Journal of Cellular Biochemistry 97:857–868 (2006)

A Promoter Element of the CD-RAP Gene is Required for Repression of Gene Expression in Non-Cartilage Tissues In Vitro and In Vivo

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Abstract The cartilage-derived retinoic acid-sensitive protein (CD-RAP) gene is expressed predominately in cartilage. Previous studies in transgenic mice have shown that the DNA promoter segment from −2,251 bp to −2,068 bp of the CD-RAP gene contains elements critical for gene expression. Subsequent studies revealed both positive and negative regulatory motifs in this 183 bp element. Here we show that this element demonstrates activation or repression of gene expression in vitro and in vivo based on cell type and content of transcription factors. The distribution of Sox (positive) and C/EBP (negative) transcription factors in cell lines and in mouse tissues is consistent with their positive and negative roles. In transgenic mice, when the 183-bp element was removed from a 3,345-bp cartilage-specific CD-RAP promoter, expression of the reporter gene became widespread, being observed in muscle, bone, lung, and liver in addition to cartilage. In vitro, mutation of the C/EBP site activated the inactive 3,345-bp CD-RAP gene promoter in myoblastic cells, suggesting that this site is responsible for (−2,079 bp) repression. These results indicate that the 183-bp element plays an important role in cartilage-specific gene expression by acting as a chondrocyte-regulatory module repressing transcription in non-chondrocytes and contributing to activation in chondrocytes. This is the first report of a functional DNA element necessary for repression in non-cartilage tissues in vivo. J. Cell. Biochem. 97: 857–868, 2006.

Key words: CD-RAP; cartilage; transcription; CCAAT enhancer binding protein; Sox9

Cartilage is a highly organized tissue that functions as a template for developing bones as well as shock absorber for joints. Chondrocytes express a number of specific genes such as types II, IX, and XI collagen, aggrecan, link protein, and CD-RAP [Sandell and Adler, 1999]. The mechanism of restricted tissue-specific gene expression has attracted intensive investigation. Among the cartilage-characteristic genes, α1(II) collagen (COL2A1) and α2(XI) collagen (Col11A2) have been studied in detail. The similar expression pattern of *Col2a1* and *Col11a2* in mice suggests that these genes are co-regulated by common mechanisms. In fact, similar cartilage-specific regulatory elements have been identified for *Col2a1* [Lefebvre et al., 1996, 1998; Zhang et al., 1996] and *Col11a2* [Bridgewater et al., 1998; Tsumaki et al., 1998; Liu et al., 2000], both genes being positively regulated by high mobility group (HMG) proteins, particularly the Sox family of transcription factors. Sox9 has been shown to be an activating transcription factor, indispensable for chondrogenesis [Lefebvre et al., 1997; Bi et al., 1999].

CD-RAP is a matrix protein expressed in cartilage. It was originally cloned as an mRNA co-regulated with *Col2a1* in cartilage and down-regulated in chondrocytes that were de-differentiated by treatment with retinoic acid [Dietz and Sandell, 1996]. Melanoma inhibitory activity (MIA), the human homologue of CD-RAP, was independently isolated from a cell line

Grant sponsor: National Institute of Health; Grant numbers: R01AR45550, R01AR36994.

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Received 2 July 2005; Accepted 27 July 2005

DOI 10.1002/jcb.20648

derived from a brain metastasis of a human melanoma [Blesch et al., 1994]. CD-RAP/MIA is expressed by various tumor cells including melanoma, chondrosarcoma, and breast cancer, but its normal physiological expression is restricted primarily to cartilage [Bosserhoff et al., 1997; Chansky et al., 1998; Bosserhoff et al., 1999]. CD-RAP/MIA is co-expressed with COL2A1, from the beginning of chondrogenesis and expressed throughout cartilage development [Bosserhoff et al., 1997]. While the function in cartilage is unknown, Stoll and colleagues have shown that CD-RAP/MIA is essentially an SH3 domain fold in solution and binds to fibronectin [Stoll et al., 2001]. The CD-RAP gene is highly conserved and compact containing four exons in 1.5 kb of DNA in both mouse and humans [Davies et al., 2004b]. Therefore, because of its small size and limited distribution, it provides a good model for the study of chondrocyte specific gene expression.

