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Identification of the cyclin D1 gene as a target of activating transcription factor 2 in chondrocytes

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ABSTRACT **Endochondral bone growth is regulated by the rates of chondrocyte proliferation and differentiation. However, the intracellular mechanisms regulating these processes are poorly understood. Recently, interruption of the gene encoding the transcription factor activating transcription factor 2 (ATF-2) was shown to inhibit proliferation of chondrocytes in mice [Reimold, A. M., *et al*. (1996) *Nature (London)* 379, 262–265]. The target genes of ATF-2 that are responsible for this phenotype remain unknown. Here we report that the cyclin D1 gene is a direct target of ATF-2 in chondrocytes. ATF-2 is present in nuclear extracts from chondrogenic cell lines and binds, as a complex with a CRE-binding protein (CREB)/CRE modulator protein, to the cAMP response element (CRE) in the cyclin D1 promoter. Mutation of the cyclin D1 CRE caused a 78% reduction in the activity of the promoter in chondrocytes. Overexpression of ATF-2 in chondrocytes enhanced activity of the cyclin D1 promoter 3.5-fold. In contrast, inhibition of endogenous ATF-2 or CREB by expression of dominant-negative inhibitors of CREB and ATF-2 significantly reduced the activity of the promoter in chondrocytes through the CRE. In addition, levels of cyclin D1 protein are greatly reduced in the chondrocytes of ATF-2-deficient mice. These data identify the cyclin D1 gene as a direct target of ATF-2 in chondrocytes and suggest that reduced expression of cyclin D1 contributes to the defective cartilage development of these mice.**

Bone formation in vertebrates can occur in two different ways: intramembranous ossification, through which the bones of the skull are formed, and endochondral ossification, through which the axial and appendicular skeleton is formed. During endochondral bone formation, the precursor bones are first laid down as a cartilagenous model. In a second step, cartilage is then replaced by bone tissue (recently reviewed in ref. 1). The longitudinal growth of these endochondral bones, however, is controlled by the coordinated proliferation and differentiation of chondrocytes in the transition zone from cartilage to bone, the growth plate. Mutations in numerous genes in humans and mice disturb the balance between chondrocyte proliferation and differentiation and thereby cause many different skeletal disorders, ranging from severe lethal chondrodysplasias to early-onset osteoarthritis (reviewed in refs. 2 and 3). These diseases highlight the importance of precision in the regulation of chondrocyte proliferation and differentiation with regard to the development of a functional skeleton. Although multiple extracellular signaling molecules have been implicated in the control of chondrocyte growth and maturation (2), very little is known about the intracellular signaling pathways and transcription factor networks involved in these processes.

Reimold *et al.* (3) recently showed that disruption of the gene encoding the activating transcription factor 2 (ATF-2) in mice causes reduced proliferation of chondrocytes and chondrodysplasia. ATF-2 is a member of the ATF/cAMP response element-binding protein (CREB) family of transcription factors (4). Members of this family can bind to the cAMP response element (CRE) found in many mammalian gene promoters. They all contain a basic region-leucine zipper motif and can bind to DNA as homodimers and heterodimers in various combinations (5, 6). In addition to associating with other members of the ATF/CREB family, ATF-2 has been reported to form heterodimers with c-Jun (6–9).

CREs are found in several genes involved in the control of the cell cycle, e.g., the cyclin D1 gene (10), the cyclin A gene (11, 12), and the retinoblastoma gene (pRb; ref. 13). Progression through the cell cycle is driven by the activity of complexes of cyclin-dependent kinases (CDKs) and their specific cyclin partners (recently reviewed in refs. 14 and 15). The activity of the CDKs is regulated by at least three distinct mechanisms: (*i*) the levels of the cyclin subunit, (*ii*) the amount of inhibitor proteins of CDKs, such as $p21^{Waf1/Cip1}$ and $p16^{Ink4a}$, and (*iii*) the phosphorylation status of the CDK. The most prominent substrates of activated CDKs are the retinoblastoma protein (pRb) and the related p107 and p130 proteins. Hyperphosphorylation of pRb by different CDKs allows progression through the cell cycle, DNA replication, and finally mitosis.

