Cooperative Interactions between Activating Transcription Factor 4 and Runx2/Cbfa1 Stimulate Osteoblast-specific Osteocalcin Gene Expression*

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The role of ATF4 (activating transcription factor 4) in osteoblast differentiation and bone formation was recently described using ATF4-deficient mice (Yang, X., Matsuda, K., Bialek, P., Jaquiot, S., Masuoka, H. C., Schinke, T., Li, L., Brancorsini, S., Sassone-Corsi, P., Townes, T. M., Hanauer, A., and Karsenty, G. (2004) Cell 117, 387–398). However, the mechanisms of ATF4 in bone cells are still not clear. In this study, we determined the molecular mechanisms through which ATF4 activates the mouse osteocalcin (Ocn) gene 2 (mOG2) expression and mOG2 promoter activity. ATF4 increased the levels of Ocn mRNA and mOG2 promoter activity in Runx2-containing osteoblasts but not in non-osteoblastic cells that lack detectable Runx2 protein. However, ATF4 increased Ocn mRNA and mOG2 promoter activity in non-osteoblastic cells when Runx2 was co-expressed. Mutational analysis of the OSE1 (ATF4-binding site) and the two OSE2s (Runx2-binding sites) in the 657-bp mOG2 promoter demonstrated that ATF4 and Runx2 activate Ocn via cooperative interactions with these sites. Pull-down assays using nuclear extracts from osteoblasts or COS-7 cells overexpressing ATF4 and Runx2 showed that both factors are present in either anti-AFT4 and anti-Runx2 immunoprecipitates. In contrast, pull-down assays using purified glutathione S-transferase fusion proteins were unable to demonstrate a direct physical interaction between ATF4 and Runx2. Thus, accessory factors are likely involved in stabilizing interactions between these two molecules. Regions within Runx2 required for ATF4 complex formation and activation were identified. Deletion analysis showed that the leucine zipper domain of ATF4 is critical for Runx2 activation. This study is the first demonstration that cooperative interactions between ATF4 and Runx2/Cbfa1 stimulate osteoblast-specific Ocn expression and suggests that this regulation may represent a novel intramolecular mechanism regulating Runx2 activity and, thereby, osteoblast differentiation and bone formation.

Runx2, the bone-specific product of the Cbfa1 gene, is a runt domain-containing transcription factor that is essential for osteoblast and hypertrophic chondrocyte differentiation and bone formation during embryogenesis and postnatal life. Runx2-deficient mice die at birth and lack both skeletal ossification and mature osteoblasts (1, 2). Runx2 haploinsufficiency causes the skeletal disorder cleidocranial dysplasia, a disease characterized by defective endochondral and intramembranous bone formation. Runx2 heterozygous mice display the same deformities as cleidocranial dysplasia patients, including delayed skeletal development, hypoplastic or aplastic clavicles, and patent fontanelles (1, 2). Runx2 is expressed in mesenchymal condensations during early development at embryonic day 11.5 and acts as an osteoblast differentiation factor (3). Runx2 is also expressed in terminally differentiated osteoblasts, where it regulates expression of bone matrix proteins (4).

Although the importance of Runx2 in osteoblast differentiation and bone formation is well established, relatively less is known about how Runx2 is regulated. Runx2 expression and functional activity are controlled by a number of factors, including bone morphogenetic proteins, fibroblast growth factor-2, PTH,1 tumor necrosis factor-α, and extracellular matrix signals (5–10). Runx2 can also physically interact with a number of nuclear proteins, including Cbfβ, which forms heterodimers with all members of the Cbfa/Runx family of factors (11–13), the transcriptional corepressor TLE/Groucho and its antagonist, HES-1 (14, 15), AP-1 proteins (16–18), SMADs (19), the retinoblastoma tumor suppressor (Rb protein) (20), Stat1 (21), TAZ (transcriptional co-activator with PDZ-binding motif) (22), lymphoid enhancer factor 1 (LEF1) (23), and Twist (24).

