[Frontiers in Bioscience 3, d838-848, August 1, 1998]

TRANSCRIPTIONAL COACTIVATORS POTENTIATING AP-1 FUNCTION IN BONE

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Received 3/27/98 Accepted 4/22/98

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1. ABSTRACT

The AP-1 proteins are formed by the heterodimerization of Fos family members and Jun family members through a structural motif called the leucine zipper. The heterodimer can then bind DNA at a consensus site termed the AP-1 site and act as a transcription factor to modulate the expression of AP-1-responsive genes. All the Jun family members can also homodimerize to exert the same function. Genetic studies including gain-of-function and loss-of-function mutations have shown that AP-1 components, particularly the c-Fos protein, are essential for proper bone development.

Both Fos and Jun family members interact with coactivator molecules to activate transcription. To date, the coactivator proteins CBP (CREB-binding protein), JAB1 (Jun-activation domain-binding protein 1), and alpha-NAC (Nascent polypeptide associated complex And Coactivator alpha) have been shown to potentiate the AP-1 transcriptional activating function. We have shown that all three proteins are expressed in bone during mouse development.

These findings raise the intriguing possibility that multiple coactivators may be involved in mediating AP-1-dependent transcription and increase the specificity of target gene activation by AP-1 proteins in differentiating bone cells.

2. INTRODUCTION

2.1. bone development and the cells of bone

Bone development requires a complex series of events involving three cell types: the chondrocytes, which form cartilage; the osteoblasts, or bone-forming cells; and the osteoclasts, the bone-resorbing cells. The chondrocytes and osteoblasts are mesenchymal in origin while osteoclasts derive from the hematopoietic system. The differentiation and function of each bone cell type appear controlled by several local and systemic factors which lead to tightly regulated patterns of specific gene expression. Some of the key transcription factors involved in osteoblastic differentiation have recently been identified (1). Interestingly, abnormal regulation of the expression of members of the AP-1 family of transcription factors has also been shown to affect the differentiation and function of all three cell types of the skeleton (see below).

Two mechanisms of bone formation have classically been distinguished: intramembranous ossification (flat bones) and endochondral ossification (long bones) (2). The essential difference between them is the presence or absence of a cartilaginous phase. Intramembranous ossification occurs when mesenchymal precursor cells proliferate and subsequently differentiate directly into osteoblasts which mineralize an immature bone tissue called woven bone, characterized by irregular bundles of randomly oriented collagen fibers and an abundance of partially calcified immature new bone, called osteoid. At later stages, this woven bone is progressively remodeled to mature, lamellar bone.

Endochondral ossification entails the conversion of a cartilaginous template into bone. Mesenchymal cells condense and differentiate into chondrocytes which secrete the cartilaginous matrix. This embryonic cartilage is avascular, and during its early development, a ring of

woven bone is formed by intramembranous ossification in the future midshaft area. This calcified woven bone is then invaded by vascular tissue, and osteoclasts and osteoblasts are recruited to replace the cartilage scaffold with bone matrix and excavate the hematopoietic bone marrow cavity.

At the extremities of long bones (epiphysis), longitudinal growth occurs by a similar process of endochondral ossification at the growth plates. Growth plate chondrocytes appear in regular columns that initially proliferate and progressively hypertrophy. The cartilaginous matrix becomes mineralized just below the hypertrophic zone of the growth plate and the chondrocytes then die. The calcified cartilage is resorbed by osteoclasts, and osteoblasts differentiate from mesenchymal cells that were brought with the invading vascular tissue. Woven bone is then deposited on top of the remaining calcified cartilage remnants. Still lower in the growth plate, the mixture of woven bone and calcified cartilage is further remodeled and replaced by mature lamellar bone trabeculae.

The earliest centers of ossification to appear during murine embryogenesis are observed at 14-14.5 days postconception (p.c.) (3). These are seen in bones of the skull, which form by intramembranous ossification, but also in the ribs and limbs, where endochondral ossification begins in the midshaft region. In the distal parts of the limbs, ossification is first observed in the middle region of the cartilage primordia of the metacarpals at about 16.5 days p.c., and complete ossification of the digits of both fore- and hindlimbs is not completed until 18.5 days p.c. (3).

2.2. AP-1 and bone development

Several lines of evidence suggested early on that bone could represent a physiologically relevant target tissue for the action of the Fos protein, the product of the c-fos proto-oncogene, and a component of the AP-1 transcription factor (see section 3 below for a detailed description of the AP-1 family members). The first link between Fos and bone tissue stemmed from the observation that v-Fos, the transforming oncogene of the FBJ (Finkel, Biskis, Jinkins) and FBR (Finkel, Biskis, Reilly) murine sarcoma viruses, induces bone lesions (osteosarcomas) *in vivo* (4). Subsequently, c-Fos expression was localized to the perichondrial growth regions of murine and human fetal bone tissues as well as mesodermal web tissue (5, 6), which are areas of active osteoblastic cell proliferation and differentiation. Osteogenic differentiation of newborn mouse mandibular condyle is preceded by a burst of c-fos expression *in vitro* (7). Fracture healing has been shown to induce the expression of c-fos (8), while the c-fos promoter region tagged on to a reporter gene targets some of the expression to lower hypertrophic chondrocytes and individual osteoblastic cells beneath the growth plate in transgenic mice (9).