In a series of studies, we have identified and characterized many regulatory elements in the proximal promoter of the mouse CD-RAP gene. All of these elements have also been shown to be active in the cartilage gene *Col2a1* as well. In the CD-RAP gene, Activator protein-2 (AP-2) binds to the sequence from −463 to −456 and regulates transcription in a biphasic manner: at a low concentration, AP-2 activates transcription and at a high concentration, represses it [Xie et al., 1998]. Overexpression of AP-2 abrogates chondrogenesis [Huang et al., 2004]. In contrast, Sox9 binds at −410 to −404 and appears to activate CD-RAP and other cartilage genes via a cooperative DNA-binding mechanism [Sock et al., 2003; Davies et al., 2004a]. Upstream Stimulatory Factor and δ-crystallin/E2-box factor 1 function at an E-box located at −487 to −482 and activate or repress CD-RAP depending on the proportion of the each factor in the nucleus [Li and Sandell, 2002].

In vivo studies in transgenic mice have revealed that a 2,251-bp promoter directs tissue specific expression of an *E. coli* β-galactosidase (*lacZ*) reporter gene, consistent with the endogenous CD-RAP gene expression pattern [Xie et al., 2000]. However, a similar construct truncated to 2,068-bp was not reliably expressed [Xie et al., 2000]. These results suggested that the 183-bp fragment between −2251 and −2068 contains elements that are responsible for tissue specific expression of CD-RAP.

In a separate series of studies, we have shown that this 183-bp element has both enhancer and repressor functions [Okazaki et al., 2002] and found a functional site for CCAAT/enhancer-binding proteins (C/EBPs) binding to the 183 bp fragment that acts as a repressor in response to IL-1β [Okazaki et al., 2002]. C/EBPs are a family of basic-leucine-zipper transcription factors with six known family members: C/EBPα, β, δ, ε, γ, and ζ [Akira et al., 1990]. Among these, C/EBPβ and/or δ activate various genes related to inflammation, such as phospholipase A_2 [Massaad et al., 2000], cyclooxygenase-2 [Thomas et al., 2000; Ogasawara, 2001] and manganese superoxide dismutase [Jones et al., 1997]. C/EBP also regulates matrix proteins, such as type I collagen [Greenwel et al., 2000], matrix Gla protein [Kirfel et al., 1997] and osteocalcin [Gutierrez et al., 2002]. C/EBPβ has three major isoforms generated by alternative translation start sites: full length (38 kDa), liver-enriched activator protein (LAP, 36 kDa) and liver-enriched inhibitory protein (LIP, 20 kDa). LAP is generally considered to be an activator, while LIP, which lacks most of the *trans*-activation domain of LAP, can act as a dominant-negative inhibitor. LAP and LIP are the predominant isoforms expressed in tissues. We have previously reported that over-expression of C/EBPβ (full length, LAP or LIP) or C/EBPδ represses the CD-RAP promoter activity in rat chondrosarcoma cells [Okazaki et al., 2002]. IL-1β, an inhibitor of chondrocyte extracellular matrix gene expression, up-regulates C/EBPβ and C/EBPδ, that, in turn, represses CD-RAP gene transcription via the 183 bp element. This C/EBP site overlaps an HMG site shown to bind to Sox 9, and the balance between these factors is thought to determine whether activation or repression occurs [Okazaki et al., 2002; Imamura et al., 2005].

In the current study, we further investigated the mechanism of function of the 183-bp element. As previous studies have suggested this fragment could act as an enhancer or a repressor depending on the availability of transcription factors, we sought to test this hypothesis in vitro and in vivo. We generated transgenic mice that harbored a 3,345 bp CD-RAP promoter and those in which the 183 bp element was removed. We show that the 183-bp element acts as a repressor of reporter gene expression in non-chondrocytic cells in vivo. In vitro studies further suggest that the

mechanism of repression involves the binding of C/EBP at the site previously shown to be responsible for down regulation by IL-1β [Okazaki et al., 2002]. We propose that it is the balance of Sox and C/EBP transcription factors that leads to activation or repression of gene expression through this "chondrocyte-regulatory module." The structure and function of the CD-RAP chondrocyte-regulatory module is similar to the other cartilage-specific genes, *Col2a1*, *Col11a2*, suggesting a common role for positive and negative regulatory factors functioning through a common cassette to regulate expression of these highly restricted genes.

MATERIALS AND METHODS

Cell Culture

RCJ3.1C5.18 rat chondrogenic (C5.18), rat chondrosarcoma (RCS), and Balb3T3 mouse fibroblast cell lines were cultured under standard conditions as described before [Xie et al., 1998]. C2C12, a mouse myoblast precursor cell line, was cultured under the same conditions as Balb3T3 cells.

