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Activating Transcription Factor 2 Controls Bcl-2 Promoter Activity in Growth Plate Chondrocytes

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Abstract Activating transcription factor 2 (ATF-2) is expressed ubiquitously in mammals. Mice deficient in ATF-2 (ATF-2 m/m) are slightly smaller than their normal littermates at birth. Approximately 50% of mice born mutant in both alleles die within the first month. Those that survive develop a hypochondroplasia-like dwarfism, characterized by shortened growth plates and kyphosis. Expression of ATF-2 within the growth plate is limited to the resting and proliferating zones. We have previously shown that ATF-2 targets the cyclic AMP response element (CRE) in the promoters of cyclin A and cyclin D1 in growth plate chondrocytes to activate their expression. Here, we demonstrate that Bcl-2, a cell death inhibitor that regulates apoptosis, is expressed within the growth plate in proliferative and prehypertrophic chondrocytes. However, Bcl-2 expression declines in hypertrophic chondrocytes. The *Bcl-2* promoter contains a CRE at −1,552 bp upstream of the translation start. Mutations within this CRE cause reduced Bcl-2 promoter activity. We show here that the absence of ATF-2 in growth plate chondrocytes corresponds to a decline in *Bcl-2* promoter activity, as well as a reduction in Bcl-2 protein levels. In addition, we show that ATF-2 as well as CREB, a transcription factor that can heterodimerize with ATF-2, bind to the CRE within the Bcl-2 promoter. These data identify the Bcl-2 gene as a novel target of ATF-2 and CREB in growth plate chondrocytes. J. Cell. Biochem. 101: 477–487, 2007.

Key words: ATF-2; chondrocytes; Bcl-2; promoter activity; ChIP; luciferase reporter assays; cyclic AMP response element (CRE)

Endochondral bone formation is initiated by mesenchymal cell condensations that subsequently undergo chondrogenesis, resulting from the actions of multiple regulatory proteins. These include transforming growth factor β (TGF-β), parathyroid hormone-related peptide (PTHrP), bone morphogenetic proteins (BMPs), and hedgehog family proteins [Cancedda et al., 1995; Lefebvre, 2001], as well as the transcription factors L-Sox5, Sox6, Sox9 [Lefebvre et al., 1998; Bi et al., 1999; Smits et al., 2001], and Cbfa1 [Ducy et al., 1997; Komori et al., 1997]. Chondroblasts secrete a cartilage matrix that consists primarily of type II collagen and aggrecan, as well as other extracellular matrix proteins. Chondroblasts, stimulated by growth hormone and insulin-like growth factor 1 (IGF-1) [Nilsson et al., 1994], proliferate in a clonal manner to support longitudinal growth. Proliferation is maintained in part by the actions of the Indian Hedgehog (Ihh) pathway, in conjunction with secretion of PTHrP from the resting zone into the growth plate [Lanske et al., 1996; Vortkamp et al., 1996]. Expression of PTHrP diminishes as it moves through the proliferating zone of the growth plate, resulting in chondroblast cell-cycle exit, when PTHrP is no longer present. The ensuing chondrocytes (no longer engaged in the cell cycle) undergo hypertrophy, eventually achieving a substantial increase in volume as well as cell diameter [Cancedda et al., 1995]. The large increase in cell diameter resulting from hypertrophy

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contributes significantly to the length of the growing bone. The hypertrophic chondrocytes secrete a hexagonal lattice-like type X collagen that surrounds the hypertrophic chondrocytes and migrates into the extracellular space [Jacenko et al., 1991; Kwan et al., 1991]. Mineralization of the cartilage matrix and vascular invasion induced by secretion of vascular endothelial growth factor (VEGF) from hypertrophic chondrocytes occur within the late hypertrophic zone, the latter introducing both marrow and osteoblast precursors [Mundlos and Olsen, 1997a,b]. Osteoblasts secrete bone matrix and proteases that overlay the mineralized cartilage and degrade the cartilage matrix, respectively. At the same time, the oldest hypertrophic chondrocytes undergo apoptosis, leaving behind bony trabeculae that contribute to the microenvironment of the marrow [Cancedda et al., 1995].

Activating transcription factor-2 (ATF-2) is a member of the ATF/CREB (cAMP-response element-binding protein) transcription factor family, and is characterized by a b-zip domain made up of basic amino acids and a leucine zipper region that acts as a DNA-binding region. Transcription factors in this family dimerize with themselves or with other family members by means of the leucine zipper. Homo- or heterodimers bind to the cyclic AMP response element (CRE), found in many gene promoters [Hai and Curran, 1991]. ATF-2 phosphorylation controls its own histone acetyltransferase activity as well as CRE-dependent transcription [Kawasaki et al., 2000]. ATF-2 targets the CRE in many known genes, including (but not limited to) *c-jun* [van Dam et al., 1995], *c-fos* [Fisch et al., 1989], and *Retinoblastoma* [Park et al., 1994]. Other genes, such as *osteopontin* [Craig and Denhardt, 1991], *osteocalcin* [Schule et al., 1990], *alkaline phosphatase* [Matsuura et al., 1990], and *fibronectin* [Bowlus et al., 1991] harbor CRE motifs within their respective genes, but we have not tested their ability to bind to ATF-2. Our own data have shown that the genes for *cyclin D1* [Beier et al., 1999, 2001] and *cyclin A* [Beier et al., 2000] are also targets of ATF-2 in growth plate chondrocytes. ATF-2 expression in the growth plate is limited to the resting and proliferation zones. Mice that are deficient in ATF-2 in the skeletal system (ATF-2 m/m mice) display a hypochondroplasia-like dwarfism that is manifested by shortened growth plates [Reimold et al., 1996; Maekawa et al., 1999]. Protein levels of both cyclin D1 and cyclin A are reduced by approximately 70–80% in growth plate chondrocytes of ATF-2 m/m mice [Beier et al., 1999, 2000]. These mice also lack ATF-2 in the brain, displaying enlarged ventricles, reduced brain size, and cerebellar defects [Reimold et al., 1996]. The ATF-2 m/m (deficient) mice are hypomorphs, that is, they express a mutant gene that displays only a partial reduction in the activity it influences (e.g., skeletal growth). These mice are not to be confused with the ATF-2 null mice that die at birth from meconium aspiration syndrome, a common human neonatal problem that portrays symptoms of severe respiratory distress [Maekawa et al., 1999].