Various studies have looked at mitogens that induce Fos expression as well as the expression of its dimerization partner Jun in numerous cell types. Growth hormone and parathyroid hormone stimulate the expression of c-fos in osteoblasts (10, 11). In addition, insulin-like growth factors I and II (IGF I and IGF II) have also been shown to induce c-fos in osteogenic cells and primary osteoblast cultures (12). Retinoic acid (13) and vitamin D (14) were also shown to induce c-fos, c-jun and jun-B mRNA levels in preosteoblastic cells.

In diseased bone, Fos expression appears abnormally elevated in both murine and human osteosarcomas (15), in lesions from patients with Paget's disease (16), and in patients with fibrous dysplasia showing increased rates of bone turnover (17). Taken together, the bulk of these studies implicated Fos in bone cell differentiation and the regulation of bone cell function. This circumstantial evidence was confirmed by elegant genetic manipulations generating both gain-of-function and loss-of-function mutations for Fos.

Transgenic mice overexpressing a c-fos transgene under the control of the human metallothionein promoter first demonstrated that deregulated c-fos expression interferes with normal bone development (18). In these initial studies, the phenotype of the transgenic animals appeared related to the level of expression of the transgene: very high levels of c-fos expression induced osteosarcomas (19) and it was later demonstrated that the osteoblasts are the transformed cells in these c-fos transgenic mice (20). Moreover, the observation that Fos-Jun double transgenic mice develop osteosarcomas at a higher frequency than Fos transgenic mice, coupled with the analysis of Jun expression levels in Fos-induced osteosarcomas (21), strongly implicate Jun as the dimerizing partner of Fos in the transformed bone cells of Fos transgenic mice.

Interestingly, when the expression of the c-fos transgene remained low, the bone lesions did not readily progress to form osteosarcomas but rather showed evidence of marrow fibrosis and increased bone turnover (18). The appearance of the lesions closely resembled bone defects observed in patients with fibrous dysplasia of bone, a condition that affects skeletal development and structure. It is characterized histologically by the persistence of chondro-osseous tissue where normally lamellar bone would develop. We have demonstrated that Fos is overexpressed in bone lesions from patients with fibrous dysplasia (17). The aberrant Fos expression is most likely a downstream consequence of the mutation in the gene encoding the stimulatory G (Gs alpha) protein resulting in the activation of signal-transduction pathways generating cAMP (22) which was demonstrated in patients with McCune-Albright syndrome and fibrous dysplasia (23).

The above-mentioned phenotypes were observed when the c-fos transgene was expressed post-natally. To investigate the consequences of aberrant Fos expression during development, the c-fos transgene was introduced into embryonic stem cells which were subsequently used to generate chimeric mice (24). These studies revealed that deregulated expression of c-fos during embryogenesis targets a different bone cell population for Fos-induced oncogenesis, namely, chondroblasts (25). A high frequency of chondrogenic tumors developed in the chimeric mice

(24), and stable chondrogenic cell lines were derived from the Fos-induced cartilage tumors (26). Overexpression of Jun family members has also been shown to perturb the maturation of chondrocytes (27), suggesting that several members of the AP-1 family of transcription factors are important for the proper differentiation and function of both the cartilage-forming cells and bone-forming cells of the skeleton.

The advent of the gene targeting methodology allowed to contrast the effects of an increased expression of c-fos with those of the lack of Fos function. Two laboratories independently developed strains of mice targeted at the c-fos locus (28, 29), and the analysis of the phenotype of these mice demonstrated that Fos is essential for the differentiation of the third cell type of bone, the osteoclast. Mice deficient for Fos function develop a phenotype of osteopetrosis, a disorder of bone remodeling characterized by impaired osteoclast function, resulting in a net increase in skeletal mass. Elegant studies involving co-culture assays for measuring osteoclastogenesis as well as bone-marrow transplant rescue experiments firmly established that osteoclast differentiation is inhibited in the absence of Fos whereas osteoblastic function remains normal (30). The lack of Fos also caused an accumulation of bone marrow macrophages (30). These elegant genetic manipulations clearly confirmed the hematopoietic origin of osteoclasts and identified Fos as a key regulator of the differentiation of the osteoclast-macrophage lineage.