ATF4, also known as CREB2 (cAMP-response element-binding protein 2) (25), is a basic leucine-zipper transcription factor that is a member of the ATF/CREB protein family. This family includes CREB, cAMP-response element modulator, and ATF1, -2, -3, and -4 (26–30). These proteins bind to DNA via their basic regions and dimerize via their leucine domains to form a large variety of homodimers and/or heterodimers that allow the cell to coordinate signals from multiple pathways (26–30). An in vivo role for ATF4 in bone development was recently described using Atf4-deficient mice (31). Skeletal preparations of Atf4−/− animals showed a marked reduction or delay in min-

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1 The abbreviations used are: PTH, parathyroid hormone; GST, glutathione S-transferase; CREB, cAMP-response element-binding protein; BMSC, bone marrow stromal cell; CMV, cytomegalovirus; aa, amino acids; YFP, yellow fluorescent protein; luc, luciferase; β-gal, β-galactosidase; ATF4, activating transcription factor 4.
eralization of bones including frontal and parietal bones, clavicles, and long bones. At birth, the delay of mineralization in the skull was still present as shown by widening of the fontanelles. Postnatally, ATF4-deficient mice have a severe reduction in bone volume and in the number and thickness of trabeculae that persists throughout life. Furthermore, ATF4-deficient mice have normal levels of Runx2 and Osterix, both required factors for osteoblast differentiation. However, the expression of both bone sialoprotein and osteocalcin, markers for terminally differentiated osteoblasts, was markedly reduced in ATF4-deficient osteoblasts. Although ATF4 mRNA is ubiquitously expressed, ATF4 protein is present only in osteoblasts. This tissue specificity is explained by proteasome-mediated degradation of ATF4 in non-osteoblastic cells (32). Furthermore, its transcriptional effects on the bone-specific Ocn gene are mediated by binding to a specific cis-acting element known as OSE1. This element was previously shown to be of equal importance to OSE2, the binding site for Runx2, in stimulating transcription through interactions with OSE1 and OSE2 sites in the promoter of Ocn mRNA (31). These observations clearly demonstrate that ATF4 is required for osteoblast differentiation and bone formation during development as well as postnatal life. However, the mechanism of action for ATF4 in bone remains poorly understood.

This study determined the functional relationship between ATF4 and Runx2 by examination of their independent and mutual effects on osteoblast-specific Ocn expression and mO2g promoter activity. As will be shown, ATF4 enhanced Runx2-dependent Ocn mRNA expression and mO2g promoter activity. ATF4 and Runx2 cooperatively regulated Ocn transcription through interactions with OSE1 and OSE2 sites in the promoter. Furthermore, pull-down assays suggested that ATF4 and Runx2 form a functional complex. Deletion analysis of Runx2 identified two separate regions required for ATF4 binding and activation.

**EXPERIMENTAL PROCEDURES**

Reagents—Tissue culture medium and fetal bovine serum were obtained from Invitrogen. Rabbit anti-mouse ATF4 antibody (for Western blot analysis) was described previously (31). Other reagents were obtained from the manufacturer, according to the manufacturer's instructions. pCMV/ATF4 was described previously (31). To generate pCMV/ATF4 expression plasmids expressing truncated forms of ATF4 (aa 1–333, aa 1–290, aa 1–276) with a stop code (TAA, TAG, or TGA), that results in a premature stop of ATF4 protein at the indicated amino acid residues was introduced into ATF4 cDNA by PCR, as described above, using pCMV/ATF4 as a template. pCMV/Runx2 expression plasmids (wild type, aa 1–410, aa 1–330, aa 1–286, and aa 1–258) containing cDNAs encoding either wild type Runx2 or deletions under CMV promoter were constructed by substituting full-length ATF4 cDNA into pAdllox plasmid followed by cAMP-response element-mediated recombination, as previously described (36). All sequences were verified by automatic DNA sequencing. Expression of ATF4 protein was confirmed by Western blot analysis (data not shown).