Integration of proliferative and antiproliferative signals appears to occur during growth phase 1 of the cell cycle (14). The D-type cyclins, which are the first cyclins expressed in growth phase 1, are responsive to these signals. For example, proliferative signals activating tyrosine kinase receptors, nuclear hormone receptors, and G protein-coupled receptors converge in the activation of cyclin D-dependent CDKs in several cell types (16). Because ATF-2 appears to be necessary for chondrocyte proliferation and because recent evidence shows that the cyclin D1 gene promoter contains a potential binding site for ATF-2 (10), we investigated the possibility that ATF-2 is involved in the regulation of the cyclin D1 promoter in chondrocytes.

MATERIALS AND METHODS

Cell Culture and Transfections. RCS cells, which were derived from a rat chondrosarcoma (17), were cultured at 37°C under 5% CO_2 in DMEM medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (50 units/ml), and streptomycin (50 μg/ml). Chondrogenic MCT cells (mouse chondrocytes immortalized with a temperature-sensitive simian virus 40 large T antigen; ref. 18) were cultured as described (18, 19) in the identical medium at 32°C under 5% CO_2. Six hours (RCS) or 15 h (MCT) before transfections, 6 ×

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Abbreviations: ATF, activating transcription factor; CRE, cAMP response element; CREB, CRE-binding protein; CREM, CRE modulator; CDK, cyclin-dependent kinase; pRb, retinoblastoma protein.

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10^4 cells were seeded into each well of a 24-well plate. Transfections were performed with Lipofectin (GIBCO) according to the manufacturer's protocol. Briefly, each well was transfected with 1.0 μg of a reporter gene construct and 0.15 μg of pRlSV40 (Promega) as a control for transfection efficiency with 1.5 μl of Lipofectin for 4 h at 32°C (MCT cells) or 37°C (RCS cells). Cotransfections with expression plasmids were performed with 1.5 μg of cyclin D1 promoter construct, 2 μg of expression plasmid or empty expression vector, and 0.15 μg of pRlSV40. Cotransfections with dominant-negative expression plasmids for ATF-2 and CREB together were carried out with 1.0 μg of each expression plasmid, and controls were carried out with 1.0 μg of each empty vector. After transfection, cells were cultured for an additional 30 h and then lysed with passive lysis buffer (Promega) according to the manufacturer's protocol. Every transfection was repeated at least three times with each of two different plasmids preparations.

Isolation of Primary Mouse Chondrocytes. Chondrocytes were isolated from the ventral parts of the rib cages of 2-day-old mice from heterozygote ATF-2 $\times$ null crosses by sequential digestion with Pronase (Boehringer Mannheim) and collagenase D as described (20). In parallel, genomic DNA was isolated from the tail of each mouse for determination of genotype as described (3). Isolated chondrocytes were cultured in suspension over 1.5% agarose in PBS to maintain their chondrogenic phenotype. After 3 days the chondrocyte clusters were digested with collagenase D (3 mg/ml), and cells were plated in monolayer culture at subconfluency. After 12 h cell were harvested for Western blot analyses as described below.

Western Blot Analyses. Exponentially growing RCS cells, MCT cells, and primary chondrocytes were lysed in SDS sample buffer. Total protein from 2–5 $\times$ 10^5 cells was resolved by SDS/PAGE and transferred to Hybond C membranes (Amersham). Antibodies against cyclin D1 were from Santa Cruz Biotechnology (HD11) and NeoMarkers (Fremont, CA) (DCS-6). The antibody against ATF-2 (C-19) was from Santa Cruz Biotechnology, the antibody against CREB was from New England Biolabs (catalog no. 9192), and the actin antibody was from Boehringer Mannheim (catalog no. 1378 996). Reactive proteins were detected with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and visualized with an enhanced chemiluminescense detection kit (ECL, Amersham). Densitometric measurement of signals was performed with CAMSCAM software and an Astra 1220S scanner (UMAX, Fremont, CA).

