Activating Transcription Factor 2 Is Necessary for Maximal Activity and Serum Induction of the Cyclin A Promoter in Chondrocytes*

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Endochondral bone growth is regulated through the proliferation and differentiation of growth plate chondrocytes. Mice deficient for the activating transcription factor 2 (ATF-2) gene show reduced proliferation of chondrocytes. Here we demonstrate that the cyclin A gene is a target of ATF-2 in chondrocytes. Serum stimulation of chondrogenic rat chondrosarcoma cells induces cyclin A expression. A cyclic AMP response element (CRE) is necessary for optimal activity and serum inducibility of the cyclin A promoter and confers regulation by ATF-2. Phosphorylation and activity of ATF-2 are enhanced dramatically upon serum stimulation of rat chondrosarcoma cells. Mutation of the CRE or over-expression of dominant-negative ATF-2 inhibits serum induction of the cyclin A promoter. Chondrocytes from ATF-2-deficient mice display reduced and delayed induction of cyclin A upon serum stimulation. The ATF-2-related transcription factor CRE-binding protein contributes to the activity of the cyclin A CRE in chondrocytes, whereas c-Jun and c-Fos regulate the promoter independently of the CRE. Our data suggest that the reduction in cyclin A levels in chondrocytes from ATF-2-deficient mice contributes to their phenotype of reduced chondrocyte proliferation and dwarfism.

Growth of endochondral bone is controlled by the coordination of proliferation and differentiation of growth plate chondrocytes (for recent review, see Refs. 1–3). Disruption of these processes by gene mutations commonly results in chondrodysplasias that are characterized by dwarfism, skeletal deformities, and early onset osteoarthritis (2, 3). Although the intracellular mechanisms involved in the regulation of chondrocyte proliferation and differentiation are poorly understood, recent evidence suggests that cell cycle genes play an important role in these processes (for review, see Ref. 4).

Progression through the mammalian cell cycle is controlled by the sequential activation of several complexes of cyclins and cyclin-dependent kinases (CDKs) (for review, see Ref. 5). Activation of CDKs is in part controlled by the expression levels of their respective cyclin partners. Therefore, much attention has been paid to the elucidation of the mechanisms controlling cyclin gene expression. The cyclin A gene is induced at the transition from the G1 to the S phase of the cell cycle and is necessary for progression through the cell cycle from this point on, first in a complex with CDR2, later with CDR1 (5, 6). Induction of cyclin A is caused, at least in part, by transcriptional activation. At least two cis-active sites in the cyclin A promoter have demonstrated importance for the induction of cyclin A transcription: a binding site for E2F transcription factors (7) and a cyclic AMP response element (CRE; 8–10).

One of the transcription factors binding to the CRE is ATF-2 (activating transcription factor 2; 11). ATF-2 is a member of the ATF/CREB family of transcription factors capable of forming homodimers and heterodimers with other family members as well as with c-Jun (11–16). ATF-2-deficient mice display reduced chondrocyte proliferation, causing dwarfism and skeletal deformities (17). We have recently identified the cyclin D1 gene, which is involved in progression through the G1 phase of the cell cycle, as one target of ATF-2 in chondrocytes (18). Reduced levels of cyclin D1 in the ATF-2-deficient mice likely contribute to the phenotype of these mice. However, the skeletal phenotype of cyclin D1-deficient mice (19) is much less severe than that of the ATF-2-deficient mice, suggesting that additional target genes of ATF-2 are involved. Here we demonstrate that the cyclin A gene is a second target of ATF-2 in chondrocytes and that reduction in cyclin A levels as a result of the absence of ATF-2 may also be in part responsible for the phenotype of ATF-2-deficient mice.
Regulation of the Cyclin A Promoter in Chondrocytes

RESULTS

Serum Induction of Cyclin A Protein Expression and Promoter Activity in Chondrocytes—Chondrogenic RCS cells were serum starved for 3 days and restimulated with 10% FBS to examine cyclin A expression and serum inducibility. Cells were harvested at 4-h intervals, and cyclin A protein expression was investigated using Western blot analyses. Whereas cyclin A protein was hardly detectable in serum-starved cells and in the first hours after serum stimulation, cyclin A levels began to rise sharply after 4 h of serum stimulation and reached maximal levels at 12–16 h (Fig. 1). This was paralleled by an increase in DNA replication as measured by bromodeoxyuridine incorporation. 2 In contrast, medium changed to fresh serum-free medium induced only a very slight increase in cyclin A protein after 24 h (data not shown).