**RESULTS**

ATF4 Activates Runx2

**ATF4 Stimulates Ocn Expression and Promoter Activity in Osteoblasts—**ATF4-null mice exhibit markedly reduced expression of osteoblast marker genes, such as those encoding osteocalcin and bone sialoprotein, indicating that ATF4 is critical for...
osteon-specific gene expression (31). However, the direct effects of ATF4 over expression have not been previously examined. To test this, we studied the effect of ATF4 on mouse Ocn expression in MC3T3-E1 subclone 4 (MC-4) cells. MC-4 cells were infected with AdCMV/β-gal and/or AdCMV/ATF4 vectors. After 48 h, the cells were harvested for RNA isolation and Northern blot analysis using Ocn cDNA as a probe. As shown in Fig. 1, overexpression of ATF4 increased Ocn mRNA in MC-4 cells in a dose-dependent manner. To determine the effect of ATF4 on mOG2 promoter activity, MC-4 cells were transiently transfected with p657mOG2-luc (reporter) and pRL-SV40 (for normalization) in the presence or absence of ATF4 expression plasmid. After 48 h, the cells were harvested for dual luciferase assay. Firefly luciferase was normalized to R. reniformis luciferase to control the transfection efficiency. As shown in Fig. 2A, ATF4 stimulated the 647-bp mOG2 promoter activity in MC-4 cells. However, introduction of a mutation of the OSE1 core sequence TTACATTA to TTAGTACA in the context of either 657-bp mOG2 promoter completely abolished ATF4 stimulation. Furthermore, an artificial promoter containing four copies of OSE1 fused to a minimal mOG2 promoter (p4OSE1-luc) was also stimulated by ATF4, whereas the same construct containing mutated OSE1 was not in MC-4 cells (Fig. 2B). Gel mobility shift assay revealed that nuclear extracts from MC-4 cells and CO3-7 cells overexpressing ATF4 formed a complex with intact OSE1 DNA that was supershifted with an anti-ATF4 antibody but not by control IgG. This complex was not formed with mutant OSE1 DNA (data not shown).

**ATF4 Stimulation of Mouse Ocn Expression Is Runx2-dependent—**Studies with Atf4 knock-out mice clearly demonstrated that ATF4 is required for osteoblast-specific Ocn and Bsp expression (31). Furthermore, as shown above, overexpression of ATF4 in both MC-4 cells and BMSCs stimulated endogenous Ocn mRNA expression and mOG2 promoter activity. Both cell types are in the osteoblast lineage and are known to contain the osteoblast-related transcription factor Runx2, a master regulator for osteoblast and hypertrophic chondrocyte differentiation and bone formation. One important question to ask is: can ATF4 also stimulate endogenous Ocn expression in cells that do not contain Runx2 protein? To address this question, we determined the functional relationship between ATF4 and Runx2 by examination of their independent and mutual effects on Ocn expression and promoter activity in non-osteoblastic cells that lack a significant amount of Runx2 protein. As shown in Fig. 3A, C3H10T12 cells transfected with β-gal expression plasmid exhibited a very low basal level of Ocn mRNA. As previously demonstrated (3, 5), Runx2 alone increased endogenous Ocn expression, consistent with its role as a master gene of osteoblast differentiation. Surprisingly and in contrast to the results from osteoblasts and BMSCs, ATF4 alone did not significantly stimulate Ocn mRNA expression in vitro and in vivo (34, 41). To gain further insight into the mechanism through which ATF4 activates Runx2, point mutations were created in OSE1 alone, both OSE2 sites, or OSE1 plus the OSE2 sites in the 657-bp