Electrophoretic Mobility-Shift Assays. Electrophoretic mobility-shift assays were performed as recently described (10, 21–24) with nuclear extracts from RCS and MCT cells. Supershifts were performed with polyclonal antibodies against ATF-2 (C-19X, Santa Cruz Biotechnology), CREB/CREM (HM93, a gift from J. Habener, Harvard Medical School; ref. 25), Jun proteins (a gift from R. Tjian, University of California, Berkeley; refs. 22 and 26; and Ab-2, Oncogene Science; ref. 22), and c-Fos (K-25X, Santa Cruz Biotechnology; ref. 24).

Plasmids. −1745CD1LUC and −141 CD1LUC have been described (24). The constructions −66 CD1LUC and −66 mut CD1LUC were created by PCR from the −141 CD1LUC plasmid with specific primers, and the region including the ATF/CRE site was mutated from 5′-T AAC GTC ACA CGG AC-3′ to 5′-TcgC GTC cCc CGG gCc-3′ (−66 mut CD1LUC; (23). Mouse wild-type ATF-2 cDNA was kindly provided by L. Glimcher (Harvard School of Public Health) and cloned into the expression vector pcDNA3 (InVitrogen) by using *Xho*I sites to yield the vector pcDNA-ATF-2. For construction of the dominant-negative ATF-2 expression plasmid, the coding sequence of the plasmid KH-M2 (provided by Dr. L. Glimcher; ref. 27) was cut out with *Bgl*II and cloned into the *Bam*HI site of pcDNA3 to yield the plasmid pcDNA-dn ATF-2. The expression plasmid for dominant-negative CREB (A-CREB) was generously provided by C. Vinson (Johns Hopkins University) (28).

Luciferase Assays. Luciferase assays were performed with the dual luciferase assay kit (Promega) according to the manufacturer's instructions 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.

RESULTS

ATF-2, CREB, and Cyclin D1 Are Coexpressed in Chondrogenic Cell Lines. We performed Western blot analyses for cyclin D1 and ATF-2 proteins in chondrogenic RCS (17) and MCT cells (18) to determine whether they are coexpressed. Cyclin D1 and ATF-2 were expressed in both cell lines (Fig. 1). In addition, CREB was also expressed in both cell lines. All subsequent experiments were performed in parallel in both cell lines. Because the results in the two lines were very similar, we present only the data obtained with the MCT cells.

The Cyclin D1 CRE Is Necessary for Promoter Activity in Chondrocytes. We investigated whether the CRE is necessary for the activity of the cyclin D1 promoter in chondrocytes by using reporter plasmids containing a wild type promoter fragment (−66 CD1LUC; ref. 10) or a mutant in which the CRE has been destroyed (−66 mut CD1LUC) in transiently transfected MCT cells (Fig. 2). Mutation of the CRE caused a 4.5-fold reduction in promoter activity, suggesting that a functional CRE is required for maximal activity of the cyclin D1 promoter in chondrocytes.

ATF-2 and a CREB/CREM Protein from Chondrocyte Nuclear Extracts Bind to the Cyclin D1 CRE. Next we examined whether chondrocyte nuclear proteins were able to bind to the cyclin D1 CRE. Electrophoretic mobility-shift assays were performed with nuclear extracts from MCT cells and ^{32}P-labeled double-stranded oligonucleotides corresponding to the cyclin D1 CRE sequence (Fig. 3). MCT nuclear proteins formed two specific complexes with the CRE (lane 1), both of which could be blocked by 100-fold excess of unlabeled CRE (lane 2). Addition of an antibody against ATF-2 resulted in the formation of a supershift and reduced the electrophoretic mobility of the upper complex (lane 4), suggesting the presence of ATF-2 in this complex. Antiserum recognizing CREB and CREM proteins resulted in a faint supershift and reduced the abundance of both complexes (lane 5), suggesting that one of these proteins is present in these complexes and that binding of the antibody to the protein inhibits formation of the complex. This antiserum does not perturb unrelated DNA/protein complexes (data not shown). Antibodies against c-Fos (lane 6) or various Jun proteins (lanes 7, 8) did not affect