The Bcl-2 family controls apoptosis, and is divided into two subgroups that comprise, (1) the anti-apoptotic proteins Bcl-2, Bcl-xL, Bcl-w, and Mcl-1 and (2) the pro-apoptotic proteins that include Bax, Bak, Bok, Bik, Blk [Merry and Korsmeyer, 1997]. Pro- and anti-apoptotic family members have the capacity to heterodimerize, and their relative concentrations may play a key role in the control the apoptosis program [Oltvai et al., 1993]. Bcl-2 functions as an anti-apoptotic factor, via the inhibition of caspase-9 activation [Adams and Cory, 1998] and possibly the restraint of cytochrome C release from the mitochondria [Murphy et al., 2000; Mikhailov et al., 2001]. In addition, Bcl-2 promotes both the exit of proliferating cells from the cell cycle and the restraint of quiescent cells into cell cycle [Gil-Gomez et al., 1998]. The ability of Bcl-2 to suppress cell-cycle progression can be inhibited by a deletion in the nonconserved loop or mutation of tyrosine-28 [Huang et al., 1997]. These related functions of Bcl-2 are distinct from its more prominent role of inhibiting apoptosis.

Bcl-2 expression is subject to both developmental and tissue-specific control. It is expressed in chondrocytes throughout the growth plate, predominantly in the late proliferative and prehypertrophic regions, and at diminishing levels in the late hypertrophic region [Amling et al., 1997]. Experimental data regarding the role of phosphorylation of Bcl-2 are conflicting. Results from Ito et al. [1997] suggest that phosphorylation supports suppression of cell death, while experiments from other investigators [Ling et al., 1998] support an association of Bcl-2 phosphorylation with mitosis, particularly M-phase arrest.

The Bcl-2 regulatory region (approximately 3.9 kb) contains two promoters. P1 is the more active promoter, spanning −3,924 to −1,286 bp and including the CRE at −1,552, while P2 is farther downstream and includes the TATA box [Heckman et al., 2002; Duan et al., 2005]. We show herein that Bcl-2 promoter activity is curtailed in growth plate chondrocytes when either the CRE is mutated or when ATF-2 activity is absent. Chondrocytes from the growth plates of ATF-2 m/m mice produce approximately 50–60% less Bcl-2 protein. The data support our hypothesis that the absence of ATF-2 inhibits the expression and activity of Bcl-2. The results suggest that ATF-2 is essential for normal expression and activity of Bcl-2 in growth plate chondrocytes.

EXPERIMENTAL PROCEDURES

Mice and Genotyping

Normal mice carry both 70 kDa and 62 kDa ATF-2 polypeptides. ATF-2 m/m mice carry only one ATF-2 protein at 66 kDa that is missing amino acids 277–326. Mice carrying wild-type or inactivated alleles of the ATF-2 gene were genotyped as described [Reimold et al., 1996]. Genomic DNA was isolated from the distal 2 mm segment of tail from each mouse and genotyped by polymerase chain reaction (PCR).

Plasmids

Bcl2LUC and muBcl2LUC were kind gifts from Linda Boxer. Bcl2LUC extends from −1,640 to −1,287 bp and includes the CRE at −1,552. The CRE site was mutated from GTGACGTTA to GGGCCTTTA to produce muBcl2LUC [Wilson et al., 1996]. Wild-type and dominant-negative ATF-2 expression plasmids have been described [Beier et al., 1999]. Dominant-negative CREB expression plasmid was a kind gift from C. Vinson (Johns Hopkins University). The pET22b(+) vectors, expressing His6-CREB-S133A and His6-CREB-S142A fusion proteins [Wu and McMurray, 2001] were generously provided by CT McMurray (Mayo Clinic, Rochester, MN).

Cell Culture

Mice carrying both wild-type and mutant alleles (i.e., heterozygotes) were crossed, producing offspring with genotype ratios approaching 1:2:1 (+/+): (+/m): (m/m). Chondrocytes from these offspring were isolated from the ventral parts of the ribcages of newborn to 2-day-old mice by sequential digestion with Pronase (Roche) and Collagenase D (Roche), as described [Lefevbre et al., 1994]. Isolated chondrocytes were cultured in suspension over 1.5% agarose in phosphate buffered saline (PBS) to maintain their chondrogenic phenotype. After 3 days, the chondrocyte clusters were digested with Collagenase D (3 mg/ml), and the cells were plated in monolayer culture at 37°C under 5% CO_2 in Dulbecco's Modified Eagles Medium (DMEM), supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml). These cells were subsequently used for immunoblots, transfections, luciferase reporter assays, and ChIP. We also used rat chondrosarcoma (RCS) cells, cultured as described [Beier et al., 1999] for electrophoretic mobility shift assays (EMSAs).