We addressed the question of whether increased levels of cyclin A are caused by enhanced transcriptional activity by transfecting the plasmid pycycAluc707, which contains 707 nucleotides of the rat cyclin A promoter fused to the firefly luciferase gene (9), into RCS cells. After transfection, cells were serum starved for 3 days, restimulated with 10% FBS, and measured for promoter activity at 4-h intervals. The low basal activity of the cyclin A promoter began to increase after 4 h of serum stimulation and reached its maximum at 12 h (Fig. 2). Medium changed to fresh serum-free medium induced only slight activation of the cyclin A promoter, suggesting that the serum inducibility of the cyclin A gene is caused, at least in part, by increased transcription.

The Cyclin A CRE Is Necessary for Maximal Promoter Activity in Chondrocytes and Confers Activation by ATF-2—The 707-base pair cyclin A promoter fragment has been shown to contain a functional CRE that can confer binding of transcription factors of the CREB/ATF family (9). We used primary mouse chondrocytes (Fig. 3A) and RCS cells (Fig. 3B) to examine the involvement of the CRE in the regulation of the cyclin A promoter in chondrocytes. Cells were transfected with the plasmids pycycAluc707 and pycycAluc707m (in which the CRE has been mutated; 9). Mutation of the CRE caused a reduction of 70–80% in promoter activity in both cell types. The transcription factor ATF-2 is expressed in proliferating chondrocytes and necessary for chondrocyte proliferation (17, 18). pycycAluc707 and pycycAluc707m were cotransfected with expression vectors for wild type or dominant-negative ATF-2, or empty expression vector pcDNA3 into RCS cells (panel B). 36 h after transfection, cells were harvested, and firefly luciferase activity was determined and standardized to Renilla luciferase activity.

2 F. Beier, A. C. Taylor, and P. LaValle, unpublished observations.
responses to overexpression of both forms of ATF-2.

Serum Enhances Phosphorylation and Activity of ATF-2—Transcriptional activation by ATF-2 is enhanced by phosphorylation of NH$_2$-terminal threonine residues through several mitogen-activated protein kinases (22, 23). We tested whether ATF-2 phosphorylation is increased by serum stimulation by Western blot with an antibody recognizing ATF-2 which is phosphorylated on threonine 71 (Fig. 4). After 12 h of serum stimulation (the time point where maximal activation of the cyclin A promoter is observed; Fig. 2), the amount of phosphorylated ATF-2 is 6-fold higher in stimulated cells than in control cells (as determined by densitometry). In contrast, the amount of total ATF-2 is very similar in stimulated and non-stimulated cells.

To determine whether enhanced phosphorylation of ATF-2 in response to serum corresponds to increased transcriptional activation, we used a fusion protein of the GAL4-DNA binding domain and the ATF-2 transcriptional activation domain (Fig. 5A). This fusion protein can activate transcription from promoters containing GAL4-binding elements only when the ATF-2 domain is activated. Serum stimulation for 12 h caused a 10-fold increase in the activity of a GAL4-responsive promoter by GAL4-ATF-2, whereas a control plasmid encoding the DNA binding domain alone showed no response to serum (Fig. 5B).

The CRE Is Necessary for Serum Induction of the Cyclin A Promoter—To determine whether the CRE is involved in the serum induction of the cyclin A promoter, pcyAluc707 and pcyAluc707m were transfected into RCS cells, and cells were serum starved for 3 days and restimulated with medium containing 10% FBS. Wild type promoter activity was induced in cells cultured in both media, the amount of phosphorylated ATF-2 is increased 6-fold in cells cultured in the presence of 10% FBS.

ATF-2-deficient Chondrocytes Display Delayed Serum Induction of the Cyclin A Promoter and Reduced Cyclin A Levels—We next addressed the function of ATF-2 in the control of cyclin A expression in chondrocytes in more detail by examining the regulation of the cyclin A gene in primary chondrocytes isolated from homozygous ATF-2-deficient mice (−/−; which display reduced chondrocyte proliferation; 12) and their heterozygous littermates (−/+; which are phenotypically normal). Wild type cyclin A promoter activity was reduced by 50% in chondrocytes from −/− animals compared with promoter activity in chondrocytes from −/+ littermates (Fig. 7A). Mutation of the CRE caused a further 40% reduction of promoter activity in −/− cells, whereas the same mutation repressed the activity of the cyclin A promoter by 78% in −/+ cells.
sion of ATF-2 rescued both of these phenotypes.

Next we analyzed cyclin A protein levels in homo- and heterozygote ATF-2-deficient mice (Fig. 8). Whereas heterozygote chondrocytes express the 70- and 62-kDa isoforms of ATF-2, but not the recently described 66-kDa form (24), none of the three isoforms could be detected in $^{1/2}/^{1/2}$ chondrocytes. Densitometric analyses revealed that cyclin A levels in subconfluent, unsynchronized ATF-2 $^{1/2}$ cells (cultured in the presence of 10% FBS) were 2.8-fold lower than in ATF-2 $^{1/1}$ cells (after standardization to actin signals).