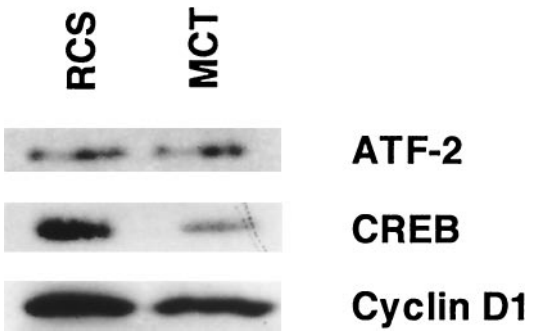

FIG. 1. ATF-2, CREB, and cyclin D1 are coexpressed in chondrocytes. Expression of ATF-2, CREB, and cyclin D1 was examined in RCS and MCT cells with Western blot analyses and specific antibodies. All three proteins were expressed in both cell types.

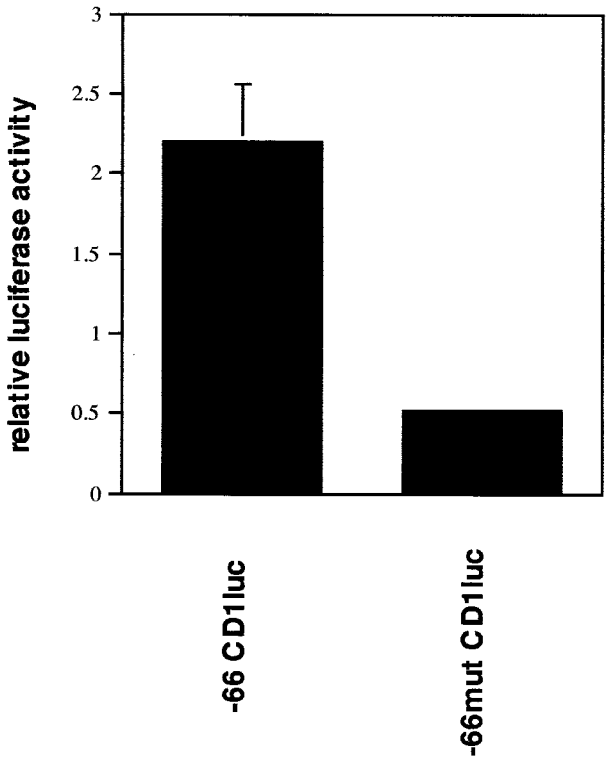

FIG. 2. The CRE is necessary for the activity of the cyclin D1 promoter in chondrocytes. MCT cells were transiently transfected with −66CD1LUC (wild-type promoter) or −66 mut CD1LUC (containing a mutated CRE) and pRlSV40 (for standardization). after transfection (30 h), cells were harvested, firefly luciferase activity was measured, and standardized to Renilla luciferase activity to yield the relative luciferase activity. −66 mut CD1LUC conferred 22% of the activity of −66 CD1LUC.

the formation or migration of the complexes, suggesting that these proteins are not present.

ATF-2 and CREB Are Necessary for Activity of the Cyclin D1 CRE in Chondrocytes. We tested the ability of the cyclin D1 CRE to confer transcriptional activation by ATF-2 by cotransfecting the reporter plasmids with an expression plasmid for ATF-2 (Fig. 4). Overexpression of ATF-2 resulted in a 3.5-fold induction of promoter activity (compared with controls that were cotransfected with empty expression vector). In contrast, overexpression of ATF-2 did not influence the activity of the promoter fragment containing the mutated CRE. These data show that ATF-2 can specifically stimulate the activity of the cyclin D1 CRE in chondrocytes.

Next, we wanted to know whether endogenous ATF-2 and CREB are required for the activity of the cyclin D1 CRE in MCT cells. The cyclin D1 reporter constructs were cotransfected with either dominant-negative ATF-2 (Fig. 5*a*) or CREB (Fig. 5*b*) expression plasmids. Wild-type promoter activity was reduced 2.5- and 3.5-fold, respectively, but promoter activity was still higher than that of the mutated promoter. Cotransfection with dominant-negative ATF-2 and CREB plasmids together reduced the activity of the wild-type promoter to the level of the promoter fragment with the mutated CRE (Fig. 5*c*). The mutated promoter was not affected by overexpression of dominant-negative ATF-2 or CREB (or both). These data suggest that both transcription factors are necessary for the optimal activity of the cyclin D1 CRE in chondrocytes.

