. Because our

2 D. M. Willis, A. P. Loewy, N. Charlton-Kachigian, J.-S. Shao, D. M. Ornitz, and Dwight A. Towler, unpublished data.
protein is 100 kDa in mass because of an additional 272 amino acids at the amino terminus, we named our variant Tubedown-100 or Tbdn100 (Fig. 3; GenBankTM number AY112670). Sequence analysis was performed using ProfileScan software accessible from the ISREC Bioinformatics home page (Swiss Institute for Experimental Cancer Research; www.isrec.isb-sib.ch/index.html). Partial DNA sequence data were (36) accessible from the Washington University Genetics website (pfam.wustl.edu/hmmsearch.shtml), and DNAMAN version 4.0 software (36) accessible from the Washington University Genetics website (pfam.wustl.edu/hmmsearch.shtml), and DNAMAN version 4.0 software for Windows purchased from Lynnon BioSoft (Vandreuil, Quebec, Canada).

Electrophoretic Mobility Shift Assays—MC3T3-E1 murine calvarial osteoblasts were used as described previously to determine DNA binding properties of the proteins. Cells between passages 6 and 16 from our frozen stocks. Crude nuclear extracts were prepared from control or FGF2- (3 nM) and forskolin (10 μM)-treated MC3T3-E1 calvarial osteoblasts essentially as previously described by et al. (21); however, 1 nM okadaic acid and 10 mM sodium orthovanadate were added to inhibit phosphatase phosphatases during extraction. The radiolabeled OCFRE duplex oligo (see above) was used to detect DNA-protein interactions. Gel supershifts and competition experiments were carried out as previously detailed (20, 31).

Antibodies, Western Blot Analyses, and ChIP Assays—The Ku70: Ku80 heterodimer-specific monoclonal antibody clone 162 (37) was obtained from Room Markussen (Fredomm, CA). Anti-Tubedown-100 antibodies were commercially prepared (Zymed Laboratories Inc.) using a conjugated synthetic peptide corresponding to the amino acids 947–1000 of the human Ku70 as the immunogen; nonspecific polyclonal antibodies were prepared from immune serum using purified, recombinant Tbdn100 (expressed in pEVT23d; Novagen) conjugated to cyrogan bromide-activated Sepharose; these techniques have been previously detailed (11). Western blot analyses were carried out on crude or fractionated cellular extracts essentially as previously detailed (11), using commercially available polyclonal anti-Ku70 (Santa Cruz, San Diego, CA, sc-14877/M19 and sc-1486/C19), anti-Ku80 (Santa Cruz number sc-14855), antitubulin, or anti-Tubd100 antibodies as indicated. Proteins were resolved by SDS-PAGE (4% stacking gel, 11% resolving gel), transferred to polyvinyldiene difluoride membrane, and blocked with 0.2% 1-Bloc (Tropix, Bedford, MA) in Tris-buffered saline. The membranes were then probed with polyclonal antibodies at 1:250 to 1:1000 dilutions. The bands were visualized using chemiluminescent detection as previously described (18) using alkaline phosphatase-conjugated rabbit anti-goat or goat anti-rabbit secondary antibodies as appropriate. ChIP assays (see Ref. 38, for method review) were carried out with differentiating MC3T3-E1 osteoblasts essentially as previously described by (38, for method review) were carried out with differentiating MC3T3-E1 osteoblasts essentially as previously described by (38). Non-MC3T3-E1 control cultures served as a control. ChIP analyses were performed with the OCFRE probe using methods previously detailed (44). Primary mouse calvarial osteoblasts were isolated from newborn mice as previously detailed (45), and cultured in α-minimal Eagle’s medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml ascorbic acid, and 3 mM β-glycerophosphate to induce differentiation. Cells were passaged once, plated in 10-cm culture dishes, and maintained under the above mineralizing culture conditions for 0, 5, 15, or 25 days, with refeeding every 2 days. RNA was extracted as previously described (44). An Applied Biosystems GeneAmp 5700 sequence detection system using Sybr Green fluorescence PCR products was used to quantify osteocalcin mRNA accumulation at day 0 of wild-type calvarial cell cultures. Data are presented as the mean ± S.D. obtained from two independent experiments (4 replicates performed at each time point).
OF CRE Binding Protein Purification Scheme