Plasmid Construction

Two different 5′ upstream constructs of the mouse CD-RAP gene were subcloned into placF, the expression vector that contains the *lacZ* reporter gene described previously [Xie et al., 2000]. Briefly, the placF vector contains the *lacZ* gene followed by a sequence of the murine protamine gene that supplies an intron and a polyadenylation signal. The sequence from −3,345-bp to −3-bp relative to the CD-RAP translation start site was subcloned upstream of the *lacZ* gene of placF. To make the deleted 3,345-bp construct, the sequence from −3,345-bp to −2,252-bp was generated by PCR and cloned upstream of the −2,068-bp construct into the placF vector [Xie et al., 2000]. As a result, the deleted 3,345-bp construct (3,345-bp Δ183), lacks the 183-bp element between −2,251-bp to −2,068-bp. After confirmation by sequencing, the 3,345-bp-placF and the 3,345-bpΔ183-placF were digested with *Xba I* and *Hind III* to release the fusion gene from the vector sequence (3,345-bp-*lacZ* and 3,345-bpΔ183-*lacZ*).

The 3,345-bp CD-RAP promoter and the 3,345-bpΔ183 promoter were also subcloned into pGL3-basic vector (pGL3b, Promega, Madison, WI) for transfection assays. To make the C/EBP mutant, 3,345-bp-pGL3b, containing a two base pair mutation at −2,078-bp and −2,079-bp, a site-directed mutagenesis kit (Sigma, St. Louis, MO) was used. The abrogation of C/EBP binding to this mutation was confirmed previously [Okazaki et al., 2002]. To add the 183-bp element upstream to the SV-40 promoter, the fragment between −2,251-bp and −2,045-bp (24-bp longer than the 183-bp to rescue the junction at −2,068-bp) was made by PCR, then cloned into pGL3-promoter vector (pGL3pr, Promega) to make 183-pGL3pr.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts from C5.18, RCS, and Balb3T3 cells were isolated using the Nu-Clear Extraction Kit™ (Sigma, St. Louis, MO) according to the manufacturer's instructions. All oligonucleotides were synthesized by Invitrogen (Carlsbad, CA) and the complementary oligonucleotides were annealed to make double-stranded oligonucleotides. Various double-stranded oligonucleotides were end-labeled using T4 polynucleotide kinase and [γ-^{32}P] dATP. Band shifts were performed as described [Lefebvre et al., 1996]. The antibodies for C/EBPs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The DNA probe for EMSA spanned nucleotides −2094 to −2069 of the 183 bp fragment. DNA protein complexes were resolved on a 5% polyacrylamide gel at 100 V for several hours. The gels were dried and autoradiography performed.

RNA Extraction and RT-PCR

Total RNA was isolated from cultured cells using Qiagen RNeasy mini kit™ (Qiagen, Inc., Valencia, CA) and 1 μg of total RNA was reverse transcribed as described before [Okazaki et al., 2002]. For semi-quantitative RT-PCR, several pilot studies were performed in advance for every gene in order to determine the linear range of the PCR reaction. The primers used in this study were as follows: Sox5: 5′-GGCAGCTTAGCGGATGTGGT-3′ (sense) and 5′-TGCTGGCGCTGTTTCTCTATC-3′ (antisense); Sox6: 5′-CTTTGAGAACCTGGGTCCCC-3′ (sense) and 5′-GTCAGGGAAGGCCTGAAGAAT-3′ (antisense); Sox9: 5′-GAGAACACACGGCCCCAG-3′ (sense) and 5′-TCCAGAGCTTGCCCAGAGTC-3′ (antisense); C/EBPα: 5′-AAGCCAAGAAGTCGGTGGA-3′ (sense) and 5′-CAGTTGACGGCTCAGCTGTT-3′ (antisense); C/EBPβ: 5′-GGCGCGAGCGCAACAACATC-3′ (sense) and 5′-

GCTCGGGCAGCTGCTTGAAC-3′ (antisense); C/EBPδ: 5′-ACGAGAAGCTGCATCAGCGT-3′ (sense) and 5′-GCTGCAATGGTAATAAGACG-TAGAAAA-3′ (antisense); MyoD: 5′-ACAGCC-GGTGTGCATTCC-3′ (sense) and 5′-CCTCC-GGTTTCAGGAGGG-3′ (antisense); CD-RAP: 5′-TCCTATCTCCATGGCTGTGGCCCT-3′ (sense) and 5′-GCTACTGGGGAAATAGCCC-AG-3′ (antisense); and GAPDH: 5′-GGTGTGA-ACCACGAGAAATA-3′ (sense) and 5′-TGAAG-TCGCAGGAGACAACC-3′ (antisense). All primers recognize both mouse and rat transcripts.