Reduced Levels of Cyclin D1 Protein in ATF-2-Deficient Mice. We compared the levels of ATF-2 and cyclin D1 protein in homozygous ATF-2-deficient mice with those in normal heterozygous littermates to determine whether ATF-2 is nec-

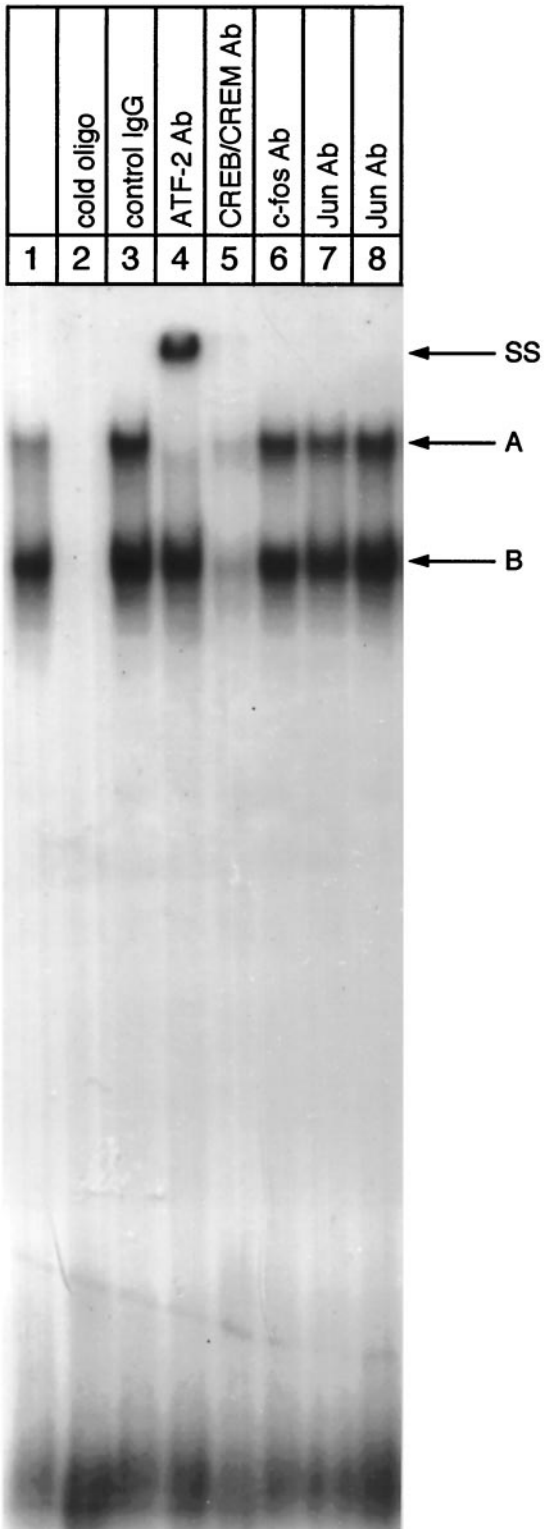

FIG. 3. ATF-2 and a CREB/CREM protein bind to the cyclin D1 CRE in chondrocytes. Nuclear extracts from MCT cells were incubated with ^{32}P-labeled double-stranded oligonucleotides corresponding to the cyclin D1 CRE sequence. The resulting complexes (lane 1) could be blocked by a 100-fold excess of unlabeled CRE oligonucleotides (lane 2). Incubation of extracts with an unrelated oligonucleotide did not produce a complex (data not shown). Addition of an ATF-2 antibody (lane 4) resulted in slower migration of the upper complex (ss, supershift), whereas incubation with a CREB/CREM antiserum (lane 5) reduced the abundance of both complexes. Addition of control IgG (lane 3) or antibodies (Ab) against c-Fos (lane 6) or different Jun proteins (lanes 7 and 8) did not affect the formation or migration of the complexes.