Immunoblots

Primary chondrocytes, isolated from newborn mouse ribs as described above were lysed in sodium dodecyl sulphate (SDS) sample buffer, following 12 h in culture. Total protein from 2–4×10^5 cells was resolved by SDS–PAGE and transferred to Hybond membranes (Amersham). Antibodies against Bcl-2 were purchased from Santa Cruz Biotechnology (catalog no. sc192).

Transfections

Two hours prior to transfection, 1.25×10^5 cells were seeded into each well of a 24-well plate. Transfections were performed with Fugene 6 (Roche), according to the manufacturer's protocol. Each well was transfected with 1.0 μg of reporter gene construct, 0.1 μg pRISV40 (Promega) (control for transfection efficiency), and 2 μl of Fugene 6. Co-transfections of expression plasmids were performed with 1.0 μg of Bcl-2 promoter construct, 0.1 μg expression plasmid or empty expression vector, and 0.1 μg of pRISV40. After transfection, cells were cultured for an additional 48 h and lysed with passive lysis buffer (Promega), according to the manufacturer's protocol. Each transfection was done in triplicate and repeated at least three times.

Luciferase Assays

Luciferase assays were performed with the dual luciferase assay kit (Promega), according

to the manufacturer's protocol in a Turner TD-20e luminometer (Promega). Lysate (10 μl) was assayed first for Firefly luciferase and then for Renilla luciferase activity. Firefly luciferase activity was normalized to Renilla luciferase activity.

Chromatin Immunoprecipitation (ChIP)

The ChIP procedure is used to determine if a given protein localizes or binds to a specific DNA sequence in vivo. Primary chondrocytes were plated at a near-confluent density, crosslinked with 1% formaldehyde for 20 min at room temperature, and quenched for 5 min with glycine (125 mM). Crosslinked cells were washed once with cold PBS, followed by liberation of the adherent cells utilizing Trypsin/ethylinediaminetetraacetic acid (EDTA) at 37°C. Dispersed cells were washed three times with serum-containing medium and resuspended at 10^6 cells per ml concentration in immunoprecipitation (IP)/lysis buffer (50 mM triethanolamine: HCl pH 7.4, 500 mM NaCl, 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.4% SDS supplemented with protease inhibitors). Chromatin in the resulting lysate was sheared to an average size of 500 bp through sonication on ice. Sonicated extracts were brought up to 2% v/v Triton X-100 and cellular debris was removed by centrifugation at 14,000*g* for 5 min. Chromatin extracts were diluted to 10 OD/ml with IP/Lysis buffer, and immunoprecipitation was undertaken with 2 μg ATF-2 antibody (Santa Cruz sc-187) and 10 μl protein A agarose beads (Santa Cruz sc-2001), incubated overnight at 4°C with constant rotation. Immuno-complexes were washed five times with IP buffer. Crosslinks were reversed and protein was digested by overnight incubation at 65°C (20 μg/ml Proteinase K in 50 mM Tris pH 8, 500 mM NaCl, 0.2% SDS). DNA was isolated by standard phenol extraction. A 115 bp fragment of the *Bcl-2* promoter that included the CRE element was amplified, along with a 190 bp fragment of the coding region of the ATF-2 locus to serve as a control. Primer sequences for the *Bcl-2* promoter fragment were P1: 5′-GTGGCTCAGAGGAGGGCTCTTTC-3′; P2: 5′-GGCTGTGGTGCCTGTCCTCTTAC-3′. Primer sequences for the ATF-2 locus were P1: 5′-GTTCCTTGGCAAGCACTGTCTTTGGGT-3′; P2: 5′-GACTGAGTCCCTAACCAATCCACTG-CCA-3′. PCR conditions were 94°C for 1 min, 64°C for 1 min, and 72°C for 1 min, 35 cycles.

The same methods were applied to ChIP of CREB, utilizing an antibody against CREB (New England Biolabs #9121) for immunoprecipitation.

Electrophoretic Mobility-Shift Assays (EMSA)

EMSA requires significant amounts of cells in order to procure enough nuclear protein. The amount of primary chondrocytes from newborn fetuses is limited by the number of fetuses of specific genotypes we can procure from a given litter. Due to limited litters, we performed EMSA using RCS cells. We isolated nuclear extracts from RCS cells using NE-PER nuclear (NER) and cytoplasmic extraction reagents (CER) (Pierce), according to the manufacturer's protocol. In brief, chondrocytes were rinsed once and scraped in PBS. After centrifugation, the cell pellet was resuspended in ice-cold CER I and incubated on ice for 10 min. The cells were lysed by mixing with ice-cold CER II. After centrifugation, the resultant pellet was resuspended in ice-cold NER. The mixture was incubated on ice for 40 min and centrifuged at maximum speed in a microcentrifuge for 10 min. The supernatant (nuclear extract) was saved for EMSA assay. The amount of protein was determined by using the Bio-Rad DC protein assay kit.