Transient Transfection and Luciferase Assay

DNA transfections were performed using FuGENE 6^{TM} (Roche Molecular Biochemicals, Indianapolis, IN) transfection reagent as described previously [Okazaki et al., 2002]. Cells were plated at the following densities: 1×10^5 of C5.18 or ROS 17/2.6; 2.2×10^5 of RCS cells; or 1.5×10^5 of C2C12 cells /well of a 12 well dish and cultured overnight. Transfection mixture containing 3 μl of FuGENE 6^{TM}, 400 ng of various promoter constructs, and 100 ng pCMV-β-galactosidase were then added and the cells were further cultured for 48 h. In some experiments, various expression vectors were co-transfected as indicated. The total amount of DNA used was adjusted using empty vector. The cells were then harvested and the lysate was analyzed for luciferase activity. The β-galactosidase activities were also measured to normalize variations in transfection efficiency. Each transfection experiment was performed in triplicate and repeated at least twice.

Statistical Analysis

Statistical analysis was performed using one-way ANOVA with post-hoc test (Bonferroni/Dunn).

Generation of Transgenic Mice

The fusion genes, CD-RAP 3,345-*lacZ* and 3,345-bpΔ183-*lacZ*, were purified and microinjected into the pronuclei of fertilized eggs from B6SJL hybrid to generate transgenic mice, as described previously [Xie et al., 2000]. The surviving eggs were implanted into pseudo-pregnant foster mothers. Founder mice were identified by PCR of the genomic DNA extracted from tails. The *lacZ*-specific primers were as follows: 5′-GCATCGAGCTGGGTAATAAGCG-TTGGCAAT-3′ (sense) and 5′-GACACCAGAC-CAACTGGTAATGG-3′ (antisense), which are expected to amplify an 822-bp fragment. Four founder mice were obtained for each transgenic line. Transgenic mice lines were established and maintained by out-breeding with wild type mice. To analyze β-galactosidase expression, the positive founders or F1 males were mated with the wild type females and embryos were isolated at 13.5 or 14.5 days of gestation. The day of the vaginal plug was designated day 0.5 of gestation. Analysis was performed in at least five different litters for each transgenic line.

LacZ Staining and Immunohistochemistry

To assess the spatio-temporal expression pattern of the transgene, β-galactosidase activity was detected in the whole embryo as described previously [Xie et al., 2000]. Briefly, embryos were liberated from the uterus and fixed in 4% paraformaldehyde at 4°C for 10 to 15 min. After overnight incubation at 30°C in the staining solution (1 mg/ml X-gal, 4 mM $MgCl_2$, 0.15 M NaCl, 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, and 0.1% Triton X-100 in 0.1 M Phosphate Buffer), the positive embryos were photographed and post-fixed in 4% paraformaldehyde overnight at 4°C. In some experiments, the fixed embryos were embedded in paraffin and serial sections of 10 μm thick were prepared for immunohistochemistry. Immunohistochemistry was carried out using antibodies against β-galactosidase (Rockland, Gilbertsville, PA), human CD-RAP [Bosserhoff et al., 1997] and C/EBPβ (Santa Cruz). Briefly, sections were incubated in 3% hydrogen peroxide at room temperature for 30 min followed by digestion with 1% hyaluronidase at 37°C for 30 min. For detection of CD-RAP, the sections were incubated in 2% β-mercaptoethanol at room temperature for 20 min. After blocking with 10% normal goat serum, the sections were incubated with primary antibodies (1 μg/ml for anti-β-galactosidase and all of C/EBPs, 1:1,000 dilution for anti-CD-RAP) at 4°C overnight. Normal rabbit serum (1:1,000 dilution) or 1 μg/ml of purified normal rabbit IgG (Santa Cruz) was used for negative controls of primary antibodies. The primary antibodies were detected using a DAKO EnVision^{TM} System HRP kit (Dako Corp, Carpinteria, CA) and the sections were counterstained with Hematoxylin or Methyl green.