These studies unequivocally demonstrate that bone is a physiologically relevant target tissue of Fos, and by extension AP-1, action. It is interesting to note that all three cell types of bone are affected by abnormal Fos activity, although the osteoclast is the bone cell type that is strictly dependent on the presence of Fos for its differentiation. As Fos is an obligate heterodimer for its function, it is most likely that the members of the Jun family, the dimerizing partners of Fos, are important for bone cell differentiation and function. Overexpression of c-Jun in bone does not affect skeletal development, although, as previously mentioned, a c-jun transgene can cooperate with the c-fos transgene to increase the frequency of osteosarcoma formation (21). Two members of the Jun family, c-Jun and JunD, have been shown to suppress the maturation of chondrocytes when overexpressed (27).We have previously shown that c-jun is expressed in osteoblasts (14); indeed, expression of c-jun in bone cells has been reported both during early embryogenesis (31) and at all stages of osteoblastic differentiation (32). Thus it is probable that the c-Jun activator plays a physiological role in the control of gene expression in bone cells.

3. AP-1

The acronym AP-1 stands for ‘activating protein-1’ and was first coined by the group of Robert Tjian to identify proteins regulating the expression of the human metallothionein promoter (33). Purification of these proteins using DNA affinity chromatography revealed that several proteins co-purified following the sequence-specific chromatographic step (34), suggesting that more than one protein could recognize the regulatory DNA binding site. A series of elegant biochemical studies by several laboratories subsequently identified these proteins as members of the fos and jun proto-oncogene families.

3.1. the fos family

The c-fos proto-oncogene is the cellular homolog of the transforming oncogene of the FBJ and FBR murine sarcoma viruses (35). These viruses induce osteosarcomas *in vivo*, hence the name of the gene, fos, for FBJ/FBR osteosarcoma. The Fos protein is a nuclear phosphoprotein of around 55000 Da molecular weight (36). Immunoprecipitation using Fos-specific antibodies demonstrated that a second nuclear protein of Mr 39 000 Da (p39) co-precipitate with the Fos protein (37). The p39 protein was subsequently identified as the c-Jun protein (38).

The antisera raised against Fos-derived peptides allowed to identify two additional members of the family, fos-related antigen-1 (fra-1) and -2 (fra-2) (39). Yet another member of the family, fosB, was cloned by screening a library of serum-inducible genes with a probe corresponding to the DNA-binding domain of c-Fos (40). All fos family members are expressed in osteoblasts, although the composition of osteoblastic AP-1 complexes appears to vary as a function of bone cell proliferation and differentiation (32).

There is extensive sequence similarity between the different family members within the basic DNA binding domain and the dimerization interface, a protein motif that has been dubbed the leucine zipper (41). This motif consists of heptad repeats of leucine residues which align along one face of an alpha helix. When aligned in parallel, the hydrophobic faces of two complementary helices form a coiled coil (42). Leucine zipper dimerization serves to juxtapose adjacent regions of each of the dimer ’s partners that are rich in basic amino acid residues and that serve as the DNA binding domain of the dimer (43, 44). Thus Fos is recognized as the paradigm of the bZIP (basic region — leucine ZIPper) class of transcription factors, which include many different subfamilies with distinct dimerization and DNA binding properties.

A number of dimerizing partners for c-Fos have been identified. These include the jun family members (45, 46), ATF (activating transcription factors) proteins (47, 48), and the recently identified Maf (the cellular homolog of the transforming oncogene from the AS42 avian transforming retrovirus), and Nrl (retina specific gene) gene families (49). All of these fos heterodimers can bind the canonical AP-1 site (see below), although with different affinities (46-49). Moreover, the c-fos/ATF heterodimers can bind to the CRE site (cAMP response element; ref. 47).

3.2. the jun family

The v-jun oncogene was identified as the transforming oncogene of avian sarcoma virus-17 (jun is the Japanese word for 17) (50). The observed sequence similarity between v-jun (and its cellular counterpart c-jun) and the DNA-binding domain of the yeast transcription

factor GCN4 immediately suggested that the c-jun protein could function as a DNA-binding transcriptional regulator (51). Several laboratories were quick to recognize that the GCN4 DNA binding site was very similar to the DNA sequence utilized to purify AP-1 proteins by DNA-affinity chromatography. This observation launched a series of studies which culminated in the identification of the Fos and Jun proteins as components of the AP-1 complexes (38; 52).

Differential screening of cDNA libraries from serum-stimulated cells allowed the identification of two additional family members which were named junB and junD (53, 54). Jun family members are also bZIP transcription factors and heterodimerize with fos family members through their leucine zippers. The diversity of active AP-1 complexes is further increased by the fact that jun family members can homodimerize and heterodimerize amongst themselves (46; 55). Additional dimerization partners for jun family members include the CREB/ATF (cAMP response element binding protein/activating transcription factor), Maf, and Nrl families of bZIP proteins (49; 56).