EMSA assays were performed as described [Beier et al., 1999]. The nuclear extracts (5–10 μg) were incubated with 50 fmol of biotin-labeled probe in the binding buffer, containing 10 mM Tris (pH 7.5), 50 mM KCl, 1 mM DTT at RT for 20 min. The oligo sequence of the Bcl-2 CRE (underlined) was 5′-GAACCGTGTGACG-TTACGCA-3′. The Bcl-2 CRE mutant (lower case) sequence was 5′-GAACCGTGgGcCt-TTACGCA-3′. The complementary oligos of wild-type and mutated Bcl-2 CREs were end-labeled with biotin, as per the Biotin 3′ End DNA Labeling Kit (Pierce), followed by annealing. The protein-DNA complexes were analyzed by electrophoresis in a 6% polyacrylamide gel, with 0.5 × Tris borate, EDTA (TBE) buffer. Supershifts were performed by incubating nuclear extracts with antibodies to ATF-2 (N-96, Santa Cruz Biotechnology), and to CREB (48H2, Cell Signaling Technology) in binding buffer at 4°C overnight, before the probe was added to the reaction system. The signal was analyzed by the Chemiluminescent Nucleic Acid Detection Module (Pierce).

RESULTS

Optimal Bcl-2 Expression Requires ATF-2 and the CRE

We initially wanted to examine changes in the concentrations of Bcl-2 protein, relative to ATF-2 activity. We used total protein from primary chondrocytes isolated from the ribs of newborn wild-type (+/+), ATF-2 heterozygous (+/m), and ATF-2 homozygous mutant (m/m) mice from three different litters, each of which produced all three genotypes, to perform immunoblot experiments as described in the Experimental Procedures. Our results showed that levels of Bcl-2 protein, isolated from ATF-2 wild-type chondrocytes, were notably higher than those from ATF-2 m/m chondrocytes. Figure 1A represents one of the three sets of experiments. Our densitometry determinations indicated that levels of Bcl-2 protein in wild-type chondrocytes were significantly higher (2.011) than those from ATF-2 m/m chondrocytes (1.045), indicating an approximate 50% reduction in Bcl-2 protein. Heterozygous chondrocytes displayed an intermediate level of Bcl-2 (1.796). Figure 1B illustrates the mean and SD of all three sets of experiments, and indicates that the densities of (+/+) and (+/m) Bcl-2 protein were not significantly different, while the densities of the ATF-2 m/m protein were significantly reduced with *P*-values of 0.003. We also determined that Bcl-2 mRNA levels in wt and m/m ATF-2 chondrocytes showed no differences when analyzed by RT-PCR (data not shown). This would suggest that the effect of ATF-2 on Bcl-2 protein may be at the post-transcriptional level.

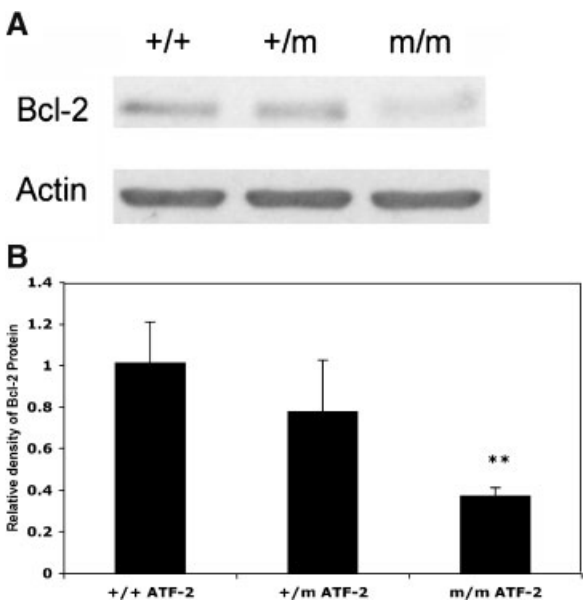


Fig. 1. Total protein from growth plate chondrocytes isolated from wildtype (+/+), heterozygous (+/−), or homozygous mutant (m/m) ATF-2 mice were analyzed by Western blot. Three different litters were used, each containing all three genotypes. Bcl-2 protein levels from one litter were shown in **A**. Loading of equal amounts of protein was demonstrated with an antibody against actin. The levels of Bcl-2 protein as determined by densitometric scan were significantly reduced in the ATF-2 m/m mouse, compared to that in wild type (**B**). $^{**}P < 0.01$, when compared to Bcl-2 protein levels in wildtype (+/+) ATF-2 mice.

Since the *Bcl-2* promoter (Fig. 2) has been shown to contain a CRE [Wilson et al., 1996] at −1,552, we analyzed the effects of ATF-2 on *Bcl-2* promoter activity in primary growth plate chondrocytes, using transient transfection assays. Figure 3 showed that, in the presence of ATF-2 (+/+ conditions), mutations in the CRE reduced *Bcl-2* promoter activity by approximately 50%, compared to activity when the CRE was intact. (Fig. 3A). However, reporter activity was severely reduced in the absence of ATF-2 (i.e., under m/m conditions), regardless of whether the CRE was intact. Our results in Figure 3B showed that overexpression of ATF-2 more than doubled the activity of the *Bcl-2* promoter in the presence of endogenous ATF-2 (vector) in wt cells. In contrast, a dominant-negative form of ATF-2 (dnATF-2) diminished the activity of the wild-type *Bcl-2* promoter by more than twofold. Inclusion of the mutated CRE in place of wild-type CRE in the *Bcl-2* promoter (muBcl2LUC; see Experimental Procedures) reduced luciferase activity to background levels, independent of overexpression of wt or dn ATF-2. Figure 3C indicated that the addition of ATF-2 to primary chondrocytes that do not express ATF-2 (i.e., ATF-2 m/m chondrocytes) increased *Bcl-2* promoter activity approximately threefold, thus rescuing activity of the *Bcl-2* promoter. However, the absence of an intact CRE in the *Bcl-2* promoter (muBcl2-LUC) rendered the transcription factor unable to produce any increase in luciferase activity, compared to that of the vector alone. Our results suggested that ATF-2 has the capability to stimulate *Bcl-2* promoter activity, provided that an intact CRE is present in the promoter, and to stimulate promoter activity in vitro when endogenous transcription factor is unavailable.