![Fig. 1. ATF4 increases Ocn mRNA expression in osteoprogenitor cells.](image)
mOG2 promoter. These constructs were then transfected into COS-7 cells with or without ATF4 and Runx2 expression vectors. As shown in Fig. 4B, mutations in either ATF4- or Runx2-binding sites slightly decreased the basal activity of the mOG2 promoter. As expected, the mutation of both OSE2 sites completely abolished the Runx2-dependent induction of promoter activity. Interestingly, mutation of the OSE1 site also markedly reduced Runx2-dependent transcription. Furthermore, mutation of either OSE1 or the two OSE2 sites dramatically reduced ATF4-stimulated Runx2 activity. Finally, mutation of OSE1 and OSE2 sites completely abrogated ATF4 regulation. It should be noted, however, that, although mutation of OSE1 greatly reduced ATF4-dependent stimulation of promoter activity in the presence of Runx2, it did not abolish it. To further explore the requirement for OSE1 in the ATF4 response, COS-7 cells were transfected with p6OSE2-luc (which contains six copies of OSE2 fused to a minimal mOG2 promoter) or p4OSE1-luc (which contains four copies of OSE1) and ATF4/Runx2 expression plasmids (Fig. 4, C and D). As expected, Runx2 stimulated transcription from p6OSE2-luc in an OSE2-dependent manner. However, ATF4 also stimulated transcription from this plasmid in the presence of Runx2, even though there were no OSE1 sites (Fig. 4C). In contrast, Runx2 was unable to enhance ATF4-dependent (OSE1) transcriptional activity in the absence of a Runx2 DNA-binding site (OSE2) (Fig. 4D). These results suggest that some of the effects of ATF4 may occur in the absence of DNA binding (to OSE1).

**Protein-Protein Interaction Studies**—Based on the functional data presented above, we next tested whether ATF4 and Runx2 can interact. Nuclear extracts from COS-7 cells overexpressing FLAG-ATF4-YFP and Runx2 were immunoprecipitated with an M2 antibody. As shown in Fig. 5A, ATF4 protein was present in an anti-Runx2 immunoprecipitate. Likewise, Runx2 protein was present in an anti-ATF4 immunoprecipitate (Fig. 6, B and C). As shown in Fig. 5, B and C, Runx2 was not immunoprecipitated by
anti-ATF4 antibodies in the absence of ATF4, and vice versa, ATF4 is not immunoprecipitated by anti-Runx2 antibodies in the absence of Runx2. To determine whether ATF4 and Runx2 can interact in osteoblasts, nuclear extracts from MC-4 cells were immunoprecipitated with anti-ATF4 antibody followed by Western blot analysis using M2 antibody. A negative control (nuclear extracts from COS-7 cells overexpressing β-gal) was added to show the antibody specificity. Similar results were obtained regardless of the GST-Runx2 ratio used, incubation strength, or incubation time (results not shown). Based on these results, we conclude that accessory factors present in nuclear extracts (but absent from bacterial cell lysates) are likely required to stabilize Runx2 ATF4 complex formation.

**Deletion Analysis**—As a first step to identifying ATF4-domain(s) necessary for complex formation with Runx2, several deletion mutants of Runx2 were made and tested for their ability to be activated by ATF4 in COS-7 cells using p657mOG2-luc as a reporter. As shown in Fig. 6A, the basal activity of Runx2 did not dramatically change with progressively larger C-terminal deletions until the entire proline-serine-threonine (PST) domain (aa 528–258) was deleted. However, a major drop in ATF4 activation was observed when the Runx2 protein was deleted from aa 330 to 286. Further deletion to aa 258 essentially eliminated both basal and remaining ATF4-induced activity. To identify the ATF4-binding domain, ATF4 and Runx2 deletion mutant expression vectors were transfected into COS-7 cells. 48 h later, nuclear extracts were prepared for immunoprecipitation using anti-ATF4 antibody and Western blot analysis for Runx2. As shown in Fig. 6, B and C, deletion of Runx2 from aa 528 to 286 did not abolish ATF4 binding. However, further deletion from aa 286 to 258 completely abrogated ATF4-Runx2 complex formation. Taken together, these data indicate that the aa 330–258 region of Runx2 is required for ATF4 activation, and the aa 286–258 region is necessary for ATF4 binding.