The heterodimers formed between the different Jun and Fos proteins have an increased binding activity compared to the Jun/Jun homodimers (55). The binding affinities of the different Jun dimers vary as follows: c-Jun/c-Jun > JunD/JunD > JunB/JunB (55). It is interesting to note that in certain assays, JunB has been shown to act as a negative modulator of c-Jun function (57, 58). Thus several parameters can affect the expression of an AP-1 responsive gene: the response element itself (see below), the relative binding affinities of the various AP-1 complexes, as well as their specific composition at any given time.

3.3 the AP-1 binding site

The consensus AP-1 binding site is the palindrome TGA(C/G)TCA (33, 34). The AP-1 sites are responsible for mediating induction by tumor promoters such as 12-*O*-tetradecanoylphorbol-β-acetate (TPA) and are therefore sometimes confusingly labeled TRE, for TPA-response element. Many genes contain AP-1 sites within their promoter region. The AP-1-responsive genes that are of particular relevance to bone cell biology include collagenase (59) and osteocalcin (60).

Biochemical analysis using *in vitro*-translated AP-1 proteins has revealed that the sequences adjacent to the core binding site can influence the stability of the Jun/DNA complexes (55). The AP-1 site is also very similar to the cAMP response element (CRE): TGACGTCA. Indeed, Jun family members have been shown to bind CREs alone (55) or when heterodimerized with ATF/CREB factors (49; 56).

3.4 post-translational modulation of AP-1 function

AP-1 activation is caused by: (i) increased expression of Fos and Jun proteins; and (ii) post-translational modification of Fos and Jun by phosphorylation (61, 62). The complex subject of the regulation of c-fos expression is adequately summarized in the review by Hipskind and Bilbe in this issue of *Frontiers in Bioscience* (63). Jun family members are similarly induced by a variety of signals including serum, cytokines, tumor promoters, and genotoxic stresses such as treatment with alkylating agents, ultraviolet (UV) light and ionizing radiation. It is the analysis of the Jun response to UV irradiation that led to the cloning of a member of the stress-activated protein kinases (SAPKs), the c-Jun amino-terminal kinase (JNK) (64, 65).

The JNK protein kinases phosphorylate the NH_2-terminal activation domain of c-Jun on residues Ser63 and Ser73 (65). The transcriptional activity of c-Jun is increased following this post-translational modification (61). Site-directed mutagenesis confirmed that phosphorylation of these serine residues is essential for stimulation of c-Jun-dependent transcription (61; 66). Thus any transcriptional co-factor implicated in mediating the c-Jun transcriptional activation function must be able to interact with the phosphorylated form of the protein.

4. COACTIVATORS POTENTIATING AP-1 ACTIVITY

AP-1-specific gene expression is regulated by a combination of the various AP-1 activators, general or basal transcription initiation factors, and associated cofactors. Accurate transcription initiation by RNA polymerase II requires at least six general transcription initiation factors: TFIIA (Transcription Factor IIA), TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (reviewed in 67). In a search for proteins interacting with c-Jun and implicated in c-Jun-mediated transcriptional activation, Franklin et al. (68) have shown that the C-terminus of the c-Jun protein associates directly with TBP (TATA box-binding protein) and TFIIB *in vitro*. While these interactions may contribute to the molecular mechanisms regulating c-Jun-driven transcription, they were measured as relatively weak interactions (c-Jun dissociated from TBP in 0.2 M salt) (68).

While the general initiation factors are sufficient for basal-level transcription, enhancement of transcription by AP-1 proteins bound to their cognate response elements requires the presence of additional mediator proteins, known as transcriptional coactivators (69, 70). Coactivators are defined functionally by their ability to selectively potentiate the stimulatory activity of specific subsets of enhancer binding transcriptional activators. Among the best characterized coactivators are the TAFs (TBP-associated factors) (70), which are subunits of TFIID. Purification and molecular cloning of several TAFs have confirmed that they provide protein interfaces to link the sequence-specific factors to the basal transcriptional machinery, and some exhibit specific enzymatic functions essential for activated gene transcription (69, 70). Coactivators distinct from the TFIID complex have also been identified and cloned. Three of these, CBP (CREB-binding protein), JAB1 (Jun-activation domain-binding protein 1), and alpha-NAC (Nascent polypeptide associated complex And Coactivator alpha) were recently shown to interact with AP-1 family

members. We shall now review their role in AP-1-mediated gene transcription and their putative involvement in bone cell differentiation and function.