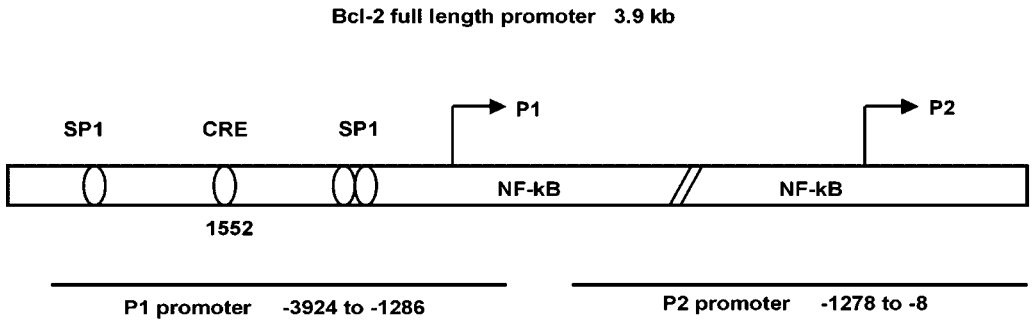


Fig. 2. The Bcl-2 promoter region. The *Bcl-2* regulatory region contains two promoters. The TATA-less P1 promoter spans nucleotides −3924 to −1286, and includes the CRE beginning at nucleotide −1552. The CRE is flanked by Sp1 binding sites. The P2 promoter is located downstream in the second exon, and lacks a CRE motif.

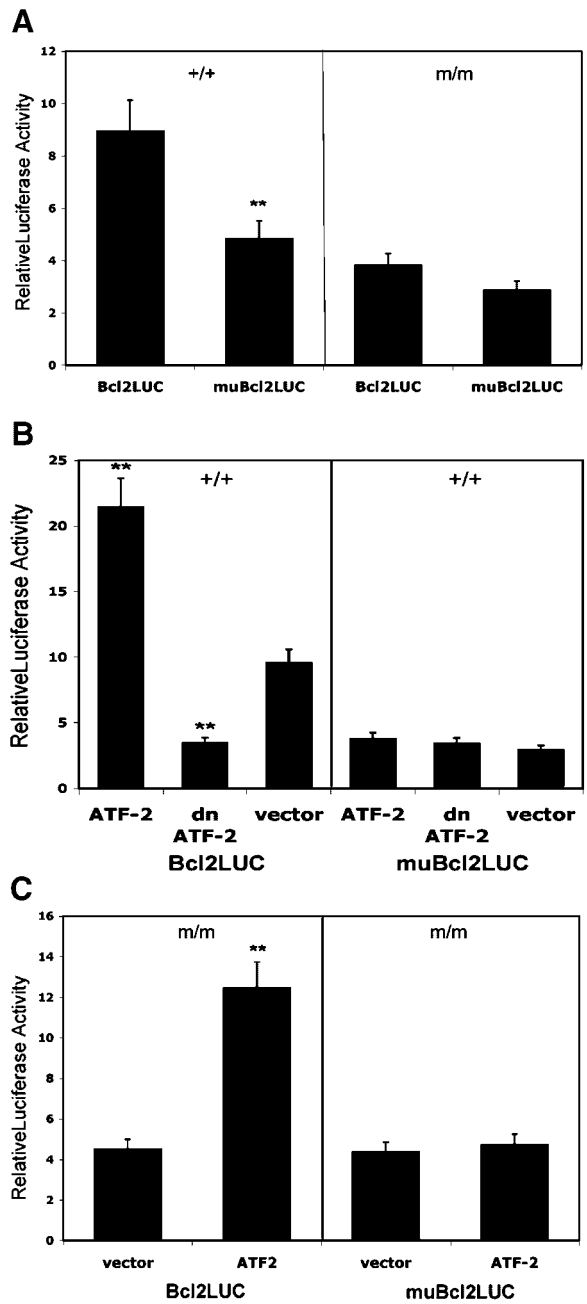
CREB Alone Cannot Adequately Stimulate *Bcl-2* Promoter Activity

Our data in Figure 4A showed that exogenous CREB, as well as the 142A activating mutation of CREB, were proficient in stimulating *Bcl-2* promoter activity close to twofold in wild-type (ATF-2 +/+) murine chondrocytes, using luciferase reporter assays. Point mutations at amino acid 133A, on the other hand, reduced the stimulation to background levels (i.e., far below that of the *Bcl-2* promoter (vector) alone). However, overexpression of CREB in the absence of ATF-2 (i.e., under m/m conditions; Fig. 4B), had no significant effect on *Bcl-2* promoter activity compared to vector alone, nor did the 133A and 142A mutations. In contrast, co-transfection of both ATF-2 and CREB in ATF-2 m/m chondrocytes provided a significant increase in *Bcl-2* promoter activity, but only equal to that of the addition of ATF-2 alone, as shown in Figure 4C. Overexpression of the 133A-mutation of CREB in the presence of supplementary ATF-2 stimulated activity above that of the *Bcl-2* promoter alone (vector), suggesting that CREB, unlike ATF-2, could not by itself stimulate *Bcl-2* promoter activity, relegating it to expression of the same basal activity as that of the vector (*Bcl-2* promoter) alone. Thus, CREB could not enhance ATF-2-stimulated *Bcl-2* promoter activity, nor could it rescue that activity in the absence of ATF-2. Interestingly, the addition of CREB to exogenous ATF-2 in ATF-2 m/m chondrocytes did not show any further *Bcl-2* promoter activity, compared to stimulation by ATF-2 alone.

