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Activating Transcription Factor 2 Targets c-Fos, but not c-Jun, in Growth Plate Chondrocytes

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ABSTRACT

Activating transcription factor 2 (ATF-2), c-Fos, and c-Jun belong to the bZIP family of transcription factors. Promoters of c-Fos, c-Jun, cyclin D1, and cyclin A are targets of ATF-2 in primary mouse chondrocytes. An ATF-2 expression vector was co-transfected with either c-Fos or c-Jun promoters in mutant ATF-2 chondrocytes in order to show by luciferase assay that ATF-2 increased promoter activity of c-Fos, but not c-Jun. Chromatin immunoprecipitation (ChIP) assays revealed that ATF-2 bound with the c-Fos promoter at the −294 cyclic AMP response element (CRE) site, but did not bind to the TPA responsive element (TRE) or activating protein-1 (AP1) sites of the c-Jun promoter. Dominant-negative (dn) c-Fos inhibited cyclin D1 promoter activity. However, dn c-Jun had minimal effect on this same promoter activity. c-Fos was capable of interactions with both the cyclin D1 CRE and AP1 sites, while c-Jun co-operated specifically with the cyclin D1 CRE site. Neither c-Fos nor c-Jun had any effect on cyclin A promoter activity. c-Fos was unable to bind to the cyclin A AP1 or CRE sites. In contrast c-Jun was competent in interactions with cyclin A AP1-2 as well as the CRE. J. Cell. Biochem. 112: 211–216, 2011. © 2010 Wiley-Liss, Inc.

KEY WORDS: ACTIVATING TRANSCRIPTION FACTOR 2 (ATF-2); bZIP FAMILY; c-FOS; c-JUN; CHROMATIN IMMUNOPRECIPITATION (ChIP); CYCLIC AMP RESPONSE ELEMENT (CRE); TPA RESPONSE ELEMENT (TRE)

C-Fos and c-Jun are components of the dimeric transcription factor AP1, and have crucial regulatory roles in a variety of biological processes. These include aspects of embryonic development and differentiation, from organogenesis to bone growth as well as cell proliferation [Glover and Harrison, 1995] and tumor development [Jochum, 2001]. c-Fos proteins (c-Fos, FosB, Fra-1, and Fra-2) are limited to the formation of heterodimers with Jun family members. In contrast, c-Jun displays more promiscuous interactions is capable of forming both homodimers and heterodimers. The latter may involve associations with both activating transcription factor 2 (ATF-2), and CREB proteins as well as c-Fos [Benkoussa et al., 2002].

c-Fos and c-Jun are characterized by a common DNA binding region that consists of a bZIP domain and a leucine zipper region. Homodimers of c-Jun, as well as heterodimers of c-Fos and c-Jun, have the capacity to interact with the AP1 consensus sequence [$T^{G}{}_{T}A^{C}{}_{G}$TCA], although Jun homodimers are less likely to bind DNA efficiently. In contrast, c-Jun/ATF-2 heterodimers, as well as ATF-2 homodimers, have been demonstrated to have a binding affinity for the consensus ATF-2/CREB sequence(s) [$T^{G}{}_{T}$ACGTCA]; [Hai and Curran, 1991] and/or [TGACGT$^{CG}_{AA}$] [Hai et al., 1989]. Jun proteins as either homo- or heterodimers modulate the differentiation and proliferation of chondrocytes at least in part by controlling the expression of key cell cycle regulators. The interaction between ATF-2 and the cyclic AMP response element (CRE) in regulatory regions of the genes for cyclin D1 and cyclin A are capable of promoting a significant enhancement of cyclin expression in growth plate chondrocytes [Beier et al., 1999, 2000]. c-Fos and c-Jun are also capable of acting either directly or indirectly on the expression of cyclins D1 and A. Cyclin D1 promoter activity is dependent upon both c-Fos and FosB to support cyclin D expression which in turn is required for progression into S-phase [Brown et al., 1998]. Cyclin A plays roles in both the control of S-phase entry and mitosic progression and its promoter is a target for c-Jun [Henglein et al., 1994]. In turn, c-Jun has the capacity to bind with c-Fos, FosB, Fra-1 and 2, as well as ATF members that include ATF-1, -2, -3, -4, CREB, and CREM [Katabami et al., 2005]. c-Jun is able to transform cells in conjunction with Ras [Johnson et al., 1996] and is required for transformation induced by c-Fos, Raf, c-Myc, Mos, and Abl [Rapp et al., 1994].