4.1 CBP

CBP and its homologue p300 are huge nuclear proteins of more than 2,400 amino acids that can interact with a variety of sequence-specific DNA binding transcriptional activators as well as with components of the basal transcriptional machinery such as TBP and TFIIB (71). CBP also interacts with RNA polymerase II via RNA helicase A (72). These interactions are thought to provide the necessary protein scaffold bridging the sequence-specific factors to the basal transcriptional machinery. CBP does not merely provide an inert link as biochemical studies have demonstrated that it possesses an intrinsic histone-acetylase enzymatic activity (73, 74). The acetylation of the histone subunits of the nucleosomes is hypothesized to facilitate binding of nuclear factors to their DNA target sites by destabilizing promoter-bound nucleosomes.

CBP was initially identified as a coactivator interacting with the phosphorylated form of CREB that mediates cAMP-regulated gene transcription (75, 76). Because c-Jun also requires post-translational phosphorylation for its transactivating function, the role of CBP in AP-1-mediated transcription was examined. Protein interaction assays revealed that CBP interacts with the phosphorylated form of c-Jun (77). Functional assays using transient transfection confirmed that CBP stimulates the activity of c-Jun, JunB and v-Jun *in vivo* (78, 79). The protein-protein interaction between the two molecules involves the N-terminal domain of c-Jun (which contains the phosphorylated Ser63 and Ser73 residues) and the CREB binding domain of CBP (residues 461 to 661) (78).

A different domain of CBP (residues 1621 to 1877) is necessary for interaction with the C-terminus of c-Fos (amino acids 250 to 380) and potentiation of c-Fos/c-Jun-mediated transcriptional activity (80). Phosphorylation of the c-Fos protein is not required for its interaction with CBP (80). Interestingly, CBP cooperates with the ternary complex factors (TCFs) Elk-1 and SAP-1a to stimulate the expression of the c-fos gene (81, 82). Thus CBP is involved in both upstream and downstream events in the control of AP-1-dependent transcriptional regulation.

Yet another region of the CBP molecule interacts with members of the nuclear hormone receptor superfamily such as the retinoic acid receptor, glucocorticoid receptor, and thyroid hormone receptor (71; 83). Nuclear receptors have been shown to inhibit AP-1 activity (84). Overexpression of CBP or P300 abrogates the inhibition of AP-1 function by liganded nuclear receptors, suggesting that competition for CBP may be involved in the cross-talk between different signaling pathways (85). Because it interacts with so many different molecular targets and is involved in key steps of transcriptional regulation, CBP has been termed a co-integrator of multiple signal transduction pathways within the nucleus (85).

The diversity of transcription factors with which CBP interacts suggests that CBP might be ubiquitously expressed. We have used immunohistochemistry to analyze CBP expression in developing mouse embryos (figure 1) and detected widespread expression. The CBP protein was detected in osteoblasts lining the developing calvaria, a site of intramembranous bone formation (figure 1B). Interestingly, the CBP protein was also present in differentiated hypertrophic chondrocytes at the onset of endochondral bone formation (17.5 days p.c.) (figure 1D), while its expression could not be detected in the chondrocytes from the resting and growth zones (figure 1D). It is currently unknown whether CBP is expressed in osteoclasts, where it could be involved in mediating Fos-dependent transcription.

These results suggest that CBP might be involved in the control of gene expression during bone development. It is interesting to note that deletions or mutations in one copy of the CBP gene cause Rubinstein-Taybi syndrome (86), a well-defined pathology with facial abnormalities, broad thumbs, broad big toes and mental retardation as the main clinical features. This finding supports the notion that CBP could play a significant role during cranio-facial and appendicular skeleton development.

4.2 JAB1

The c-Jun protein must be phosphorylated at residues Ser63 and Ser73 to transactivate (61). The laboratory of Michael Karin thus utilized the N-terminal transactivation domain of c-Jun as a bait in the yeast two-hybrid system to screen for proteins interacting with c-Jun and involved in mediating c-Jun-dependent gene transcription. This strategy led to the identification, cloning and characterization of JAB1, an evolutionary conserved nuclear protein potentiating c-Jun and JunD activity (87). The interaction of JAB1 with c-Jun is not phosphorylation-dependent. Biochemical analysis provided insight on the molecular mechanisms through which JAB1 stimulates the transcriptional activity of specific Jun family members: JAB1 stabilizes the binding of c-Jun or JunD homodimeric complexes on their cognate AP-1 DNA binding sites while not affecting binding of either JunB or v-Jun (87). Thus JAB1 will contribute to the diversity and specificity of AP-1 responses which cannot be accounted for by the nearly identical DNA sequence-recognition properties of the Jun proteins.

JAB1 homologues were identified in *Caenorhabditis elegans* and *Sarcharomyces pombe* (*S. pombe*) by computer screen (87). Interestingly, the yeast homologue, *pad1+* , is necessary for target-gene activation by the *S. pombe* AP-1 transcription factor Pap1, a homologue of c-Jun (87). Transfection studies in wild-type and mutant yeast revealed that JAB1 can perform some, but not all, of the functions attributed to Pad1, demonstrating some degree of functional redundancy between the yeast and the mammalian genes (87).