| Protein extract from 3 × 10^6 MG63 human osteosarcoma cells |
|-------------------------------------------------------------|
| Ammonium Sulfate Precipitation                              |
| 0% - 35%                                                   |
| < 100 kDa                                                  |
| 35% - 70%                                                  |
| > 70%                                                      |
| S300 Size Exclusion Chromatography                          |
| Flow through                                               |
| 0.1 M NaCl                                                  |
| 0.2 M NaCl                                                  |
| 0.3 M NaCl                                                  |
| 0.5 M NaCl                                                  |
| 1.0 M NaCl                                                  |
| DE52 Chromatography                                         |
| Flow through                                               |
| 0.1 M NaCl                                                  |
| 0.2 M NaCl                                                  |
| 0.3 M NaCl                                                  |
| 0.5 M NaCl                                                  |
| 1.0 M NaCl                                                  |
| Heparin Sepharose Chromatography                            |
| Flow through                                               |
| 0.5 M NaCl                                                  |
| 1.0 M NaCl                                                  |
| 2.0 M NaCl                                                  |
| 3.0 M NaCl                                                  |
| 5.0 M NaCl                                                  |
| Mono Q FPLC                                                 |
| Flow through                                               |
| 0.1 M NaCl                                                  |
| 0.2 M NaCl                                                  |
| 0.3 M NaCl                                                  |
| 0.5 M NaCl                                                  |
| 1.0 M NaCl                                                  |
| HiTrap Heparin FPLC                                         |
| Flow through                                               |
| 0.5 M NaCl                                                  |
| 1.0 M NaCl                                                  |
| 2.0 M NaCl                                                  |
| 3.0 M NaCl                                                  |
| 5.0 M NaCl                                                  |

**FIG. 1. Purification scheme for isolating the OF CRE DNA-binding protein complex.** Cellular protein was extracted from 2 × 10^6 human MG63 osteosarcoma cells and OF CRE DNA binding activity purified using standard protein chromatography techniques, applying OF CRE binding in gel shift as an assay (17, 20). Starting with 1 g of protein extract, ~4 µg of purified OF CRE protein was obtained as eluted from HiTrap heparin-Sepharose HPLC. The binding complex eluted with an apparent molecular mass of 200 kDa from the Sepheracyl S300 gel filtration chromatography.

**RESULTS**

**Purification of OF CRE-binding Complex from MG63 Osteosarcoma Cells and Identification of Ku70, Ku80, and Tbdn100**—Previously we described the characterization of an FGF2- and Msx2-regulated DNA-protein interaction at the OF CRE (17). We wished to identify the transcription factor complex binding to the OF CRE. Gel filtration analyses indicated that the native binding activity was ~200 kDa (data not shown) and was likely to be a multimer protein complex. Therefore, we purified OF CRE binding activity 25,000-fold using classical biochemical techniques (32, 47), starting with 2 × 10^6 MG63 human osteosarcoma cells and applying sequential ammonium sulfate precipitation, gel filtration, diethylaminoethyl-Sepharcl chromatography, Mono Q FPLC, and heparin-Sepharose FPLC techniques, following DNA binding in gel shift as an assay (Fig. 1). This resulted in about 4 µg of purified protein. As shown in Fig. 2A, Coomassie Brilliant Blue staining of SDS-PAGE-fractionated protein reveals the presence of four constituents of ~50, 70, 80, and 100 kDa in the fraction with peak binding activity. Notably, the pattern of elution for the 70-, 80-, and 100-kDa proteins (arrows, panel A) coincides with the elution of OF CRE DNA binding activity from the heparin-Sepharose FPLC column (Fig. 2B). Therefore, the heparin-Sepharose column fraction containing the greatest OF CRE binding activity and amounts of these three constituents was concentrated 50-fold and proteins were resolved by SDSPAGE, the individual bands were excised and digested in-gel with trypsin, and tryptic peptides were analyzed by mass spectrometry. The 70- and 80-kDa proteins were identified as Ku70 (GenBank accession number P1409556) and Ku80 (GenBank accession number P135010), the heterodimeric constituents of nuclear Ku antigen. Initially, the 100-kDa protein (Fig. 3; GenBank accession number AY112670) was completely novel, i.e. did not match any of the known proteins in the nonredundant GenBank data base. However, this transcript recently was reported as a shorter variant called Tubedown-1, a nuclear protein N-acetyltransferase (28). Using 5′ and 3′-rapid amplification of cDNA ends, we cloned the 100-kDa form of this novel acetyltransferase from mouse and human heart cDNA. Sequencing of both the human (886 codons) and mouse (865 codons) heart cDNAs encoding Tbdn100 confirm the predicted protein sequence of the longer variant (Fig. 3). Importantly, mass spectroscopy analysis identified 23 polypeptides encoded by the human Tbdn100 cDNA, including polypeptides found in the extreme end of the novel NH2 terminus. This confirms that the sequence predicted by the cDNA sequence does encode a protein containing these sequences and that the full-length, 100-kDa form of Tbdn is expressed both in the MG63 osteoblasts (by LC-MS/MS protein sequencing) and heart (by cDNA sequencing). We call the novel, longer variant we identify in bone and heart “Tbdn100,” reflecting its larger size of Mr = 100,000 and relationship to the murine Tbdn sequence. The novel subunit, Tbdn100, is glutamate- and lysine-rich protein, has a putative bipartite nuclear localization signal (48, 49) at residues 612–629, and multiple NH2-terminal tetratricopeptide repeat motifs (Fig. 3). Tbdn100 also shows distant homology to the GCN5 family of N-acetyltransferases (50, 51), consistent with its role in transcriptional regulation (see below). Northern blot analyses of poly(A) mRNA (Fig. 4) confirms the robust expression of Tbdn (see below) in mouse tissues. Four transcripts of about 7, 5, 4, and 3 kb were visualized. The 5-kb transcript was widely expressed, but robustly so in heart, liver, kidney, testis, and MC3T3E1 murine calvarial osteoblasts (Fig. 4); in situ hybridization studies also confirm the expression of Tbdn in craniofacial osteoblasts (data not shown). The expression of the 7-kb transcript was selective for heart and kidney, whereas the smaller transcripts were most prominent in heart and testis (Fig. 4). Larger messages can be additionally identified in total RNA from MC3T3E1 cells, possibly representing incompletely processed nascent transcripts (not shown). Thus, Ku70, Ku80, and Tbdn100 represent the proteinaceous constituents of the purified osteoblast OF CRE binding activity. Northern blot (Fig. 4) and Western blot (Fig. 5, see below) analyses confirm expres-
The cDNA sequence and predicted protein sequence of human Tbdn100.