JunB targets the cyclin A gene to promote cell proliferation [Andrecht et al., 2002]. The importance of the role of AP1 in the cell cycle then becomes twofold. JunB represses the cyclin D1 promoter, while c-Jun promotes its activation. The implication is that an increase in cyclin D1 promoter activity provides the motivation for G1 progression via a temporal increase in transcription of cyclin D1 [Brown et al., 1998]. Clearly these networks of transcription factors have an essential function in modulating cell proliferation by

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controlling the expression of key cell cycle regulators. Skeletal development and bone growth are exquisitely sensitive to the proliferation status of chondrocytes. In this paper we explore how the activities of c-Fos, c-Jun, and their ancillary molecules are tailored to meet the proliferative needs of growth plate chondrocytes.

METHODS

CELL CULTURE AND TRANSFECTION

Primary mouse chondrocytes were isolated and cultured for 3 days. The chondrocyte clusters were digested with collagenase D, followed by cells plated in 24-well plates at 5×10^4 cells/well. Subconfluence was achieved within 12 h. Transfections were performed with Effectene (Qiagen), according to the manufacturer's protocol. Briefly, each well was transfected with 0.2 μg of the reporter gene construct and 0.02 μg of pRLSV40 (Promega) as a control for transfection efficiency with 1.76 μl Enhancer and 2.2 μl Effectene. Co-transfections with dominant-negative (dn) expression plasmids for ATF-2, c-Fos, and c-Jun were carried out with 0.06 μg for each expression plasmid, while controls were carried out with 0.06 μg for each empty vector. After a 24 h transfection, cells were serum-starved for 3 days and then stimulated with medium containing 10% FBS for 12 h.

LUCIFERASE ASSAYS

Luciferase assays were performed with the dual luciferase assay kit (Promega) according to the manufacturer's instructions. Lysate (10 μl) was assayed first for Firefly luciferase and then for *Renilla* luciferase activity. Firefly luciferase activity was normalized to Renilla luciferase activity.

WESTERN BLOTS

Primary mouse chondrocytes were plated at sub-confluent density. Attachment was achieved after an overnight incubation. Cells were then serum-starved for 3 days, followed by a 12-h stimulation with medium containing 10% FBS. Isolation of protein was achieved by lysing cells in SDS sample buffer. Total protein from 2 to 5×10^5 cells was resolved by SDS/PAGE and transferred to Hybond C membranes (Amersham). Antibodies against c-Fos and c-Jun were purchased from Santa Cruz Biotechnology. Reactive proteins were detected with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and visualized with an enhanced chemiluminescence detection kit (ECL, Amersham). Densitometric measurement of signals was performed with CAMSCAM software and an Astra 1220S scanner.

CHIP ASSAY

Primary mouse chondrocytes were plated at subconfluent density, subjected to serum stimulation, cross-linked with 1% formaldehyde for 20 min at room temperature, and quenched for 5 min with glycine (125 mM). Cross-linked cells were then washed twice with cold PBS, followed by collection of cells in cold swelling buffer (5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% NP-40). The cells were briefly pelleted and then resuspended in SDS lysis buffer. Chromatin in the resulting lysate was sheared to an average size of 500 bp via sonication twice on ice for 2 min at output 2 and duty circle 40 with a Branson (model 102) sonicator. Cellular debris was removed by centrifugation at 14,000*g* for 5 min. Chromatin integrity and yield was monitored by agarose electrophoresis. Immunoprecipitation was undertaken with 2 μg of the appropriate antibody and 10 μl protein A sepharose beads incubated overnight at 4°C with constant rotation. Immune complexes were washed five times with low salt (0.1% SDS, 1% Triton X-100, 2m M EDTA, 200 mM Tris–HCl, pH 8.1, 150 mM NaCl); followed by a high salt wash (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 200 mM Tris–HCl, pH 8.1), and finally a 0.25 M LiCl wash that included 1% NP-40, 1% sodium desoxycholate, 1mM EDTA, 100 mM Tris–HCl, pH 8.1 and 2× TE buffers. Protein was then digested by overnight incubation with 20 μg/ml Proteinase K at 65°C. DNA was isolated by standard phenol extraction and used for PCR.