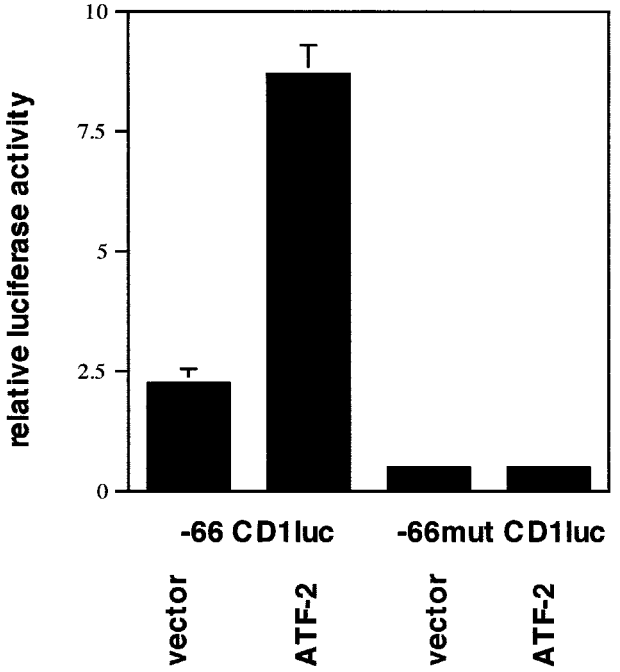

FIG. 4. Overexpression of ATF-2 increases the activity of the cyclin D1 CRE. MCT cells were cotransfected with −66CD1LUC or −66 mut CD1LUC, either empty expression vector or an expression vector for ATF-2, and pRlSV40. After transfection (30 h), cells were harvested, and firefly luciferase activity was measured and standardized to Renilla luciferase activity to yield the relative luciferase activity. Overexpression of ATF-2 increases the activity of −66 CD1LUC 3.5-fold, whereas it had no effect on the activity of −66 mut CD1LUC.

essary for expression of the cyclin D1 gene *in vivo* (Fig. 6). As expected, ATF-2 was detected only in chondrocytes from heterozygous mice. Chondrocytes from homozygous ATF-2-deficient mice contained only 17% of the cyclin D1 protein found in their heterozygous littermates, as determined by densitometry. Western blot analyses for actin protein revealed that equal amounts of protein were loaded in both samples.

DISCUSSION

As demonstrated by the large number of mutations causing skeletal defects, the proper regulation of chondrocyte proliferation and differentiation (the latter requires exit from the cell cycle) is essential for the formation of a functional skeleton (1, 2). Because the intracellular control of proliferation is executed by the genes of the cell cycle machinery, it is likely that these genes play an important role during endochondral bone formation. This view is supported by recently published experiments in which the gene encoding the CDK inhibitor $p57^{Kip2}$ or the pRb-related p107 and p130 genes have been disrupted in mice (29, 30). In both cases, chondrocytes display delayed exit from the cell cycle and differentiation, leading to severe skeletal defects. The data presented here address the regulation of expression of a cell cycle gene in chondrocytes.

The results of the transfection experiments clearly demonstrate the importance of the CRE in the cyclin D1 promoter for its activity in chondrocytes. Electrophoretic mobility-shift assays demonstrated that chondrocyte nuclear proteins form two distinct, specific complexes with the cyclin D1 CRE. As shown by supershift assays, the slower migrating complex contained ATF-2. Antiserum against CREB/CREM proteins clearly reduced the abundance of both complexes. This could be because of the same protein in both complexes, or to different forms of CREB or CREM in the two complexes. Because CREB is expressed in chondrocytes and has been