5'/H11032- and 3'/H11032-rapid amplification of cDNA end reactions were carried out with human heart cDNA to obtain the sequence used to clone human Tbdn100 by PCR as described in the text. The GenBank accession number for human Tbdn100 is AY112670. The nuclear localization consensus at residues 612–628 is in **bold italics**. The 23 unique peptides identified by micro LC-MS/MS from Tbdn100 purified from human MG63 osteosarcoma cells are **underlined**. Affinity purified antipeptide antibodies were generated against the COOH-terminal 15 amino acids of Tbdn. Seven tetratricopeptide repeat motifs are encoded by residues 46–79, 80–113, 114–147, 148–181, 224–257, 374–407, and 408–441.

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|--------------------------------------------------|
| FIG. 3. The cDNA sequence and predicted protein sequence of human Tbdn100. 5'- and 3'-rapid amplification of cDNA end reactions were carried out with human heart cDNA to obtain the sequence used to clone human Tbdn100 by PCR as described in the text. The GenBank accession number for human Tbdn100 is AY112670. The nuclear localization consensus at residues 612–628 is in **bold italics**. The 23 unique peptides identified by micro LC-MS/MS from Tbdn100 purified from human MG63 osteosarcoma cells are **underlined**. Affinity purified antipeptide antibodies were generated against the COOH-terminal 15 amino acids of Tbdn. Seven tetratricopeptide repeat motifs are encoded by residues 46–79, 80–113, 114–147, 148–181, 224–257, 374–407, and 408–441. |
Expression of Tbdn100, Ku70, and Ku80 in MC3T3E1 Osteoblasts—We wished to verify the expression of OCFRE constituents in osteoblasts. Therefore, affinity purified anti-Tbdn polyclonal antibodies were prepared, commercially available Ku70 and Ku80 antibodies were obtained, and MC3T3E1 cellular extracts were prepared for Western blot analyses as outlined under “Experimental Procedures.” The accumulation of 100- and 50-kDa proteins selectively immunoreactive for affinity-purified anti-Tbdn100 antibody was detected in the nuclear fraction of MC3T3E1 calvarial osteoblasts (Fig. 5A, lanes 1 and 2). Notably, this confirms that the longer Tbdn100 variant protein is indeed expressed in MC3T3E1 calvarial osteoblasts; the more rapidly migrating form may represent either a proteolytic fragment encompassing the COOH-terminal domain (antibody directed to the COOH terminus) or a variant arising from alternative mRNA processing (Fig. 4). Immunoreactivity for the cytoplasmic marker tubulin was present predominantly in the cytoplasmic fraction (Fig. 5B, lanes 3 and 4); no immunoreactive proteins were observed in the absence of primary antibody (lanes 5 and 6). Western blot analyses also confirm the expression of Ku70 and Ku80 in MC3T3E1 calvarial osteoblasts (Fig. 5B). As also shown in Fig. 5B, FGFR treatment of MC3T3E1 osteoblasts for >20 h up-regulates Ku80 and Ku70 protein accumulation 2–3-fold, as suggested from previous binding data (20). By contrast, total Tbdn protein accumulation assayed by Western blot is not affected by FGF treatment (data for Tbdn100 is shown in Fig. 5B). Thus, Ku70, Ku80, and Tbdn100 represent the proteinaceous constituents of the purified osteoblast OCFRE binding activity. The novel, lengthier Tubedown variant Tbdn100 is expressed in MC3T3E1 calvarial osteoblasts, and localizes to the nuclear fraction as predicted from the primary structure of its cDNA.