PCR

Genomic DNA was isolated from the distal 2 mm segments of mice tails, followed by genotyping via polymerase chain reaction (PCR). There were three CRE sites in the transcriptional control region of the c-Fos promoter: −342 CRE (CCCGTCA), −294 CRE (TGCGTCA), and −67 CRE (CTGACGTAG). There was also a JUN1 (AP1) site (TGACATCAT) in addition to a JUN2 (TRE) site (TTACCTCA) in the transcriptional control region of the c-Jun promoter. An AP1 site (TGTCTCA) as well as a CRE site (TAACGTCA) in the transcriptional control region of the cyclin D1 promoter were also evident. Two AP1 sites that included an AP1-1 (TAAGTCAC) and an AP1-2 (TCATTCAG), as well as one CRE (TGACGTCA) in the transcriptional control region of the cyclin A promoter were also present. The PCR primers that identify the different binding sites are described in Table I. The conditions for PCR were: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, followed by a 72°C amplification for 1 min, at 40 cycles.

TABLE I. Binding Sites in Transcription Control Regions, and Primers for PCR and CHIP

c-Fos	−67CRE	Forward 5′-CCCCCTAAGATCCCAAATGT-3′
	CTGACGTAG	Reverse 5′-GTCGCGGTTGGAGTAGTAGG-3′
	−294CRE	Forward 5′-TCCCTCCCTCCTTTACACAG
	TGCGTCA	Reverse 5′-CCCGTCTTGGCATACATCTT-3
	−342CRE	Forward 5′-GGGTCCACATTGAATCAGGT-3
	CCCGTCA	Reverse 5′-TGTAAAGGAGGGAGGGATTG-3′
c-Jun	Jun1 (AP1)	Forward 5′-GGGTGACATCATGGGCTATT-3′
	TGACATCAT	Reverse 5′-AAGTCCGTCCGTCTGTCTGT-3′
	Jun2 (TRE)	Forward 5′-GAACACAAGCCGAAGCTGAG-3′
	TTACCTCA	Reverse 5′-ATTGGCTTGCGTCGTTCTC-3′
Cyclin D1	AP1	Forward 5′-GTGGTCTGGTTCCTGGAAGG-3′
	TGTCTCA	Reverse 5′-CAGTATCCCCCTCCTCCACT-3′
	CRE	Forward 5′-CCGGCTTTGATCTCTGCTTA-3′
	TAACGTCA	Reverse 5′-GCTGTACTGCCGGTCTCC-3′
Cyclin A	AP1-1	Forward 5′-GACACGCCTTTAATCCCAGA-3′
	TAAGTCAC	Reverse 5′-TGCCATACAACACAGGGAAA-3′
	AP1-2	Forward 5′-CAGCGTTTCCCTGTGTTGTA-3′
	TCATTCAG	Reverse 5′-GCCTCAGCTAAGGGGTGAAG-3′
	CRE	Forward 5′-GATCCACTGAGCAGCAGAGA-3′
	TGACGTCA	Reverse 5′-AAGTAGCCCGCGACTATTGA-3′

RESULTS

ATF-2 TARGETS c-Fos BUT NOT c-Jun

We analyzed the effects of ATF-2 in c-Fos and c-Jun promoter activities in ATF-2 m/m primary mouse chondrocytes. Cells were transiently transfected with either c-Fos or c-Jun promoters as well as the ATF-2 expression vector for 24 h, followed by serum starvation for 3 days. Cells were then stimulated with fresh medium containing 10% FBS for 16 h. The promoter activities were determined by Firefly luciferase activity standardized to Renilla activity. Figure 1 revealed that over-expression of ATF-2 was approximately doubled compared to the c-Fos promoter activity in ATF-2 m/m cells. However, over-expression of ATF-2 had no effect on c-Jun promoter activity. These data imply that although c-Fos requires ATF-2, c-Jun promoter activity does not require this transcription factor.