ATF-2 and CREB Bind to the CRE in the Bcl-2 Promoter

Given our conclusion that the actions of CREB might be minimal with regards to supporting Bcl-2 promoter activity, while ATF-2 activity appeared to be requisite, we determined to establish whether ATF-2 alone, or ATF-2 and CREB together, physically interacted with the *Bcl-2* promoter at the CRE motif. We employed the technique of Chromatin Immunoprecipitation (ChIP, an assay that allows DNA-bound proteins such as transcription factors to be crosslinked via formaldehyde to the adjacent chromatin) as shown in Figure 5. We amplified a 115 bp fragment of the Bcl-2 promoter that included the CRE (lane 2) as well as a 190 bp coding fragment of ATF-2 as a control (lane 3). Immunoprecipitation with antibody against ATF-2 revealed a visible *Bcl-2* promoter fragment containing the CRE in lane 6, corresponding with the same in lane 2. Our controls for the *Bcl-2* promoter (lane 4), unrelated DNA (lane 5), and the coding fragment of ATF-2 (lane 7) were not visible in the absence of antibody against ATF-2 (Fig. 5A). After sequencing the DNA fragments from lanes 2 and 6, we confirmed that those sequences represented the portion of the *Bcl-2* promoter that included the CRE. We repeated these experiments using the same input under the same conditions, but with an antibody against CREB instead of ATF-2 after immunoprecipitation. We determined that the transcription factor CREB also interacted physically with the CRE within the *Bcl-2* promoter (Fig. 5B).

We also used an EMSA to determine if ATF-2 and CREB form complex(es) with the Bcl-2 CRE (Fig. 5C). The EMSA showed nuclear extracts from RCS cells, formed a complex with the Bcl-2 CRE (lane 2). The Bcl-2 CRE-binding site was competed by 100-fold molar excess of cold Bcl-2 CRE oligo (lane 3). Furthermore, mutant Bcl-2 CRE sequences did not form the complexes with RCS nuclear extract (lane 4). These data verify the specificity of protein and DNA binding. Antibodies against ATF-2 and CREB were used to identify proteins present in the protein-DNA complexes. An antibody can have one of several outcomes when being added to this binding reaction. If the protein bound to the antibody is not involved in formation of complex, the antibody will have no effect. If the protein in the complex is identified by the antibody, the antibody will either obstruct complex formation or result in a supershift. Our results show that the antibodies against ATF-2 and CREB blocked the complex formation, indicating that these proteins in the RCS nuclear extracts are ATF-2 and CREB (lanes 5 and 6, respectively).



DISCUSSION

Alternative Interactions With the CRE

It has become clear over the last several years that control of chondrocyte proliferation, cell-cycle exit, and hypertrophy, are essential for normal skeletal development and growth. We have identified the transcription factor ATF-2, as a vital regulator of chondrocyte proliferation via its control of cyclin D1 [Beier et al., 1999] and cyclin A [Beier et al., 2000]. The genes for both cyclins harbor CRE motifs that are targets for interaction with ATF-2 and CREB, and upon binding to that motif, promoter activity is initiated. As indicated above, the *Bcl-2* promoter also harbors a CRE. Our in vitro data demonstrate unambiguously that ATF-2 and/or CREB have no effect on *Bcl-2* promoter

Fig. 3. *Bcl*-2 promoter activity requires ATF-2 and the CRE in growth plate chondrocytes. Wild-type primary chondrocytes were co-transfected with Bcl-2 promoter constructs containing either an intact CRE (GTGACGTTA) or a mutated CRE (GGGCCTTTA). The cells were processed for relative luciferase activity as described in methods. (**A**) Mutations in the CRE (muBcl2LUC) resulted in significant reductions in reporter activity, compared to that of the wildtype Bcl-2 promoter. In the absence of ATF-2 (i.e. m/m conditions), the activities of Bcl-2LUC were reduced by 60% compared to that in the presence of ATF-2, while muBcl2LUC activity in ATF-2 m/m chondrocytes was also reduced. (**B**) Ectopic ATF-2 more than doubled the relative activity of the Bcl-2 promoter. Dominant/negative (dn) ATF-2 caused reductions of 60% in *Bcl*-2 promoter activity in the presence of the CRE. In the absence of the CRE (muBcl2LUC), luciferase activity was minimal and was not affected by ATF-2. (**C**) Ectopic ATF-2 provided a threefold increase in Bcl-2 promoter activity in the absence of ATF-2 (m/m). However, in the absence of both the CRE (muBcl2LUC) and ATF-2 (m/m), addition of ATF-2 had little effect on muBcl2LUC promoter activity. The data shown represent the mean and standard deviation of four independent experiments, each performed in triplicate. **, $P < 0.01$ when compared to Bcl2LUC vector.