Ku Antigen Complexes Assembled on OC Promoter Chromatin and the OCFRE—Our original analyses of OCFRE function and protein-DNA interactions was carried out in MC3T3E1 osteoblasts. To show that Ku binds endogenous OC chromatin in the osteoblast, we performed ChIP assays (38) using anti-Ku70 and anti-Tbdn100 antibodies as outlined under “Experimental Procedures.” As shown in Fig. 6A, anti-Ku70 and anti-Tbdn100 antibodies recruited the OC gene to the pellets in ChIP assays; by contrast, the BSP gene (encodes another extracellular matrix protein) was not found in the pellet in ChIP using either anti-Ku70 or anti-Tbdn100 antibodies. Two separate anti-Ku70 antibodies (see M19 and C19; see “Experimental Procedures”) were used that detected Ku antigen association with the OC gene in ChIP assays (Fig. 6A, lower), whereas anti-GST and anti-Gal4 DNA-binding domain polyclonal antibodies (negative controls) precipitated relatively little OC chromatin. When quantified by fluorescence PCR, a 15–30-fold enrichment of the OC gene is observed with the two distinct anti-Ku70 antibodies (Fig. 6B). The background raising in ChIP performed with nonspecific negative control antibodies (anti-FLAG, anti-Gal4, and anti-GST) is negligible; moreover, no enrichment occurs in ChIP assays with anti-Ku70 antibodies using Ku70/−/− primary calvarial cell cultures as the source of chromatin (Fig. 6B). Using either MC3T3E1 (not shown) or COS-1 crude nuclear extracts (Fig. 6C), specific DNA binding that contains Ku antigen could be seen to be associated with the OCFRE. Competition studies confirmed binding sequence-specific binding; whereas cold homologous duplex OCFRE oligo (GCAGTCA cognate) can compete for binding (Fig. 6B, lanes 2 and 3; compare with lanes 1 and 6, no competitor), the unrelated A/T-rich HOX sequence (CTAAATGG cognate) (18) is without effect (Fig. 6C, lanes 4 and 5). Notably, the addition of the heterodimer-specific anti-human Ku antibody monoclonal antibody 162 (37) (Fig. 6C, lane 7) disrupts formation of the complex. Disruption without supershift is observed with endogenous binding activity in this monkey fibroblastic cell line (Fig. 6C, lane 7). In COS-1 cells, markedly increased OCFRE binding is observed following transient co-transfection with pcDNA3-Ku70 with pcDNA3-Ku80 (human cDNAs kind gift of Dr. Westley Reeves; Fig. 6C, lane 8). Incubation of extracts from
**Panel A**

ChIP assays were carried out as described under "Experimental Procedures." Data are representative of results obtained in three independent experiments. Note that although anti-Ku70 and anti-Tbdn antibodies precipitate OC genomic DNA, BSP genomic DNA is not precipitated (upper). Two distinct anti-Ku70 antibodies (shown, lower panel) and an anti-Ku80 antibody (not shown) recruit OC genomic DNA in ChIP: anti-FLAG, anti-glutathione S-transferase (GST), and anti-Gal DNA-binding domain antibodies do not (negative controls, lower).

**Panel B**

Real time fluorescence PCR measurement of OC gene enrichment in ChIP assays as described under "Experimental Procedures." Note the 15–30-fold enrichment of the OC gene by immunoprecipitation of MC3T3E1 chromatin by Ku70 antibodies. Note that control antibodies (anti-FLAG, anti-Gal4, and anti-GST) do not immunoprecipitate OC chromatin, and give a background signal similar to that arising from transected cells with monoclonal antibody 162 results in a visible supershifted complex (Fig. 6C, lane 9). Similar results are obtained in MC3T3E1 osteoblasts (not shown). Notably, monoclonal antibody 162 does not interact nonspecifically with the radiolabeled OCFRE probe (Fig. 6C, lane 10). Thus, Ku antigen-containing complexes were detected bound to the endogenous OC gene in MC3T3E1 cells, and in sequence-specific DNA-binding complexes assembled by the OCFRE.