CYCLIN D1 ACTIVITY REQUIRES c-Fos AND c-Jun, BUT CYCLIN A IS INDEPENDENT OF AP1

ATF-2-wt primary mouse chondrocytes were transiently transfected with cyclin D1 or cyclin A promoters, as well as the expression vector for dn c-Fos and/or dn c-Jun. After a 24 h transfection, cells were serum-starved for 3 days, followed by stimulation with fresh medium containing 10% FBS. The promoter activities were determined by Firefly luciferase activity, standardized to Renilla activity. Cyclin A promoter was co-transfected with dn c-Fos, dn c-Jun, or both in order to demonstrate that neither dn c-Fos nor dn c-Jun inhibited the activity of the cyclin A promoter. Figure 2 demonstrated that neither dn c-Fos nor dn c-Jun inhibited cyclin A promoter activity, thus implying that c-Fos and/or c-Jun have the capacity to affect cyclin A promoter activity. In addition, dn c-Fos and dn c-Jun significantly inhibited the cyclin D1 promoter activity but had no effect on that of cyclin A, thus specifying that the transcription factors c-Fos and c-Jun are required for promoter activity of cyclin D1, but not that of cyclin A. Figure 3, in which the cyclin D1 promoter was co-transfected with dn c-Fos, dn c-Jun, or both, demonstrated that these dn transcription factors inhibited cyclin D1 promoter activity considerably, thus implying that both c-Fos and c-Jun affect cyclin D1 promoter activity. Figure 4 defined the relationship between cyclin D1 and cyclin A, in that cyclin D1 is required for cyclin A promoter activity.

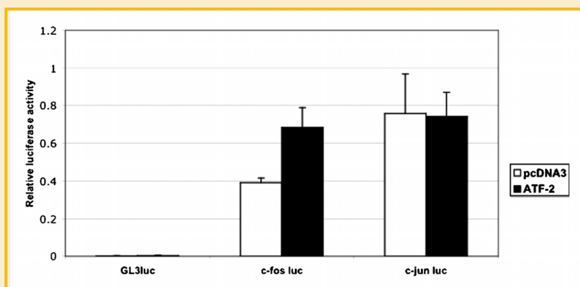

Fig. 1. c-Fos promoter activity requires ATF-2 in primary cultured chondrocytes, but c-Jun promoter activity is not necessary. Cells were transiently transfected with control promoter (GL3luc), c-fos, or c-jun promoter with the expression vector ATF-2 or control expression vector (pcDNA3). After a 24 h transfection, cells were serum-starved for 3 days. Cells were then stimulated with fresh medium containing 10% FBS for 16 h. Promoter activities were determined by Firefly luciferase activity standardized to Renilla activity.

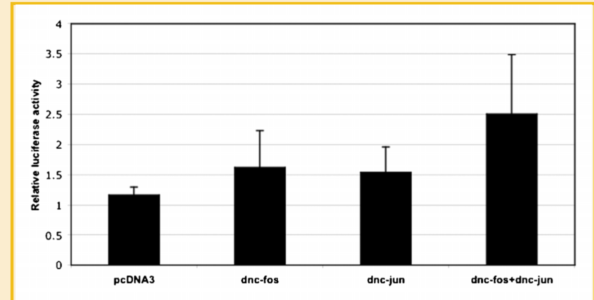

Fig. 2. Cyclin A activity does not require c-Fos or c-Jun, but neither dn Fos nor dn c-Jun are inhibited. Cells were transiently transfected with control promoter (GL3luc), c-fos, or c-jun promoter with the expression vector ATF-2 or control expression vector (pcDNA3). After a 24 h transfection, cells were serum-starved for 3 days. Cells were then stimulated with fresh medium containing 10% FBS for 16 h. Promoter activities were determined by Firefly luciferase activity standardized to Renilla activity.

ATF-2 BINDS TO THE CRE IN THE c-Fos PROMOTER BUT DOES NOT BIND TO THAT OF c-Jun

The c-Fos promoter has three CREs (−342CRE, −294CRE, and −67CRE), while the c-Jun promoter has one CRE (Jun2) and one AP1 (Jun1). We determined to establish whether ATF-2 physically interacted with the c-Fos and c-Jun promoters at those specific binding sites. We employed the technique of chromatin immunoprecipitation (ChIP) as shown in Figure 5. We amplified 155, 181, and 218 bp fragments of the c-Fos promoter that included the −342CRE, −294CRE, and −67CRE as well as a 200 bp coding fragment of ATF-2 as a control. Immunoprecipitation with antibody against ATF-2 revealed a visible c-Fos promoter fragment containing the −294CRE, corresponding with input. Our controls (no antibody samples) for the c-Fos and c-Jun promoters, unrelated