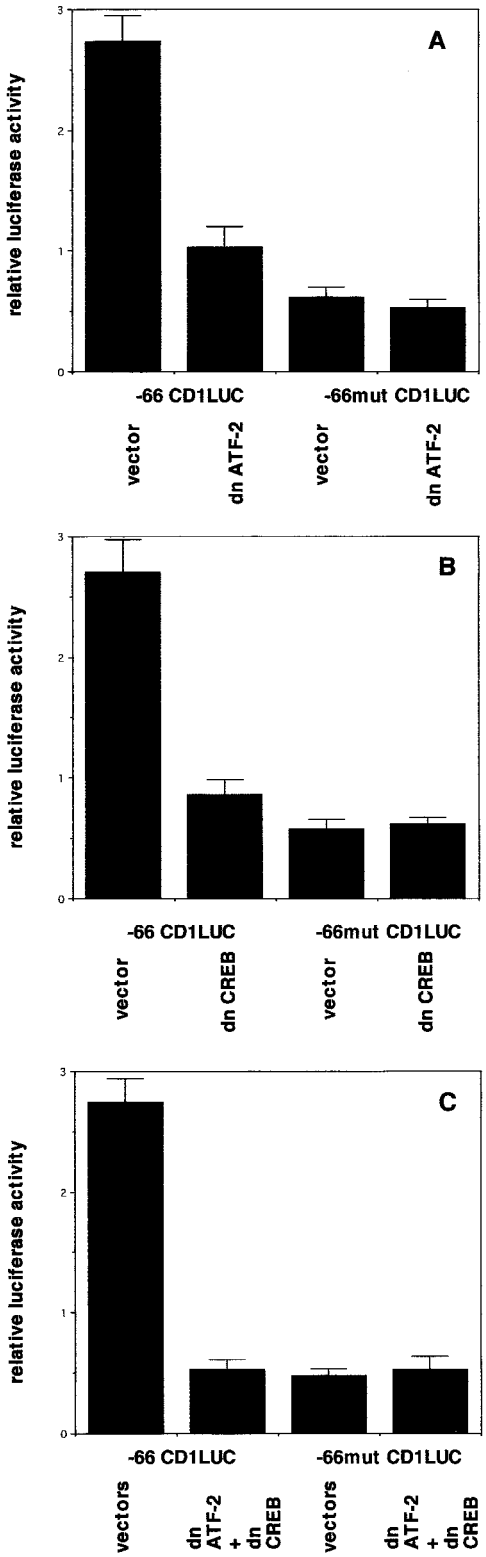

FIG. 5. ATF-2 and CREB activity are necessary for the activity of the cyclin D1 CRE in chondrocytes. MCT cells were cotransfected with −66CD1LUC or −66 mut CD1LUC, either empty expression vector or expression vectors for dominant-negative ATF-2 (*a*), dominant-negative CREB (*b*), or both (*c*), and pRlSV40. After transfection (30 h), cells were harvested, and firefly luciferase activity was measured and standardized to Renilla luciferase activity to yield the relative luciferase activity. Dominant-negative ATF-2 or CREB alone reduced the activity of −66 CD1LUC 2.5- to 3.5-fold; in combination, they reduced the activity to the levels of −66 mut CD1LUC. The activity of −66 mut CD1LUC was not affected by either dominant-negative expression vector.

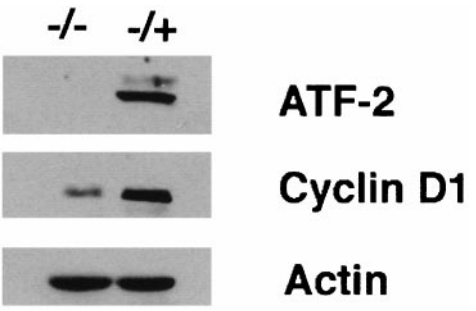

FIG. 6. Cyclin D1 protein levels are reduced in ATF-2-deficient chondrocytes. Total protein from chondrocytes isolated from heterozygous (−/+) or homozygous (−/−) ATF-2-deficent mice were analyzed by Western blot. As expected, chondrocytes from −/− mice do not express ATF-2, in contrast to −/+ chondrocytes. The level of cyclin D1 is greatly reduced in homozygote mice. Loading of equal amounts of protein was demonstrated with an anti-actin antibody.

shown to bind to this sequence in other cell types (10), it is likely that it is found in at least one of the complexes. Further mobility-shift experiments in which antibodies specific for CREB, CREM and related factors, or purified proteins were used will be necessary to determine the exact composition of the complexes. As demonstrated by the supershift assays, neither jun proteins nor c-fos appear to be part of the complexes, although ATF-2 has been shown to heterodimerize with jun proteins (6–9). Expression of wild-type or dominant-negative versions of c-Jun and c-Fos do not affect the activity of the cyclin D1 CRE in these cells (data not shown).