**Panel C**

Antibody and Chromatin Source

**Fig. 6. Association of Ku complexes with the OC promoter.** Panel A, ChIP assays were carried out as described under "Experimental Procedures." Extracts were prepared either from CV-1 cells (lanes 1–7) or COS-1 cells transfected with human Ku70 and Ku80 expression constructs. No extract was added in lane 10. Lanes 1–7, competition assays with cold oligos; lanes 6–10, immunological supershift assays. Note that although cold homologous OCFRE duplex oligo can compete for complex formation (lanes 2 and 3), the A/T-rich HOX cognate does not (lanes 4 and 5). The anti-Ku antibody disrupts formation of this endogenous complex (lane 7 versus lane 6). Extracts from COS-1 cells expressing human Ku subunits yield a partial supershift in this assay (lane 9). Note that the anti-Ku antibody alone does not interact with the probe (lane 10). See text for details.
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**A.**

![Graph](image)

**Co-Expressed Proteins**

- pcDNA3
- Ku
- Tbdn
- Ku+Tbdn

**B.**

![Graph](image)

**Protein Activity**

**Relative Light Units**

1. OC-[154/-113]-RSVLUC
2. RSVLUC

- pcDNA3
- Ku
- Tbdn
- Ku+Tbdn

**N.S.**

- 0.3 kb OCLUC Activity (RUL / pL)

**Fig. 7. Regulation of OC proximal promoter activity by Ku antigen and Tbdn100.** Cells were transiently transfected with the indicated promoter-reporter constructs and combinations of Ku70, Ku80, and Tbdn100 pcDNA3 expression constructs as detailed under “Experimental Procedures”; pcDNA3 was used to maintain uniform DNA content in each transfection. Luciferase activity was measured after 3 days as previously detailed (18, 20). Data are presented as the mean (± S.D.) activity observed in three independent replicates. Panel A, MC3T3E1 cells were transfected with the 0.2-kb OC promoter-luciferase reporter (0.2-kb OCLUC) and expression plasmids for Ku70, Ku80, and Tbdn100. Note that the combination of Ku with Tbdn100 up-regulates OC promoter activity 7-fold. Panel B, CV-1 cells were transfected with pcDNA3, Ku (Ku70 + Ku80), or Tbdn100 (Tbdn) in the indicated combinations either with RSVLUC or OC-(−154/−113)-RSVLUC (42-bp OC promoter region −154 to −113 placed upstream of RSVLUC) as described under “Experimental Procedures.” Note that although the RSV promoter is unresponsive, OC-(−154/−113)-RSVLUC is up-regulated by expression of Ku + Tbdn100. See also Fig. 8, and text for details.

**Ku-Tbdn complex and Runx2 in transient co-transfections (Fig. 8B); a 10.7-fold induction is elicited.** The heterologous RSV minimal promoter is not synergistically induced. The individual contributions of Runx2, Ku, and Tbdn100 were examined further in the presence of FGFR2-ROS signaling; both Ku and Tbdn100 augment Runx2-dependent transactivation (Fig. 8C). Whereas Ku uniformly enhances Runx2 activation of OC-(−154/−113)-RSVLUC either in the presence or absence of FGFR2-ROS, Tbdn100 augmentation is pronounced only in the presence of FGFR2-ROS (Fig. 8C and data not shown). Notably, this functional interaction is promoter-specific, because RSV (Figs. 7 and 8) and matrix metalloproteinase 1 promoter (data not shown) basal promoters are not activated. Thus, these data demonstrate the functional interactions between Ku, Tbdn100, and Runx2 in transcription driven by the 42-bp OC promoter region −154 to −113, and confirm the role of the novel Ku complex in OC promoter regulation. Ku and Tbdn100 serve to augment Runx2 transcription, enhanced in the presence of activated FGF receptor signaling.