activity, in normal growth plate chondrocytes in the absence of the CRE. When the CRE is intact, exogenous ATF-2 will enhance wild-type *Bcl-2* promoter activity in growth plate chondrocytes. Under ATF-2 m/m conditions (when ATF-2 is not expressed), *Bcl-2* promoter activity is significantly curtailed, as is Bcl-2 protein expression, but they are not absent. Three binding sites for Sp1 transcription factors flank the CRE motif within the P1 region (−3,924 to −1,286) of the *Bcl-2* promoter. In addition, two NFkB-binding sites (known to activate *Bcl-2* gene expression in lymphoma cells) are located just downstream [Heckman et al., 2002]. However, there is no evidence that they interact with the CRE. These transcription factors might support limited activation of *Bcl-2* promoter activity in the absence of ATF-2 by binding to sites other than the CRE.

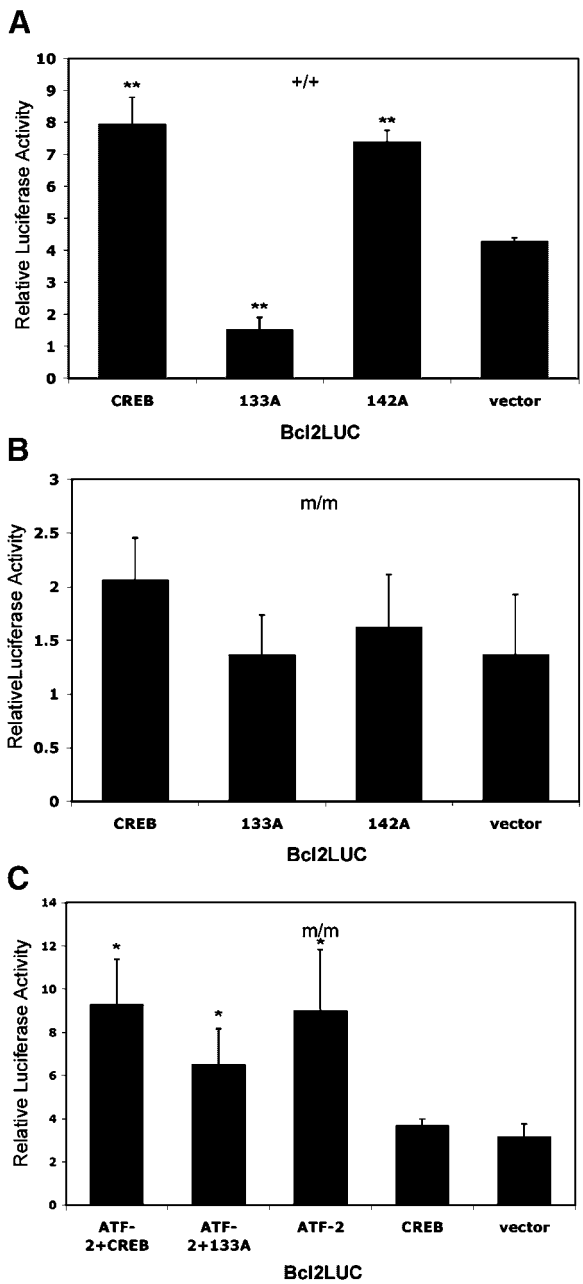


Fig. 4. CREB promotes Bcl-2 promoter activity in wild-type chondrocytes, but has little effect on ATF-2 m/m chondrocytes. Wild-type primary chondrocytes were co-transfected with Bcl2LUC alone, or together with expression vectors for CREB, dominant-negative CREB 33A, or activating mutation CREB142A, and pRLSV40. The cells were processed for relative luciferase activity as described in methods. (**A**) Over-expression of CREB and activating mutant 142A CREB increased Bcl2LUC activity close to twofold in the presence of ATF-2, while mutant 133A-CREB reduced Bcl2LUC activity almost threefold. (**B**) CREB displayed negligible activity in the absence of ATF-2 (m/m conditions). ATF-2 m/m chondrocytes were co-transfected with Bcl-2LUC alone, or with CREB, 133A CREB, or 142A CREB, as well as pRLSV40. There were no significant changes in relative luciferase activity compared to Bcl2LUC. (**C**) CREB did not contribute to the rescue of Bcl-2 promoter activity by ATF-2 under m/m conditions. ATF-2 m/m chondrocytes were transfected with either Bcl2LUC alone, or together with either ATF-2 and CREB, ATF-2 and CREB133A, ATF-2 alone, or CREB alone. Overexpression of ATF-2 and CREB, or ATF-2 alone increased *Bcl*-2 promoter activity approximately threefold, while ATF-2 plus CREB133A increased promoter activity by twofold. However, over-expression of CREB alone had little effect on *Bcl*-2 promoter activity compared to vector, while over-expression of ATF-2 plus CREB had similar effects on *Bcl*-2 promoter activity compared to over-expression of ATF-2 alone. The data shown represent the mean and standard deviation of at least four independent experiments, each performed in triplicate, for each graph. *, $P < 0.05$, **, $P < 0.01$ when compared to Bcl2LUC vector.