Finally we compared the induction of cyclin A protein expression by 10% FBS in serum-starved primary chondrocytes from homozygous and heterozygous ATF-2-deficient mice (Fig. 9). In heterozygous cells, maximal induction of cyclin A protein was observed after 16 h of serum stimulation, whereas in homozygous cells this induction was delayed, and maximal expression was clearly lower.

The Role of CREB, c-Fos, and c-Jun in Cyclin A Promoter Activity in Chondrocytes—Because ATF-2-deficient chondrocytes still displayed significant levels of cyclin A protein as well as activity of the CRE in the cyclin A promoter, we examined the possibility that other transcription factors might be responsible for this effect. The CRE is the target sequence for the transcription factor CREB, and CREB cooperates with ATF-2 in the regulation of the cyclin D1 CRE in RCS cells (18). In addition, Fos and Jun proteins have been shown to bind to the cyclin A CRE in other cell types (9, 25). To address the function of these proteins in the regulation of cyclin A transcription, we cotransfected cyclin A promoter constructs with expression vectors for dominant-negative versions of CREB, c-Fos, c-Jun, and ATF-2 into unsynchronized, subconfluent chondrocytes from homozygote (Fig. 10A) and heterozygote (Fig. 10B) ATF-2-deficient mice. All four dominant-negative constructs caused a reduction in cyclin A promoter activity in heterozygote cells. However, the inhibition by dominant-negative CREB and ATF-2 was largely dependent on the CRE, whereas dominant-negative c-Fos and c-Jun blocked the activities of the promoter plasmids with wild type and mutated CRE to a similar degree (Fig. 10A). Dominant-negative ATF-2 had no effect in ATF-2-null cells, whereas dominant-negative CREB reduced the activity of the wild type promoter to the level observed for the promoter with the mutated CRE (Fig. 10B). In contrast, dominant-negative c-Fos and c-Jun caused similar reductions in activity of wild type and mutant promoters.
DISCUSSION

We show here that the cyclin A CRE is necessary both for basal cyclin A promoter activity in asynchronously dividing chondrocytes and for serum induction of the cyclin A promoter. We have shown previously that ATF-2 is among the major transcription factors binding to the CRE in chondrocytes, thus regulating the transcription of the cyclin D1 gene (18). Here we identify the cyclin A gene as a second target gene of ATF-2 in chondrocytes. Ectopic wild type ATF-2 can activate the cyclin A promoter in chondrocytes, whereas dominant-negative ATF-2 inhibits the activity of the promoter. In addition, activity and phosphorylation of ATF-2 are strongly enhanced after 12 h of serum stimulation, when cyclin A promoter activity is maximal. ATF-2 activity is regulated through phosphorylation by several mitogen-activated protein kinases as well as protein kinase Cα (22, 23, 26). However, the protein kinase C phosphorylation site (serine 121; 23) is not present in the GAL4 fusion protein used in these studies, nor is phosphorylation of this site recognized by the phospho-specific ATF-2 antibody used. Therefore, a potential role of serine 121 in the regulation of ATF-2 activity in chondrocytes cannot be excluded. Experiments to identify the extracellular signals, intracellular pathways, and kinases regulating ATF-2 activity in chondrocytes are currently being performed in our laboratory.

The effect of ATF-2 on the cyclin A promoter is conferred mainly by the CRE; however, mutation of the CRE does not abolish the effects of ATF-2 on cyclin A promoter activity entirely. These data suggest that ATF-2 regulates the cyclin A promoter through a second cis-active element in addition to the CRE. The cyclin A gene is also a target of the cell cycle-regulated transcription factors of the E2F family (7). E2F activity is regulated by the phosphorylation status of pocket proteins, which in turn is controlled by the activity of CDKs and indirectly by cyclin levels. Because ATF-2 regulates the expression of the cyclin D1 gene in chondrocytes (18), it is likely

![Figure 8](image8.png) Cyclin A levels are reduced in chondrocytes from ATF-2-deficient mice. Exponentially growing primary chondrocytes isolated from homozygous (−/−) or heterozygous (+/−) ATF-2-deficient mice were lysed, and protein expression was examined using Western blot analyses with antibodies specific for ATF-2, cyclin A, and actin. No ATF-2 could be detected in −/− chondrocytes, whereas a clear signal was obtained in +/+ cells. Cyclin A levels were 2.8-fold higher in +/+ chondrocytes. A Western blot for actin demonstrated equal gel loading.