**Functional and Physical Interactions between Ku, Runx2, and Msx2—**The OC promoter region −154 to −113 is a target for Msx2-dependent suppression and Runx2-dependent activation. A characteristic feature of OCFLF-dependent transcription is suppression of transcription driven by the OCFLF by Msx2. To confirm this functionally important hallmark, we examined the effects of Msx2 on the activation of OC-(−154/−113)-RSVLUC by Ku/Tbdn and Runx2. As shown in Fig. 9A, Msx2 inhibits transcription via this element. As previously described (17), suppression was not dependent upon the Msx2 COOH-terminal domain, but requires the intact Msx2 homeodomian NH2-terminal arm and extension missing in Msx2-(2-208; Δ132-148) (Fig. 8A). Consistent with a role for Msx2 protein-protein interactions in this regulation, the histone deacetylase inhibitor trichostatin A does not alter Msx2-dependent suppression; moreover, the Msx2 variant Msx2(T147A) that cannot bind DNA (18) still suppresses activation (data not shown). Pull-down assays confirm protein-protein interactions between these OC regulatory factors. As shown in Fig. 9B, [35S]Met-radiolabeled Msx2 associates with T7-tagged Ku70 (lane 1) and Ku80 (lane 2) bound to the T7 affinity resin, but not to the BSA-coated anti-T7 antibody-coated Sepharose resin itself (lane 3). About 5% of input (lane 4) was recovered. By contrast, a Msx2 variant lacking the NH2-terminal arm and extension (17) does not interact with either Ku70 (lane 5) or Ku80 (lane 6). Radiolabeled Runx2 avidly binds to both Ku70 (Fig. 9B, lane 9) and Ku80 (lane 10), whereas interacting minimally with the anti-T7 antibody resin (lane 11), consistent with the functional interactions we observe in transient co-transfection assays (see above). Under these pull-down assay conditions, Tbdn100 interacts albeit less robustly with the individual Ku70 (Fig. 9B, lane 13) and Ku80 (lane 14) subunits but not at all with anti-T7 antibody-coated resin (lane 15). However, the interaction observed is similar to the heterodimeric interactions noted with the radiolabeled Ku subunits, with immobilized Ku70 (lanes 17-19) or Ku80 (lanes 20-22). USF1 also does not interact with Ku antigen in this assay, and Dlx5 selectively interacts with Ku70 but not Ku80 (data not shown). Thus, both functional (Figs. 8 and 9A) and physical (Fig. 9B) interactions exist between components of the Ku/Tbdn complex and the osteoblast transcription factors, Msx2 and Runx2.

**Calvarial Cells Isolated from Ku70−/− Mice Exhibit Profound Deficiencies in OC but Not OPN Gene Expression—**Alt and co-workers (52) recently generated Ku70-deficient mice for studies of lymphocyte biology. To characterize the effects of Ku complexes on OC expression, we isolated calvarial osteoblasts from wild-type and Ku70−/− neonatal mice, and evaluated the expression of the OC gene when calvarial cell cultures were grown under mineralizing conditions. We applied quantitative fluorescence RT-PCR to the analyses of OC (osteoblast-specific terminal differentiation marker (16)) and OPN (extracellular matrix protein expressed in osteoblasts, monocytes, and migrating vascular smooth muscle cells (53)) gene expression in these cultures. OC and OPN levels were normalized to the expression of the constitutive housekeeping gene GAPDH. As shown in Fig. 10A, with time in mineralizing conditions, expression of OC is up-regulated 600-fold in wild-type cultures, heralding osteoblast terminal differentiation. However, in Ku70−/− calvarial cell cultures OC gene expression is markedly perturbed, as demonstrated by the greater than 90% decrement in OC gene expression. By contrast, expression of the gene for OPN was not reduced; in fact, at later stages of culture, OPN expression in Ku70−/− cultures slightly exceeds...
that of wild-type cultures (Fig. 10B), indicating selective perturbation of OC expression. Thus, biochemical analyses, transient transfection studies, and quantitative evaluation of gene expression of Ku70-deficient calvarial cells converge to indicate a role for Ku antigen complexes in the expression of the OC gene in calvarial osteoblasts.

**DISCUSSION**

The OCFRE confers both positive (20, 21) and negative (17, 22) regulation on the OC promoter, and functions as a target of Msx2 action (12, 17) via the OC promoter region /H11002 to /H11002. We have identified Ku70, Ku80, and Tbdn100 as the constituents of the major homeoprotein-regulated OCFRE complex in MG63 osteosarcoma cells (12, 17) by protein purification and nanobore LC-MS/MS analysis. Applying immunological reagents in ChIP and gel shift assays, we confirm the association of Ku and Tbdn100 with the OC promoter. In transient transfection assays, we show that Ku:Tbdn expression functions to activate the OCFRE in concert with Runx2, a master regulator of osteoblast-specific gene expression in mesenchymal cells (3).
Importantly, mutation of the OCFRE precludes both basal activity and factor-stimulated transcriptional activation driven by the FGF-responsive OC promoter fragment −154 to −113 (21) (and data not shown). Profound reduction in OC gene expression is observed in cultures of calvarial osteoblasts from Ku70 −/− mice as compared with wild-type cells; the deficiency in OC expression that arises in the absence of this OCFRE-binding protein subunit provides additional evidence for its function in OC transcription. Ku participates in distinctive patterns of protein-protein interactions with Msx2, Runx2, and Tbdn100. Based upon our cumulative data, our working model of OC promoter regulation by Msx2 (Fig. 11) reflects the inhibition of Runx2 and Ku DNA binding activity via these protein-protein interactions. ChIP assays confirm that Msx2 inhibits Runx2 association with OC chromatin.2 Future experiments will identify the precise structural motifs present in Msx2 necessary for interaction with each Ku subunit, and the effects of Msx2 on formation of the Ku/Runx2 and Ku/Tbdn complexes.