A putative interaction between JAB1 and Fos family members was not tested. Similarly, the JAB1 target(s) within the basal transcriptional machinery remain unknown. Considering the importance of AP-1 factors for proper bone development, we examined whether JAB1 is

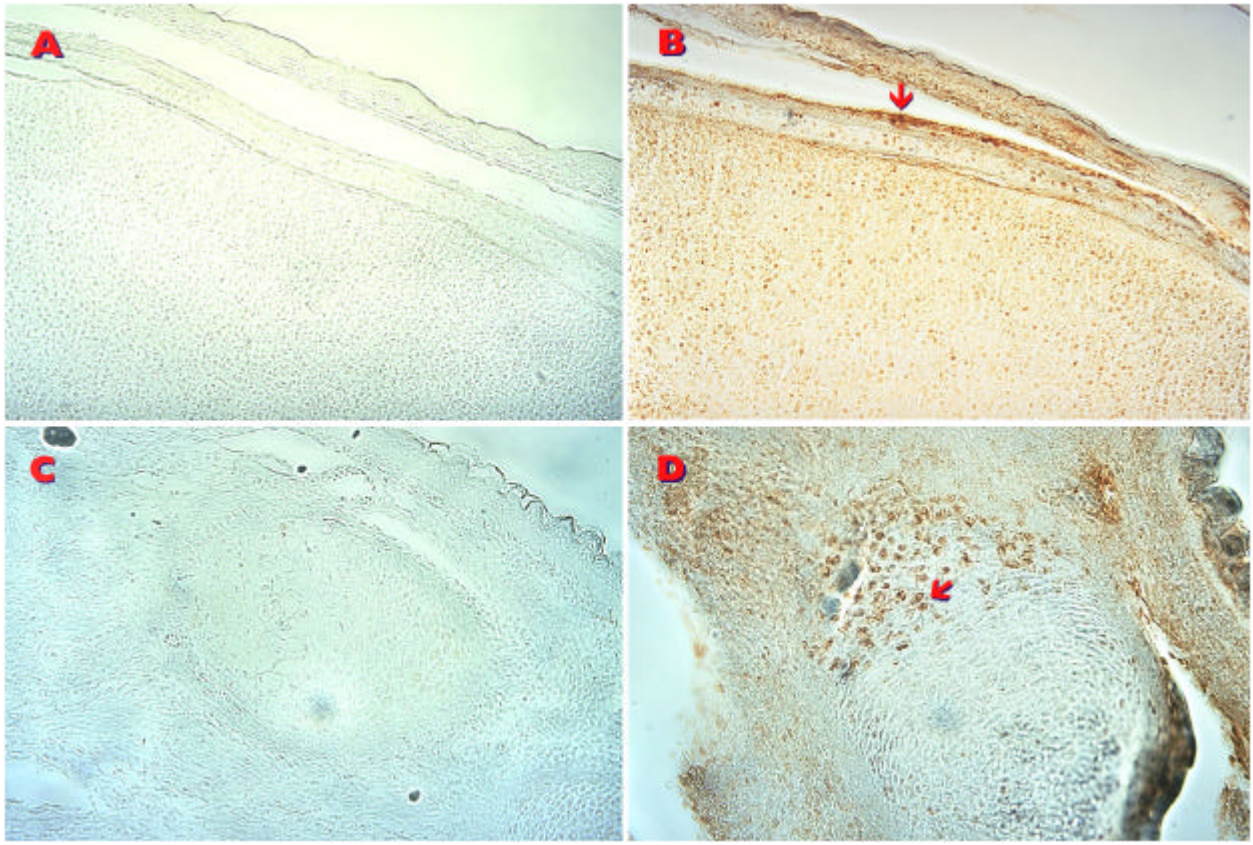

Figure 1. Immunohistochemical detection of CBP protein in developing mouse embryos. Embryos were collected at 17.5 days p.c., fixed in paraformaldehyde, and embedded in paraffin. Sagittal sections magnified 25 X (A, B, C) or 40 X (D) are presented. (A, C) control staining of head and limb, respectively. (B) Immunodetection with the anti-CBP antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Note the positive signal in nuclei from brain cells. The arrow points to osteoblasts lining the developing calvaria that express the CBP coactivator. (D) Staining of developing limb with the anti-CBP antibody. The arrow points to hypertrophic chondrocytes that show a positive CBP signal in their nucleus. Note the absence of signal in chondrocytes from the resting zone and the proliferative zone.

expressed in bone cells. We have used reverse-transcription coupled with polymerase chain reaction amplification (RT-PCR) to analyze JAB1 expression in mouse osteoblasts. figure 2 shows that JAB1 mRNA was detected in confluent osteoblastic MC3T3-E1 cells as well as in mRNA isolated from developing mouse calvaria (17.5 days p.c.). Thus JAB1 may represent an important regulator of gene transcription during skeletal development.