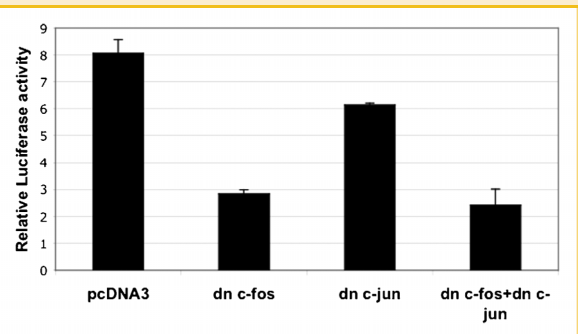

Fig. 3. Cyclin D1 promoter activity requires c-Fos and c-Jun. However, cyclin A acts independently in mouse chondrocytes. Cells were transiently transfected with control promoter (GL3luc), c-Fos, or c-Jun promoter with the expression vector ATF-2 or control expression vector (pcDNA3). After a 24 h transfection, cells were serum-starved for 3 days. Cells were then stimulated with fresh medium containing 10% FBS for 16 h. Promoter activities were determined by Firefly luciferase activity standardized to Renilla activity.

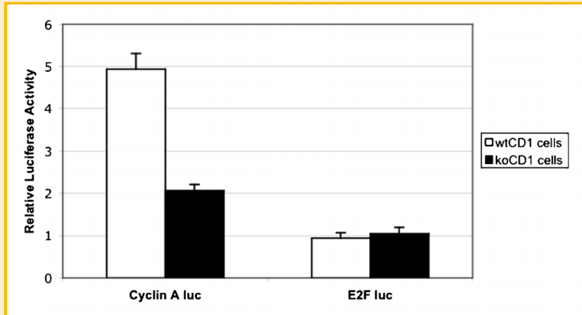

Fig. 4. Cyclin D1 is required for the promoter activity of cyclin A. The promoter was co-transfected with dn c-Fos, dn c-Jun, or both. Neither dn c-Fos nor dn c-Jun inhibited promoter activity of the cyclin A promoter. Therefore, c-Fos, c-Jun, or both are capable of promoting cyclin A activity. Results indicated that dn c-Fos, and/or dn c-Jun, significantly inhibited cyclin D1 promoter activity, thus implying that both c-Fos and/or c-Jun have considerable effects on cyclin D1 promoter activity.

DNA, and the coding fragment of ATF-2 were not visible in the absence of antibody against ATF-2. We repeated these experiments using the same input and antibody against ATF-2 under the same conditions, using primers for the c-Jun promoter instead of that of c-Fos. We amplified 150- and 180 bp fragments of the c-Jun promoter, which included c-Jun1 and c-Jun2. We determined that the transcription factor ATF-2 did not interact physically with c-Jun1 and c-Jun2 within the c-Jun promoter. However, ChIP assays demonstrated that ATF-2 bound to c-Fos at −294CRE, but did not bind to c-Jun, thus supporting our data that ATF-2 is required for c-Fos promoter activity. It is not essential for that of c-Jun. In brief: c-Fos and c-Jun target cyclin D1; c-Jun binds to cyclin A but has no effect on its promoter activity; c-Fos binds to the cyclin D1 CRE and AP1 site, but does not bind to cyclin A; c-Jun binds to cyclin D1 and cyclin A.

Fig. 5. ATF-2 binds to the −294 CRE in the c-Fos promoter. However, ATF-2 does not bind to c-Jun.

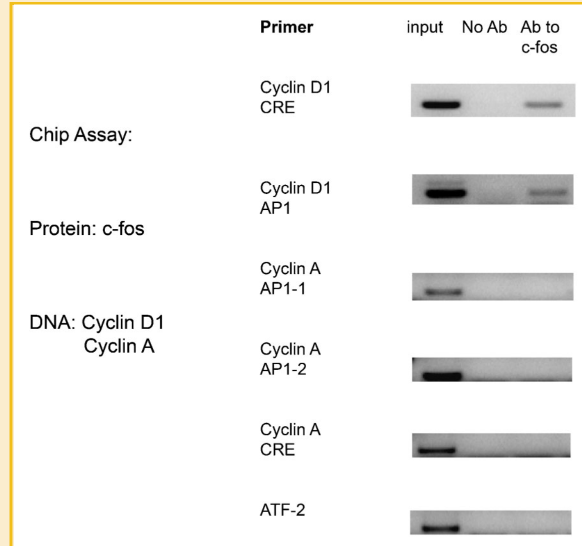

Fig. 6. c-Fos binds to the cyclin D1 CRE and AP1 sites. However, c-Fos does not bind to Cyclin A.