RESULTS

In Vitro Analysis of the 183 bp Element

We have reported that IL-1β induces C/EBPβ and δ in chondrocytes, and that the direct binding of C/EBPβ to the C/EBP motif represses the transcription of the CD-RAP gene. C/EBPs are reported to form heterodimers with different family members (C/EBPα or δ) or different isoforms of C/EBPβ (38, 36, and 20 kDa) [Akira et al., 1990]. In the C5.18 chondroprogenitor cell nuclear extract used in this study, we were able to confirm that various isoforms of C/EBPβ or δ and C/EBPα bind to the 183 bp element by supershifting with specific antibodies to C/EBPα, C/EBPβ, and C/EBPδ (Fig. 1A). The C/EBPα and δ supershifted bands cannot be seen on this exposure, but are implied from the removal of specific shifted bands. The multiple bands of C/EBPβ are considered to be caused by the different heterodimers, since all three isoforms of C/EBPβ are present in C5.18 nuclear extracts (Fig. 1B).

C/EBPs are Reciprocally Expressed With *CD-RAP*

We have shown that the DNA structure of the CD-RAP gene is such that it is available for transcription [Davies et al., 2004b], therefore, the activity of the 183 bp element is determined primarily by the presence of specific transcription factors. Consequently, we examined the mRNA levels of relevant transcription factors in the cells used in this study: the chondrocyte-like RCS; the chondroprogenitor C5.18; the fibroblast Balb 3T3; and the muscle progenitor line, C2C12 (Fig. 2). In each PCR reaction, cycle numbers and amount of template DNA were optimized to be in the linear range in order to semi-quantify the amount of transcripts between the cell types. Although Sox 5, 6, and 9 were all expressed in both chondroprogenitor cells and chondrocytes (C5.18 and RCS), CD-RAP mRNA was detected only in RCS cells. In contrast, C/EBPα and β were barely detected in RCS cells whereas they were strongly expressed in other cells. C/EBPδ was detected only in C5.18 cells. These PCR results are consistent with protein expression by Western blot analysis (data not shown). Therefore, the expression of CD-RAP in these cell lines is both positively correlated with expression of Sox 5, 6, and 9 and inversely correlated with the expression of C/EBPs. Immunohistochemistry for CD-RAP and C/EBPα, β, δ confirmed this expression pattern in vivo (data not shown). C/EBPβ and C/EBPδ were detected in the lower half of the growth plate where CD-RAP expression begins to decline [Davies et al., 2002]. C/EBPα was not detected (data not shown). Thus, the reciprocal expression of C/EBPβ and CD-RAP, both in vitro

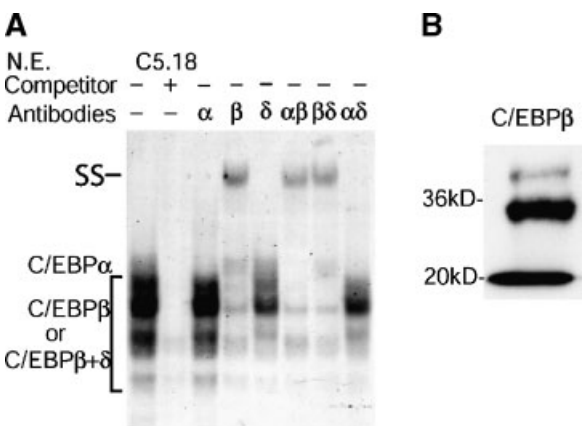

Fig. 1. C/EBP isoforms in C5.18 nuclear extracts. **A**: EMSA using a portion of the 183 spanning the C/EBP site as probe, and C5.18 nuclear extracts. Competitor 3 covers the C/EBP site. For suupershift analysis, antibodies for C/EBPα, β and δ were added in various combination as indicated for supershift analysis. C/EBPβ formed the predominant complexes. C/EBPα binding is shown as a weak band. C/EBPδ formed several bands as heterodimers with β. **B**: Western blot of C5.18 nuclear extract for C/EBPβ showing the presence of three isoforms of C/EBPβ: full length (38 kDa), LAP (36 kDa) and LIP (20 kDa).