4.3 alpha-NAC

In recent years, we have used the technique of differential display of messenger RNA amplified by the polymerase chain reaction (differential display PCR) (88) to compare genes expressed in mineralizing osteoblasts with those expressed in de-differentiated, non-mineralizing cells of the same lineage. This has allowed us to identify, clone and characterize alpha-NAC, a gene expressed in differentiated osteoblasts during development (89, 90). NAC has been previously purified as a heterodimeric complex binding the newly synthesized polypeptide chains as they emerge from the ribosome (91). The beta-NAC subunit has been identified as BTF3b (91), a protein involved in regulating transcription in yeast (92, 93) and in higher eukaryotes (94). It should be noted that the initial model for the function of the NAC heterodimer proposed by Wiedmann et al. (91) has recently been shown to be incorrect (95). Additional work will be required to reconcile the alpha-NAC function that we have described (89, 90; 96) with previously published studies (91; 95).

Based on sequence similarities between alpha-NAC and transcriptional regulatory proteins and the identification of the heterodimerization partner of alpha-NAC as the transcription factor BTF3b (91), we investigated a putative role for alpha-NAC in transcriptional control. Additional work from our laboratory characterizing the function of the muscle-specific isoform of alpha-NAC, skNAC, as a sequence-specific DNA binding transcription factor and demonstrating that alpha-NAC has inherent DNA binding activity *in vitro* (96) further supported a role for alpha-NAC in the regulation of gene transcription.

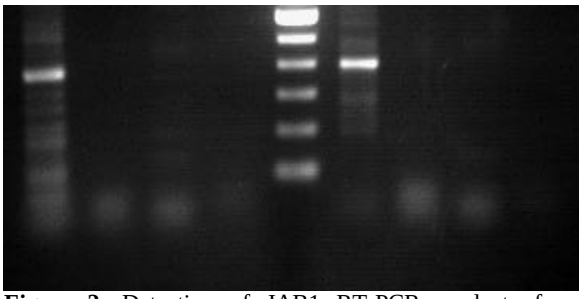

Figure 2. Detection of JAB1 RT-PCR products from MC3T3-E1 osteoblastic cells and developing mouse calvaria. RNA was isolated from both sources, reverse-transcribed, and amplified with primers derived from the human JAB1 sequence. Left-most lane shows the expected 473 bp amplimer from MC3T3-E1 osteoblasts. The center lane (lane 5) contains the 100 bp molecular size markers while lane 6 shows the amplified 473 bp fragment from reverse-transcribed RNA isolated from calvaria of 17.5 days p.c. mouse embryos. Additional lanes represent controls without template and with the forward and reverse primers alone, respectively. Forward primer: 5'-(C/T)TGGC(C/T)CTGCTGAAGATGG(G/C)T-3'; reverse primer: 5'-TC(A/G/C)A(C/T)CACCAC(A/G/T)GC(C/T)AC(A/G)(A/G)(A/C)(A/G/C/T)-3'.

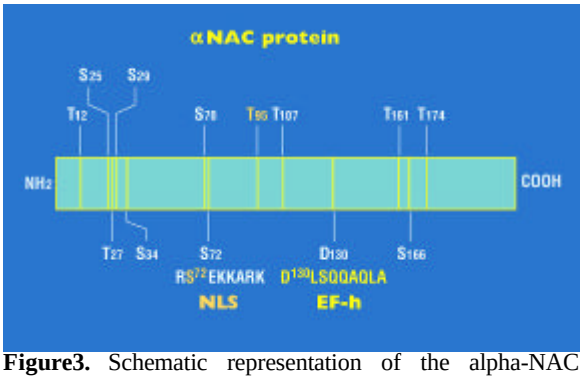

Figure3. Schematic representation of the alpha-NAC protein. Highlighted residues represent putative phosphorylation sites for casein kinase II (in yellow) or protein kinase C (in pink). Note Ser 70 and Ser 72 which flank the nuclear localization signal (NLS) and could contribute to the control of the subcellular localization of alpha-NAC. EF-h, calcium-binding EF-hand motif.

The alpha-NAC protein was shown to act as a transcriptional coactivator by potentiating the activity of the chimeric activator GAL4/VP-16 *in vivo*. Specific protein/protein interactions between alpha-NAC and the VP-16 moiety of GAL4/VP-16 were demonstrated using gel retardation, protein blotting assays, and pull-down protein assays, while interactions with TBP were detected using immunoprecipitation, affinity chromatography, and protein blotting assays (89).