![Figure 9](image9.png) Reduced and delayed serum induction of cyclin A protein expression in ATF-2-null chondrocytes. Exponentially growing primary chondrocytes isolated from homozygous (−/−) or heterozygous (+/−) ATF-2-deficient mice were serum starved for 3 days, restimulated with 10% FBS, and harvested after 0, 12, 16, 20, and 24 h. Cyclin A protein levels were detected by Western blot analyses. Cyclin A protein induction was reduced and delayed in ATF-2 −/− chondrocytes.

![Figure 10](image10.png) Role of CREB, c-Fos, and c-Jun in cyclin A transcription in chondrocytes. Primary, unsynchronized chondrocytes isolated from heterozygous (−/+; panel A) or homozygous (−/−; panel B) ATF-2-deficient mice were cotransfected with pycylAuc707 or pycylAuc707m and empty expression vector or expression vectors for dominant-negative forms of ATF-2, CREB, c-Jun, or c-Fos. 36 h after transfection, cells were harvested, and firefly luciferase activity was determined and standardized to Renilla luciferase activity.
that it also influences E2F activity and cyclin A promoter activity through E2F. Therefore we suggest a model in which ATF-2 regulates the cyclin A promoter in a dual way, directly through the CRE and indirectly through the E2F site (Fig. 11). Experiments are under way in our laboratory to examine whether the E2F site contributes to the effects of ATF-2 on the cyclin A promoter.

The regulatory relationship between ATF-2 and cyclin A exists in vivo, as shown by the reductions in cyclin A protein levels, cyclin A promoter activity, and serum induction of cyclin A expression in ATF-2-deficient chondrocytes. These data also suggest that the reduction in cyclin A protein levels is at least partially responsible for the phenotype of the ATF-2-deficient mice. However, mutation of the CRE causes a larger decrease in promoter activity than loss of ATF-2. In addition, considerable CRE activity is present in ATF-2-deficient chondrocytes, suggesting that other factors activate cyclin A transcription from this site. The ATF-2-deficient mice used in this study are still able to express one isoform of ATF-2 (24). However, expression of this isoform could not be detected in chondrocytes isolated from heterozygote or homozygote ATF-2-deficient mice. Therefore, it is unlikely that this isoform causes the observed activity of the CRE in ATF-2-null chondrocytes. In contrast, overexpression of dominant-negative CREB in the ATF-2-deficient background reduces the activity of the wild type cyclin A promoter to that of the CRE mutant promoter, suggesting that CREB accounts for this (or the majority of this) activity. We have observed a similar cooperativity of CREB and ATF-2 in the regulation of the cyclin D1 gene in chondrocytes (18). In addition, the dwarfed phenotype of CREB-deficient mice (27) suggests a role for CREB in skeletal growth. The AP-1 transcription factors c-Jun and c-Fos have also been shown to bind to the cyclin A CRE in other cell types (9, 25). Overexpression of dominant-negative forms of these proteins inhibited cyclin A promoter activity in chondrocytes; however, these effects were independent of the CRE, suggesting that they act through different promoter elements. Because both c-Fos and c-Jun are necessary for maximal activity of the cyclin D1 promoter, it is possible that they exert their effect through regulation of E2F activity.

Our data clearly show that the CRE and ATF-2 are important for serum induction of the cyclin A promoter. Deletion of the CRE, as well as loss of ATF-2, causes both a delay and decrease in serum induction. However, cyclin A promoter activity is still responsive to serum in both cases, suggesting that additional promoter elements are involved in serum induction. A likely candidate for this function is the above mentioned E2F site because E2F activity can likely be induced by mitogenic signals that do not require ATF-2 or CREB.

In summary, our data identify the cyclin A gene as a second target of ATF-2 in chondrocytes. Both cyclin D1 and cyclin A are involved in the control of progression through the G1 and S phases of the cell cycle, suggesting that ATF-2 exerts its effect on chondrocyte proliferation and endochondral bone growth through the regulation of cell cycle progression. The complete characterization of these processes will contribute significantly to our understanding of the mechanisms controlling and of the diseases associated with skeletal growth.

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FIG. 11. Model of regulation of the cyclin A promoter by ATF-2. ATF-2 regulates the cyclin A promoter in chondrocytes both in a CRE-dependent and a CRE-independent fashion. ATF-2 binds directly to the CRE and activates transcription. In addition, ATF-2 stimulates the expression of the cyclin D1 gene, likely enhancing CDK activity and phosphorylation of pocket proteins. This will likely induce transcriptional activation by E2F transcription factors and up-regulation of cyclin A promoter activity through the E2F site. In addition, c-Jun and c-Fos are necessary for maximal activity of the cyclin A promoter, possibly through regulation of E2F activity.

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