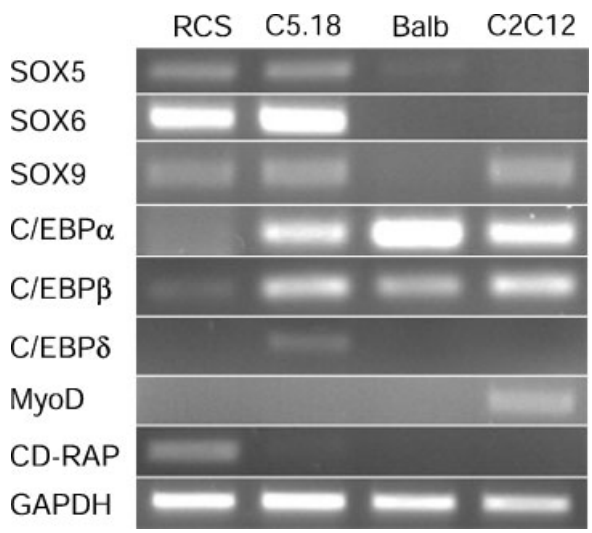

Fig. 2. Transcription factors in RCS, C5.18, Balb 3T3, and C2C12 Cells. We used RT-PCR to determine the presence of various transcription factors and CD-RAP in cell lines. Both chondrocyte-like cell lines, RCS and the chondroprogenitor cell line C5.18, express Sox5, 6, and 9. C5.18, Balb3T3, and C2C12 all strongly express C/EBPα and β whereas RCS barely express C/EBPβ and no C/EBPδ. CD-RAP mRNA is only detected in RCS cells.

and in vivo, is consistent with biochemical data, all demonstrating that the presence of C/EBP is inversely correlated with CD-RAP biosynthesis. The expression of the muscle-specific transcription factor Myo D was determined in C2C12 cells as a marker for potential muscle cell differentiation.

The 183-bp Element Acts as an Enhancer in RCS Cells and as a Repressor in C5.18 cells

Previously, we showed that site-directed mutagenesis of the C/EBP site within the 2,251-bp promoter increased the promoter activity twofold in C5.18 cells [Okazaki et al., 2002]. To test whether constructs with and without the 183 bp element containing the C/EBP site demonstrate cell-specificity, promoter constructs with (2251) and without (2068) the 183 bp element were transfected into RCS (Fig. 3A) and C5.18 cells (Fig. 3B). In RCS cells (CD-RAP positive; C/EBP negative), the 2,251 bp promoter activity was stronger than the 2,068 bp, consistent with the presence and activity of Sox proteins and absence of C/EBP in these cells (see Fig. 2). In C5.18 cells (CD-RAP repressed; C/EBP positive), the 2,251-bp promoter was weaker than the 2,068-bp. These results suggest that the 183-bp element present in the 2,251 bp promoter acts as an enhancer in RCS cells and as a repressor in C5.18 cells, and that C/EBP is responsible for the repressive effect of this element. That is, in the presence of C/EBP (C5.18 cells) and C/EBP binding sites (2,251 bp promoter) repression is observed; without C/EBP (RCS cells) expression is enhanced.

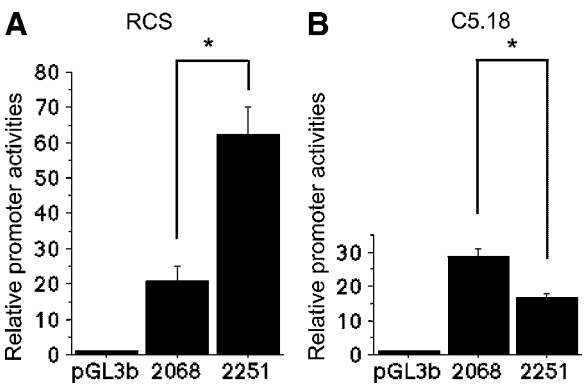

Fig. 3. Activity of the 183 bp element is dependent on cell line. The 183-bp element acts as an enhancer or a repressor depending on which cell type is transfected. The 2,251-bp-pGL3b or 2,068-bp-pGL3b was transiently transfected into RCS (**A**) or C5.18 cells (**B**). The activity of 2,251-bp promoter was stronger than that of 2,068-bp in RCS cells, but weaker in C5.18 cells. The activity of promoterless pGL3b was set as 1 ($P < 0.01$).