Preliminary structure-function analysis suggests that, in addition to the homeodomain NH2-terminal arm, the COOH terminus of Msx2 stabilizes physical interactions with Ku.

A more general theme of functional interactions between Ku antigen and homeodomain transcriptional regulators is emerging. We previously described the OCFRE as a DNA binding activity inhibited by Msx2 (12), and show physical interactions between Msx2 and Ku subunits in this report. Recently, Hache and co-workers (54) identified by the yeast two-hybrid cloning strategy that Dlx1 and Dlx2, homeoproteins highly related to Dlx5 and Msx2, interact with the COOH-terminal domain of Ku70 via protein-protein interactions with a trihelical DNA binding motif uniquely found in Ku70 but not Ku80. We also observe Dlx5 interactions with Ku70 but not Ku80 (data not shown). Because Msx2 interacts with both Ku subunits, this suggests a fundamental difference in the molecular biology of Msx and Dlx interactions with Ku antigen. It is tempting to speculate that cross-talk occurs between Ku and Msx2 signaling during osteoblast development, coordinating proliferation and the timing of terminal differentiation. The regulation of Ku activities by Msx2 during DNA repair (55), Werner protein RecQ helicase stimulation (56, 57), and telomerase targeting (58) have yet to be examined. If regulated by the osteoblast homeoproteins, these latter activities may contribute to the net actions of Msx2 on skeletal growth, osteoblast senescence, and sarcomatous transformation (59, 60).

The physical and functional interactions identified between Ku and Runx2 are intriguing. Like Runx2 (61), Ku70 is required for robust OC gene expression. The precise mechanisms whereby Ku and Runx2 synergistically activate OC transcription are now being examined. Possibilities include promotion of inward DNA transactions, stabilization of DNA binding to certain cognates, and nuclear matrix targeting. Dynan and co-workers (62) recently showed that Ku plays a distinct role in the reinitiation phase of gene transcription. Based upon their new data, one attractive mechanism for the observed synergy would be that Runx2 promotes initiation whereas Ku augments Runx2-directed re-initiation of OC gene transcription. Potential differences arising from the Runx2 variants that are differentially expressed in osteoblasts (63, 64) will be evaluated. The effects of Msx2 and Ku antigen on the assembly and stabilization of promoter-specific Runx2-chromatin complexes is being examined in ChIP assay; Msx2 selectively inhibits assembly of these complexes on the endogenous OC chromatin.2 Because Ku antigen and Runx2 appear important for both normal T-cell function (52, 65, 66) and osteoblast gene expression (63, 67), functional interactions between Ku and Runx2 may occur in lymphocytes as well.

**Fig. 10.** Selective perturbation of OC gene expression in differentiating calvarial cells lacking Ku. Primary calvarial cell cultures were prepared from wild-type and Ku70 −/− mice, and maintained under mineralizing conditions. Total RNA was extracted from these cultures at the times indicated, and analyzed for expression of OC mRNA accumulation in Ku70 −/− cultures only (panel A) and OPN (panel B) using real-time fluorescence RT-PCR as described under “Experimental Procedures.” Expression levels are normalized to GAPDH, and referenced to baseline in cultures of wild-type calvarial cells to facilitate comparisons. Note that during early culture, OC mRNA accumulation is up-regulated −600-fold (panel A), as previously identified by Northern blot analyses (10). Further note the profound deficiency in OC mRNA accumulation in Ku70 −/− cells (up-regulated only 10-fold with differentiation) compared with wild-type cell cultures. By contrast, expression of OPN is not reduced at these later stages in Ku70 −/− calvarial cells (panel B).

**Fig. 11.** Working model for transactivation of the OC promoter region −154 to −113 and inhibition by Msx2. OC promoter activity is supported by protein-DNA interactions at adjacent OCFRE and OSE2 cognates bound by Ku and Runx2, respectively. Note that unlike most Runt domain proteins, Runx2 does not dimerize with CBP-β (82). FGF receptor signaling enhances transcriptional interactions between these factors via mechanisms yet to be detailed, but may function to stabilize assembly of the complex via Tbdn. Msx2 inhibits transcription driven by this complex via inhibitory protein-protein interactions that regulate DNA binding of transactivators (17) and the basal transcriptional machinery (18). See text for details.
It is interesting to note that the extended OCFRE cognate (CTGCAGTTCACC, upper strand; GGTGACTGCAG, lower strand) closely resembles that of several other Ku responsive elements previously identified, including the NRE1 from the mouse mammary tumor virus promoter (26), and the strict late UL38 promoter element of HSV1 (68). The steps of transcriptional activation (69–71), reinitiation (62), and suppression (26, 72–74) have been shown to be regulated by Ku dependent upon promoter context. Other targets of Ku action in the osteoblast are not known; however, a Ku cognate mediating transcriptional suppression of the parathyroid hormone (75) and parathyroid hormone-related polypeptide (76) promoters have been described that mediate responses to calcium and the calcitropic hormone, vitamin D. Whether the cell-autonomous perturbations in osteoblast functions that we have identified in calvarial cell cultures from Ku70-deficient mice are additionally influenced in vivo by dysregulated calcium homeostasis remains to be tested.