THE CYCLIN D1 PROMOTER HAS ONE CRE AND ONE AP1 SITE, WHILE THE CYCLIN A PROMOTER HAS ONE CRE AND TWO AP1 SITES

We determined to establish if c-Fos and/or c-Jun physically interacted with the cyclin D1 and cyclin A promoters at those binding sites. We again employed ChIP. We amplified 150 and 155 bp fragments of the cyclin D1 promoter that included the CRE and AP1, as well as 186, 178, and 150 bp fragments of the cyclin A promoter that included the CRE, AP1-1, and AP1-2. A 200 bp coding fragment of ATF-2 was used as a control. Immunoprecipitation with antibody against c-Fos revealed two visible cyclin D1 promoter fragments containing the CRE and AP1, corresponding with the same as the input. However, there was no visible cyclin A promoter fragment. Our controls (no antibody samples) for the cyclin D1 and cyclin A promoter, unrelated DNA, and the coding fragment of ATF-2, were not visible in the absence of antibody against ATF-2 (Fig. 6). We repeated these experiments using the same input and antibody against c-Jun under the same conditions, and determined that c-Jun interacted physically with the CRE within the cyclin D1 promoter, as well as with the CRE and AP1-2 of the cyclin A promoter (Fig. 7). Our data demonstrated that c-Fos interacted with cyclin D1 promoter at the CRE and AP1 sites, but did not bind to cyclin A. These data are consistent with results indicating that c-Fos is required for cyclin D1 promoter activity, but does not affect the activity of cyclin A. As expected, c-Jun has a positive effect on cyclin D1 promoter activity.

DISCUSSION

Skeletal development and bone growth are defined to large a extent by the activities of chondrocytes as well as osteoblasts and osteoclasts. The growth of long bones in particular is exquisitely sensitive to the proliferative versus the hypertrophic status of

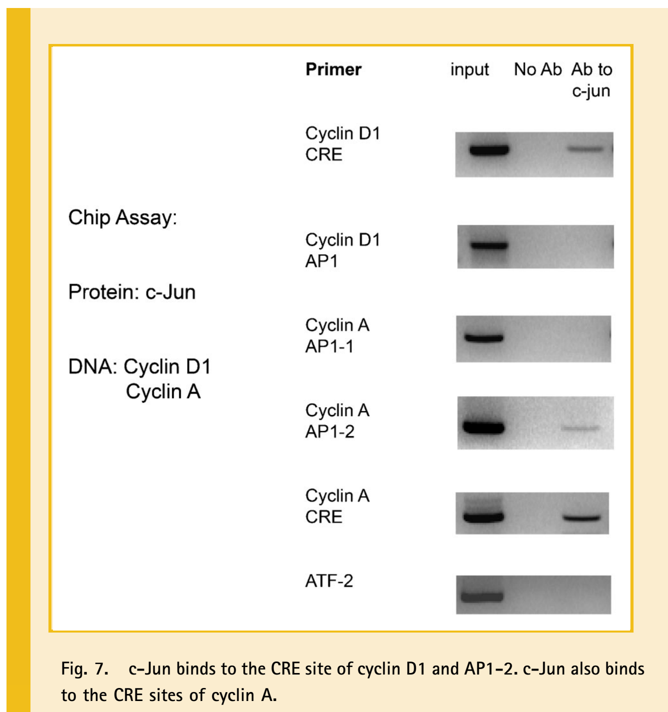

Fig. 7. c-Jun binds to the CRE site of cyclin D1 and AP1-2. c-Jun also binds to the CRE sites of cyclin A.

chondrocytes within the growth plate. In this paper we have begun to unravel networks of transcription factors and cell cycle regulators that have central role in the control of chondrocyte proliferation.