Removal of the 183 bp Element Relieves Repression of C5.18 Cells

In preparation for testing the function of the 183 bp fragment in transgenic mice, a longer promoter construct containing CD-RAP upstream DNA was tested by transient transfection in C5.18 cells. The rationale for choosing a longer construct for transgenic mice was two fold: first, merely truncating the 2,251 bp construct by 183 bp significantly reduced activity in vivo [Xie et al., 2000], and, secondly, the longer construct would contain more contextual DNA. We have recently shown that a similar construct demonstrated high activity in directing gene expression in RCS cells [Davies et al., 2004b]. In order to examine the function of the 183-bp element in the 3,345 bp promoter, the 183 bp fragment was deleted from the 3,345 bp promoter (Fig. 4A). The 3,345-bp, 3,345-bpΔ183, and 2,251-bp promoter constructs were tested by transfection into C5.18 or RCS cells (Fig. 4B,C). The 2,251-bp promoter was included so that the results could be compared to previous reports [Xie et al., 2000]. The 3,345-bp promoter had similar activity to the 2,251-bp promoter in both cell lines. Removal of the 183 bp fragment had no effect in RCS cells (Fig. 4B), but in C5.18 cells, removal of the 183 bp fragment increased expression by 2.5 fold (Fig. 4C). This result is consistent with the hypothesis that a repressive site (potentially C/EBP) was removed in the 3,345-bpΔ183 construct, thus permitting expression in cells that contain C/EBP proteins.

In Vivo the 3,345 kb Promoter Directs Cartilage-Specific Gene Expression Whereas the 3,345Δ183 kb Promoter is Widely Expressed

In order to explore the promoter activities of 3,345-bp and 3,345-bpΔ183-bp promoter in vivo, transgenic mice that harbor either 3,345-bp or the 3,345-bpΔ183 promoter linked to *lacZ* gene were generated. The X-gal staining analysis for whole embryo (day E14.5) revealed that the 3,345-bp promoter induced tissue-specific expression of the *lacZ* gene in cartilage primordium of rib, limb, scapula, nasal bone, temporal bone and caudal vertebra (Fig. 5A). In contrast, the 3,345-bpΔ183-bp promoter directed increased gene expression both in magnitude and range of tissues (Fig. 5B). Strong X-gal staining was also observed in the 3,345-bpΔ183-bp embryo in

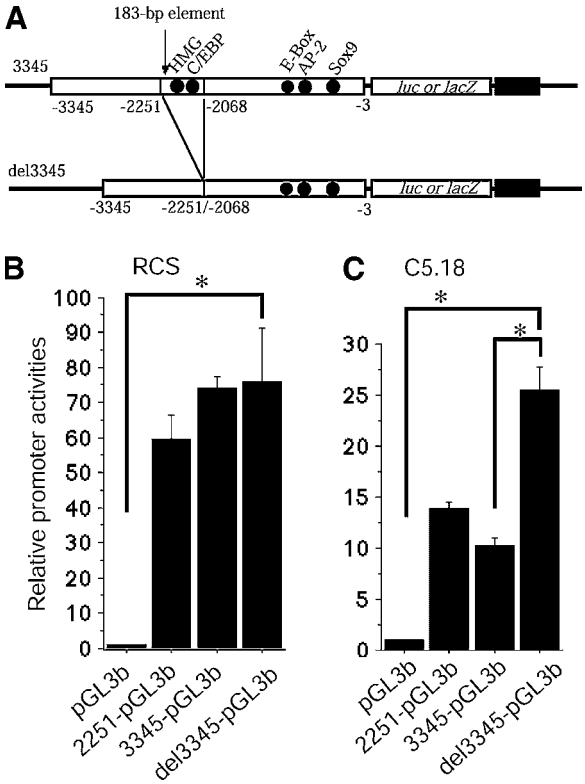

Fig. 4. Luciferase reporter constructs to test the 183 bp activity in vivo. **A**: Diagram of 3,345-bp and 3,345-bpΔ183 promoters linked to *luciferase* or *lacZ* gene reporter. The 183-bp element was removed from 3,345-bp promoter to make the deleted 3,345-bp promoter (3,345-bpΔ183). The *luciferase* gene (pGL3b) was used for transfection assays and *lacZ* gene (placF) was used for transgenic mice. The black box represents the polyadenylation site in the vector. Transfection of the 3,345-bp-pGL3b, the 3,345-bpΔ183-pGL3b and 2,251-bp-pGL3b in RCS cells (**B**) and transfection in C5.18 cells (**C**). When compared to the intact 3,345-bp construct, the expression of 3,345-bpΔ183 promoter was stronger in both C5.18 and RCS cells. The activity of promoterless pGL3b was set as 1. Each bar represents the mean ± SD ($P < 0.0001$).