CREB may not be Required for *Bcl-2* Promoter Activity

CREB can also affect *Bcl-2* promoter activity, but not independently in growth plate chondrocytes, based on our results. Under wild-type conditions, additional CREB can enhance the effect of endogenous ATF-2 and CREB on the *Bcl-2* promoter. However, CREB appears to be relatively inactive in the absence of ATF-2 in vitro. An earlier examination of the roles of ATF-2 and CREB as binding partners suggested that they both bound to the CRE in the cyclin D1 promoter [Beier et al., 1999]. We have demonstrated herein by ChIP assay that both ATF-2 and CREB interact physically with the CRE motif in the *Bcl-2* promoter.

Our promoter activity experiments suggest that CREB plays only a minor role in Bcl-2 expression in the growth plate, compared to

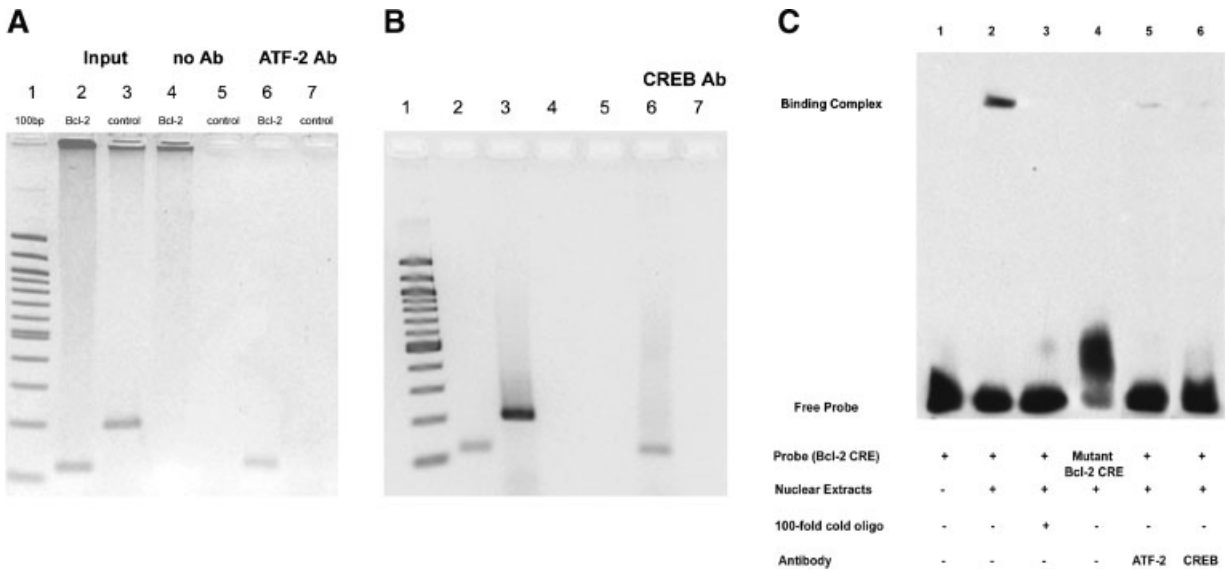


Fig. 5. ATF-2 and CREB bind to the CRE in the *Bcl-2* promoter. ATF-2 and CREB interaction with the CRE in the *Bcl-2* promoter were analyzed with Chromatin Immunoprecipitation (ChIP) (**A** and **B**). Lane 2 showed the 115 bp fragment of the *Bcl-2* promoter that included the CRE element amplified with input. Lane 3 showed the 190 bp fragment of the coding region of the ATF-2 locus with input. No antibody in the immunoprecipitation resulted in no amplification of either Bcl-2 or ATF-2 (lanes 4 and 5, respectively). Addition of antibody against ATF-2 or CREB in the immunoprecipitation resulted in an amplification of Bcl-2 (lane 6) but not of ATF-2 (lane 7). ATF-2 and CREB interact on with the Bcl-2 CRE were also determined using electrophoretic mobility shift assays (EMSA, **C**). Nuclear extracts from RCS cells were incubated with biotin-labeled double-stranded oligonucleotides corresponding to the Bcl-2 CRE sequence. Lane 1 was the free probe (Biotin-labeled Bcl-2 CRE). Lane 2 was the resulting complex of labeled Bcl-2 CRE and nuclear extracts. The complex in lane 2 was blocked by a 100-fold excess of unlabeled CRE oligonucleotides, as shown in lane 3. Nuclear extracts did not bind to the biotin-labeled mutated Bcl-2 CRE sequence in lane 4. Addition of antibody against either ATF-2 or CREB resulted in reduction of the abundance of complex as shown in lanes 5 and 6, respectively.

that of ATF-2. This is somewhat surprising. Transgenic mice, expressing a CREB mutant that cannot be phosphorylated display a phenotype of dwarfism and atrophied pituitaries [Struthers et al., 1991]. CREB-null mice die after birth resulting from respiratory distress, and exhibit reduction of the corpus callosum and anterior commissures in the brain, as well as impaired fetal ab T cell lineage development [Rudolph et al., 1998]. More recently, expression of A-CREB, a dominant-negative inhibitor of CREB, in growth plate chondrocytes of transgenic mice resulted in dwarfism and lethality, immediately after birth due to constriction of the ribcage followed by respiratory failure [Long et al., 2001]. These phenotypes strongly imply that CREB plays crucial roles in endochondral growth plate progression and skeletal growth. However, our data suggest that CREB, in the absence of its dimerization partner, does not have the capability to rescue Bcl-2 expression in vitro. Perhaps the role that CREB plays may be one required simply for interaction with ATF-2, in order to target the CRE. ATF-2 alone then, would promote expression of Bcl-2.

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