We have been able to demonstrate that ATF-2 controls chondrocyte cell cycle progression by stimulating the expression of the genes encoding c-Fos and c-Jun. We have also established that c-Fos and c-Jun in their turn act either directly or indirectly on the expression of cyclins D1 and A. In addition to these effects via c-Fos/c-Jun, however, ATF-2 has been reported to directly modulate expression of cyclin D1 in chondrocytes. Taken together, it is clear that there is a hierarchy of transcription factors that regulate cyclin expression in chondrocytes. This role for ATF-2 as both a direct and indirect regulator of cyclin D expression is consistent with the finding that the level of cyclin D1 is substantially reduced in chondrocytes from mice that are deficient in ATF-2. Whether the same transcriptional networks controlling cell cycle regulators are employed to modulate cell proliferation in other cell types has yet to be determined. It would seem likely that different parallel regulatory pathways might be emphasized to a greater or lesser extent depending upon the cell and tissue type. Certainly bone development and bone pathologies such as osteopenia and osteosarcoma, seem particularly sensitive to AP1 and ATF-2 levels.

Osteoblasts (derived from mesenchymal progenitors) generate bone via extracellular matrix and cartilage templates, which then undergoes mineralization. Osteoclasts (from the monocyte/macrophage lineage) in contrast reduce bone mass by resorbing mineralized extracellular matrix. The interplay between osteoblasts and osteoclasts thus plays an essential role in bone growth and remodeling. Both c-Fos and Fra-1 are present in these in these cell types.

A role for c-Fos in bone growth emerged from studies by Wagner and co-workers [reviewed by Wagner, 2002] who generated transgenic mice that expressed the gene for c-Fos under the control of the human metallothionein promoter [Ruther et al., 1987]. Deregulation of c-Fos expression obstructed normal bone development in the absence of induction of malignant tumors [Ruther et al., 1987] but had no effects on any other organs, suggesting that c-Fos is required for bone development. Sunters et al. [1998] later suggested that cyclin D1 might be a target for c-Fos. Indeed induction of c-Fos expression in transgenic mice led to a dramatic increase in cyclin D1 expression in chondrocytes and osteoblasts. Furthermore prolonged activation of c-Fos resulted the development of osteosarcomas in which the levels of cyclins D1 and E, as well as CDKs 2, 4, and 6 were elevated. Based on these observations Sunters et al. [1998] suggested that c-Fos overexpression in osteoblasts promoted accelerated S-phase, due to cyclin A/E-CDK activity. This notion was confirmed in later studies [Sunters et al., 2004]. Consistent with this effect on proliferation, the differentiation of osteoblasts is seemingly insensitive to disruption of the c-Fos gene by homologous recombination in mice [Wang, 1992].

While the data that we have described here makes a mechanistic link between ATF-2, the AP1 family and cyclins A and D, and the regulation of chondrocyte proliferation, there is no question that Fos family members have additional roles in bone homeostasis. Recent studies on the Fos-related protein Fra-2 suggests that Fra2/AP1 has an essential role as a regulator of bone development and maintenance [Bozec et al., 2010; Wagner, 2010]. Mice deficient in Fra-2 are deficient in chondrocytes and osteoclasts. Furthermore, osteoblasts from these mice display differentiation defects both in vivo and in vitro. This is likely related to the findings that the promoters of the osteoblast-specific osteocalcin and collagen 1 $\alpha 2$ genes are both targets of Fra-2. Evidently Fra-2/AP1 functions as a positive regulator bone growth and extracellular matrix deposition.

It is clear that c-Fos and Fra-2 are important regulators of bone cell differentiation, while c-Jun (as well as JunB and Fra-1) is essential in both embryonic and post-natal development. In the absence of c-Fos, mice are viable, but are devoid of osteoclasts, resulting in an osteopetrotic phenotype [Sunters et al., 2004]. In mice deficient in c-Jun, however, embryonic development ceases between embryonic day 12 and 13, due to heart defects that include incomplete separation of the aorta and pulmonary artery. This implies that c-Jun is crucial for the development of normal cardiac outflow [Eferl et al., 1999]. Certain Fos and Jun proteins can be dispensable (e.g., c-Fos, JunD, and FosB), but embryonically expressed AP1 proteins such as c-Jun, JunB, and Fra-1 are all essential.

Taken together, the work from several laboratories demonstrates that the AP1 family of transcription factors operates at multiple levels in bone development and growth. The data that we have presented here now indicates that ATF-2 and AP1 proteins function in a hierarchical regulatory network that controls chondrocyte proliferation by modulating cyclin D and cyclin A expression. In this way both ATF-2 and AP1 are able to modulate chondrogenesis, a key early process in bone formation.

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