Recently, the crystal structure of the Ku70:Ku80 heterodimer bound to DNA was published (77). The Ku heterodimer forms a proteinaceous ring around the co-crystallized stem-loop DNA structure, with contacts made primarily in the major and minor grooves of the double helix (77). As pointed out by the authors, this raises a number of topological conundrums that must be answered. For example, if the ring conformation identified in the crystal structure is adopted by Ku during DNA ligation reactions, how is Ku released from DNA following the ligation event? A similar question arises in the promoter-specific regulation of gene transcription and chromatin remodeling by Ku in vivo and in transient transfection assays that utilize circular, supercoiled DNA promoter-reporter plasmids. A second COOH-terminal helix-strand-helix DNA-binding domain recently characterized by nuclear magnetic resonance (78) is present in Ku70 residues 536–609 that is disordered in the above crystal structure, and does not require heterodimerization with Ku80 for interaction with DNA (40). Notably, this COOH-terminal domain interacts with homodomain proteins, and mutations as Lys residues 595 and 596 in human Ku70 abrogate heterodimerization with the homodomain (54). It is intriguing to speculate that this COOH-terminal DNA-binding module may confer a second, distinct set of DNA-protein interactions in concert with other interacting DNA-binding proteins. Future studies will detail the potential roles for OC promoter DNA configuration in the recognition by Ku, and outline the structure-function relationships for Ku-dependent transcription modulated by associated co-regulatory molecules such as Runx2 and Msx2.

In a recent report by Hasty and co-workers (79), the Ku80 (also known as Ku86) knockout mice have been shown to have osteoporosis as well as other phenotypes consistent with precocious aging, characterized by excessive tissue apoptosis. These authors point to the differences between Ku80 –/– mice and Ku70 –/– mice; specifically, they note the lower incidence of neoplasms in Ku80 –/– versus Ku70 –/– animals. Ku70 typically heterodimerizes with Ku80; however, as noted above, Ku70 also exhibits DNA binding activity as a monomer (40) in part via the unique COOH-terminal DNA-binding domain that mediates protein-protein interactions with homeoproteins of the Dlx family (54). Systematic comparisons of the unique phenotypes and osteogenic gene regulatory programs elaborated in Ku70 –/–, Ku80 –/–, and Ku70 –/–/Ku80 –/– animals will be required to determine whether the Ku subunits serve distinct functions in skeletal physiology.

Our data demonstrate for the first time that Tbdn100 is expressed in the MC3T3E1 osteoblast and functions as a transcriptional co-regulator, cooperating with Ku antigen and Runx2 to up-regulate transcription from the OC promoter. The poly(A) + mRNA for the lengthier variant we identify is widely expressed. Gendron and co-workers (28) recently identified Tbdn10-1 as an FGFR2- and leukemia inhibitory factor-regulated transcript in endothelial cells; moreover, they showed that the shorter variant of murine Tbdn can undergo auto-acetylation. Thus, Tbdn100 may function as a co-adapter that mediates transactivation in part via regulation of chromatin acetylation, as described for P/CAF, the nuclear receptor p160 co-regulators, and cAMP-response element-binding protein/p300 (51). Tbdn100 possesses an additional 4 NH2-terminal tetratricopeptide repeat motifs that are lacking in the shorter variant of Tbdn previously reported. Our Western blot analyses clearly demonstrate the nuclear localization of Tbdn100, and the accumulation of full-length Tbdn100 predicted from our cDNA sequence (100 kDa) as well as a smaller 50-kDa protein. The ability of Tbdn100 to promote activation of OC(–154/–113)-RSVLUC is pronounced in the presence of FGFR2-ROS. This suggests that post-translational modifications initiated by FGFR2 activation may promote stable association of Ku and Runx2 complexes via Tbdn. Notably, phosphorylation (80) and N-acetylation (81) have been shown to enhance functionally important transcription factor protein-protein interactions. A series of systematic structure-function studies are underway to: (a) identify the Tbdn100 domain structures necessary for transcriptional regulation, nuclear protein acetylation, protein-protein interactions, and responses to FGFR2-ROS; and (b) characterize the role and regulation of different Tbdn isoforms in osteoblast gene expression.

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