Dominant-negative ATF-2, which heterodimerizes with endogenous ATF-2, rendering it unable to bind to DNA (27), significantly reduced the activity of the wild-type cyclin D1 promoter but was not able to reduce the activity to the same levels as the mutation of the CRE. Similar results were obtained with a dominant-negative version of CREB, which acts in an analogous manner (28). In contrast, overexpression of dominant-negative ATF-2 and CREB together inhibit the activity of the CRE to the same magnitude as the mutation of the CRE. These data suggest that, although both proteins are necessary for maximal activity of the element, when only one factor is inhibited, the other protein is able to confer activation of the CRE, either as homodimer or in a heterodimeric form with other factors such as CREM or ATF-1. Inactivation of both CREB and ATF-2, however, causes a total loss of activity of the CRE. The observed effects are not specific for the basal 66-bp fragment of the cyclin D1 promoter, because dominant-negative ATF-2 and CREB have very similar effects when cotransfected with the plasmid −1745 CD1LUC (ref. 24; data not shown).

Chondrocytes from ATF-2-deficient mice contain greatly reduced levels of cyclin D1 protein, demonstrating that the observed regulatory relationship between ATF-2 and cyclin D1 is conserved *in vivo*. The effects on the protein level are greater than the 4.5-fold reduction in promoter activity observed when the cyclin D1 CRE is mutated. One possible explanation for this is that loss of ATF-2 could cause reduced levels of other transcription factors necessary for cyclin D1 gene transcription. For example, potential ATF-2-binding sites play important roles in the transcription of the c-fos and c-jun genes (31–34). Indeed, preliminary results suggest that a binding site for Fos and Jun proteins is necessary for maximal activity of the cyclin D1 promoter in chondrocytes (F.B. and P.L., unpublished observations). We have initiated experiments to explore whether levels of c-Jun, c-Fos, or related transcription factors are altered in ATF-2-deficient chondrocytes.

Homozygous deletion of the cyclin D1 gene in mice resulted in reduced postnatal growth (35). It is likely that alterations in the proliferation of chondrocytes may have contributed to this phenotype. However, the skeletal defects of these mice are clearly less severe than those of the ATF-2 null mice, possibly because of the presence of cyclins D2 and D3. This suggests that additional target genes of ATF-2 are involved in the reduction of chondrocyte proliferation in ATF-2-deficient mice. In particular, it will be interesting to determine whether other D-type cyclin genes (cyclin D2 and D3) are regulated by ATF-2 in chondrocytes. Possible ATF-2-binding sites have also been described in the cyclin A (11, 12) and retinoblastoma genes (13). The cyclin A gene is necessary for progression through the cell cycle from the S phase on; reduced expression of cyclin A could, therefore, contribute to inhibited proliferation of chondrocytes. The pRb gene acts as a negative regulator of proliferation. It remains to be seen whether its expression is reduced in ATF-2-deficient chondrocytes and how this would affect chondrocyte proliferation. These experiments are in progress in our laboratory.

The activities of both CREB (reviewed in ref. 29) and ATF-2 (36–40) are tightly regulated by a variety of kinases. Therefore, transcriptional control by these factors offers the possibility to integrate signals from different pathways and extracellular stimuli. Together with potential additional *cis*-acting elements in the cyclin D1 promoter, the cyclin D1 CRE might play an important role in the integration of proliferative and antiproliferative signals in chondrocytes. Experiments to identify the kinases and pathways controlling CREB and ATF-2 phosphorylation in chondrocytes are under way.

In summary we have identified the first target gene of ATF-2 in chondrocytes. The observed decreased expression of cyclin D1 in ATF-2-deficient mice may contribute to the described phenotype of chondrocyte proliferation in those mice. Disruption of this regulatory pathway might also be involved in the pathogenesis of human skeletal diseases, such as chondrodysplasias, osteoarthritis, or chondrosarcomas. In addition, this study is the first to address the regulation of cell cycle gene expression in chondrocytes. These results will enable us to identify the extracellular signals and intracellular pathways controlling cyclin D1 expression and cell cycle progression in the future.

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