The aa 241–442 region of Runx2 was previously shown to activate pG4SV-luc as an autonomous activation domain (42). Because this region covers the region (aa 330–258) identified above required for ATF4 binding and activation, we next tested whether this region of Runx2 could also be activated by ATF4. This region of Runx2 was fused to the heterologous DNA-binding domain (DBD) of the yeast transcription factor Gal4 (Gal4-DBD-(1–147)) and tested for its ability to trans-
activate a luciferase reporter gene driven by five copies of the Gal4 enhancer (pGal4SV-luc) in the presence or absence of ATF4. As shown in Fig. 7, the responsiveness of this region to ATF4 activation was preserved in this heterologous system, indicating that it can function as an autonomous activation domain.

As an initial step to identifying regions (domains) within ATF4 necessary for Runx2 activation, several deletion mutants of ATF4 were made and transfected into COS-7 cells with or without the Runx2 expression vector using p657mOG2-luc as a reporter. As shown in Fig. 8, deletion of ATF4 from aa 349 (wild type) to 333 did not change its ability to activate Runx2. However, deletion of the whole leucine zipper domain (aa 332–303) dramatically decreased Runx2 activation by 70%. Deletion of the basic domain (aa 302–276) did not alter Runx2 activation. Taken together, these data indicate that the leucine zipper domain (30 aa) of ATF4 is critical for ATF4 to activate Runx2, although it is clear that other region(s) near the N terminus may also be required.

FIG. 6. Deletion analysis of Runx2 cDNA. A (top), a schematic showing the domain structure of Runx2 and the deletion mutants. Runx2 contains three transcri-ptional activation domains (AD1, -2, and -3), a transcrip-tional repression domain (RD) containing a VWRPY motif at the C terminus (COOH), a RUNT domain responsible for DNA binding, a nuclear localization sequence (NLS), and a large C-terminal PST domain, which is rich in serine/threonine/tyrosine residues. A (bottom), COS-7 cells were transfected with p657mOG2-luc, pRL-SV40, ATF4 expression plasmids, and various deletion mutant Runx2 expression plasmids. After 48 h, the cells were harvested for dual luciferase assay. B, pull-down assay; COS-7 cells were transiently transfected with ATF4 expression plasmids and various Runx2 deletion mutant expression plasmids. After 48 h, nuclear extracts were prepared and immunoprecipitated (IP) with anti-ATF4 antibody followed by Western blot (WB) analysis for Runx2. C, 10 μl of nuclear extracts from each group was used for Western blot analysis using anti-Runx2 antibody.

DISCUSSION
ATF4 deficiency causes defective osteoblast differentiation and bone formation. Furthermore, expression of osteoblast-specific Ocn was dramatically reduced, suggesting that Ocn is a major target of ATF4 (31). However, the mechanisms used by ATF4 to regulate Ocn are poorly understood. DNA-protein interaction analysis and co-transfection experiments showed that ATF4 binds to OSE1 and uses this site to activate the mOG2 promoter (31). However, it is not known whether ATF4 is sufficient for osteoblast-specific Ocn mRNA expression. In this study, we found that ATF4 stimulated endogenous Ocn expression in osteoblasts that contain Runx2 protein. In contrast, although ATF4 stimulated four copies of OSE1 fused to a minimal mOG2 promoter, ATF4 was not able to induce Ocn mRNA expression or 657-bp mOG2 promoter activity in non-osteoblastic cells that lack detectable Runx2. Instead, ATF4 stimulation of both Ocn mRNA expression or the 657-bp mOG2 promoter required Runx2, a master regulator for osteoblast differentiation and bone formation.
ATF4 Activates Runx2

To specifically test for cooperativity between ATF4 and Runx2 in osteoblast-specific Ocn expression, we examined the functional relationships between ATF4 and Runx2 using p657mOG2-luc as a reporter. The 657-bp mOG2 promoter contains one ATF4-binding site and two Runx2-binding sites that are required for the osteoblast-selective expression of the Ocn gene in vitro and in vivo (34). More importantly, the 657-bp mOG2 promoter contains sufficient information to direct osteoblast-specific expression in transgenic mice (43). The results of these transfections, shown in Fig. 4B, revealed three major features: (i) mutation of OSE1 markedly reduced Runx2-dependent mOG2 transcription, (ii) mutation of either OSE1 or OSE2 dramatically reduced ATF4-stimulated Runx2 activity, and (iii) mutation of both OSE1 and OSE2 sites completely abrogated ATF4 regulation. These data strongly support the view that the cooperativity between ATF4 and Runx2 is critical for them to stimulate Ocn expression.