Alpha-NAC was also shown to potentiate the activity of the homodimeric c-Jun activator while transcription mediated by the c-Fos/c-Jun heterodimer was unaffected (90). Accordingly, alpha-NAC specifically interacted with c-Jun in pull-down protein interaction assays. Using c-Jun deletion mutants, we have determined that alpha-NAC interacts with the N-terminus of the c-Jun protein. The interaction of alpha-NAC with c-Jun is not phosphorylation-dependent. The strength of the interaction between c-Jun and alpha-NAC was measured using affinity chromatography with a step gradient of salt. The binding of c-Jun to the alpha-NAC affinity resin was disrupted with 0.4 M salt, confirming that it represents a very strong interaction. Similar to JAB1, alpha-NAC stabilized the AP-1 complex formed by the c-Jun homodimer through an 8-fold reduction in the dissociation constant (k_d) of the complex. Furthermore, the high affinity of alpha-NAC for c-Jun and TBP (89, 90) is thought to stabilize the interaction of the c-Jun homodimer with the basal transcriptional machinery. These findings allowed us to propose the following model for the potentiation of c-Jun-mediated transcription by alpha-NAC: the rate of transcription from a c-Jun-dependent promoter is increased in the presence of the alpha-NAC coactivator through the stabilization of the c-Jun dimer on its binding site, and through the recruitment of the basal transcriptional machinery by alpha-NAC which stabilizes the interaction between c-Jun and TBP (90).

It is interesting to note the similarities between the mechanism of action of JAB-1 and alpha-NAC, despite the complete absence of sequence homology between them. Both proteins interact with the N-terminal portion of c-Jun and can bind the phosphorylated as well as the unphosphorylated forms of the transcription factor (87; 90). Moreover, the two coactivators appear to act by stabilizing the interaction of the c-Jun homodimer to its cognate DNA binding site (87; 90).

Immunocytochemical analysis revealed that the nuclear localization of alpha-NAC is cell cycle-dependent (89). The nuclear form of alpha-NAC is differentially phosphorylated (not shown) and computer modeling identified several putative serine and threonine phosphorylation sites (**f**igure 3). One of the serine residues overlaps the nuclear localization sequence in alpha-NAC. We hypothesize that differential phosphorylation of specific residues within the alpha-NAC sequence controls the subcellular localization of alpha-NAC. Defining the signaling events involved in the subcellular distribution of alpha-NAC will constitute an important area of future investigations.

We studied the pattern of expression of the alpha-NAC protein during mouse development. Alpha-NAC expression was undetectable prior to 14-days p.c. (90). Subsequently, alpha-NAC was specifically detected in the nucleus of differentiated osteoblasts in the primary ossification centers of ribs and long bones at 14.5-days p.c. (90). Postnatally, expression of the alpha-NAC protein was ubiquitous (96).

5. PERSPECTIVES

The essential role played by c-Fos in bone cell differentiation and function is well documented. The expression of Jun family members in bone cells coupled to the observation that c-Fos is an obligate heterodimer strongly suggest that Jun family members are involved in

the control of gene expression in bone cells. We surmise that the early embryonic lethality of c-jun-deficient mice (97) may prevent the analysis of the role of the c-Jun protein in bone. The powerful techniques allowing to generate tissue-specific gene inactivation (98) might prove useful to investigate this role.

A critical area for our understanding of the role of AP-1 factors in skeletal development remains the identification of the physiologically relevant AP-1-dependent genes in bone. While computer analysis of promoter regions may easily identify AP-1 response elements, it is not so easy to ascertain that these elements are functional in any given tissue. Molecular phenotyping of the cell types of bone utilizing random cDNA sequencing (99) may help to shed some light in the identification of relevant AP-1 target genes.

Finally, it is becoming evident that some aspects of the specificity of the AP-1 responses involve molecular networking through coactivator and co-integrator molecules. While we have determined that all coactivators interacting with AP-1 components are expressed in bone cells, only alpha-NAC appears to be tightly restricted to osteoblasts during embryogenesis. It will be interesting to analyze the putative involvement of these coactivators during bone cell differentiation. It is anticipated that animal models deficient for the function of these molecules, which are being engineered in various laboratories including our own, will help shed light on the specific roles played by these proteins during skeletal development.

6. ACKNOWLEDGEMENTS

The authors thank Ms. Josée Prud'homme for technical advice on immunohistochemistry, M. Edwin Wan for the synthesis of oligonucleotides, and M. Mark Lepik for the preparation of the figures. This work was supported by the Shriners of North America. R. St-A. is a Chercheur-Boursier from the Fonds de la Recherche en Santé du Québec.

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Key Words: Coactivator, Transcription, AP-1, Bone development

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