It is well documented that Runx2 can interact with a number of factors. These interactions are critical for Runx2 activity and play important roles in the regulation of osteoblast differentiation and bone formation. For instance, interactions between Runx2 and Cbfβ were essential for normal bone formation in vivo (11–13). Likewise, interactions between Runx2 and Rb protein or TAZ protein were found to induce osteoblast differentiation (20, 22). Runx2 activity can also be negatively regulated via this mechanism. For example, binding of Twist to Runx2 inhibits Runx2 DNA binding and suppresses osteoblast differentiation and bone development (24). In the present study, we found that ATF4 interacted with Runx2 in osteoblast nuclear extracts or when both factors were co-transfected in COS-7 cells. However, we failed to demonstrate a direct physical interaction between ATF4 and Runx2 by GST pull-down assays. There are several possible explanations for this finding: (i) GST-ATF4 and/or Runx2 fusion proteins may not fold into the correct conformation after expression and purification from bacteria or, alternatively, the GST peptide may interfere with ATF4-Runx2 binding; (ii) ATF4 and/or Runx2 may require eukaryotic cell-specific post-translational modifications, such as phosphorylation or glycosylation before they can interact; (iii) the ATF4-Runx2 interaction requires specific accessory factors present in eukaryotic cell nuclear extracts. Further studies will be required to discriminate between these various possibilities.

ATF4 enhanced Runx2 activity by >3-fold (see Figs. 4B and 5A). The regions of Runx2 required for ATF4 complex formation and activation were also identified. Because both ATF4 and Runx2 are expressed in osteoblasts (3, 31), our data strongly suggest that ATF4 plays a critical role in the regulation of endogenous Runx2 activity and, thereby, osteoblast differentiation and bone formation during development and after birth, which may explain the dramatic bone phenotypes seen in ATF4 deficiency.

The formation of complexes between Runx2 and other factors provides a mechanism for generating an osteoblast-specific response to different signaling molecules (44). For example, bone morphogenetic proteins induce osteoblast differentiation and bone formation through SMADs-Runx2 interactions (19, 45). Likewise, PTH increases the expression of rat collagenase 3 in osteoblasts via cooperative interactions between AP-1 and Runx2 (17, 18). Our recently published work demonstrates that the OSE1 in the mOG2 promoter is necessary and sufficient for PTH induction of the Ocn gene (35). Interestingly, PTH treatment of MC-3 cells increased the binding of a nuclear factor to the OSE1 DNA that was supershifted by a specific anti-ATF4 antibody (data not shown). These results suggest that ATF4 is a major mediator of PTH actions in osteoblasts. It is likely that PTH signals up-regulate ATF4 and, thereby, ATF4-Runx2 interactions and osteoblast differentiation as well as bone formation. Ongoing studies in our laboratory are exploring this interesting possibility.

Finally, a recent study shows that ATF4 is regulated via an ubiquitin/proteasomal pathway (32). Interestingly, although Atf4 mRNA is ubiquitously expressed, ATF4 protein is only detected in osteoblasts. According to this study, this tissue
specification is explained by proteasome-mediated degradation of ATF4 in non-bone cells. For unknown reasons, this degradation pathway is less active in osteoblasts allowing ATF4 accumulation. Indeed, inhibition of the ubiquitin/proteasomal pathway by MG115, which blocks the N-terminal threonine in the active site of the β-subunit of the 26 S proteasomal complex (46, 47), leads to ATF4 accumulation and induced Ocn mRNA expression in non-osteoblastic cells (32). The effect of this proteasomal pathway on ATF4-Runx2 interactions is an important area for future study.

In conclusion, this study provided the first demonstration that ATF4 modulates Runx2 transcriptional activity. Nuclear complex formation between ATF4, Runx2, and possibly other important bone-related transcription factors.

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