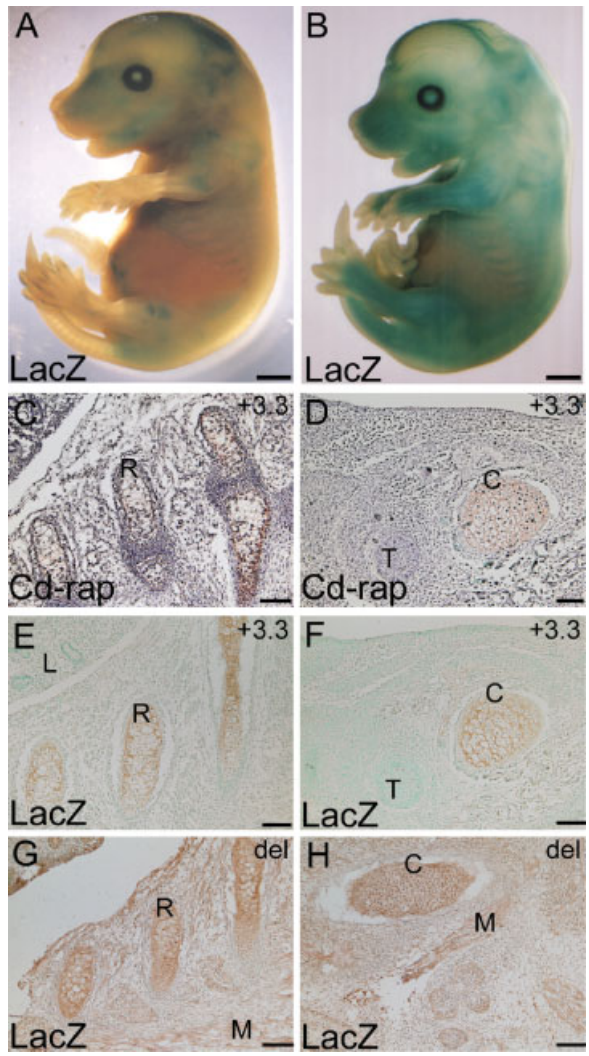

Fig. 5. Removal of the 183 bp domain in vivo. X-gal staining of embryo (day 14.5) of transgenic mice that harbors the 3,345-bp-*lacZ* (**A**) or the 3,345-bpΔ183-*lacZ* (**B**). The wild type 3,345-bp promoter generates lacZ expression in cartilage of ribs, shoulder, limbs, nose, temporal bone, and vertebra. In contrast, the 3,345-bpΔ183 promoter generates much broader expression. Note the more ubiquitous staining in limb and back suggesting the muscle staining in addition to cartilage. Immunohistochemistry for CD-RAP and LacZ in the E14.5 transgenic mice (**C**–**H**). Reddish/brown staining indicates positive immunoreaction. Sections are counterstained with hematoxylin or methyl green. Immunolocalization of endogenous CD-RAP (C, D). LacZ staining in the 3,345-bp promoter mouse (E, F). LacZ staining in 3,345-bpΔ183 promoter mouse in sections similar to those in A-D (G, H). The 3,345-bp promoter generates LacZ expression in cartilage (E, F) in the same distribution as that of endogenous CD-RAP (C, D) whereas the deleted promoter drives expression in muscle and other tissues in addition to cartilage (G, H). (C, E and G) rib cartilage; (D, F and H) Meckel's Cartilage; Cartilage (C), Tongue (T) Muscle (M). A–B: the bars represent 1 mm; (C–H) the bars represent 100 μm.

cartilages, as well as many other tissues. The limbs, face and ribs were positive for LacZ expression as well as soft tissues beneath the surface, suggesting expression in muscle. Consistent results were observed in different litters from four different founders of each transgenic line.

Localization of β-galactosidase was carried out to demonstrate the specific tissue expression pattern of the *lacZ* gene. The distribution of β-galactosidase driven by the wild type 3,345-bp promoter was detected by immunohistochemistry (Fig. 5E–H). The distribution of anti-β-galactosidase (Fig. 5E,F) was the same as endogenous CD-RAP protein detected with anti-CD-RAP antibody(Fig. 5C,D). The analysis

showed that the wild type 3,345-bp promoter directed gene expression in all cartilage primordia including limb, nasal bone, vertebra, rib (Fig. 5E), palatal shelf of maxilla, basisphenoid bone, and Meckel's cartilage (Fig. 5F, and unpublished data), but not in tongue (Fig. 5F) and surrounding tissues. In contrast, the 3,345-bpΔ183-bp promoter induced the expression of β-galactosidase in cartilages and muscle (Fig. 5G,H), as well as epithelial cells of the lung, myoblasts in muscle, and hepatocytes in the liver (Fig. 6A,B and data not shown).

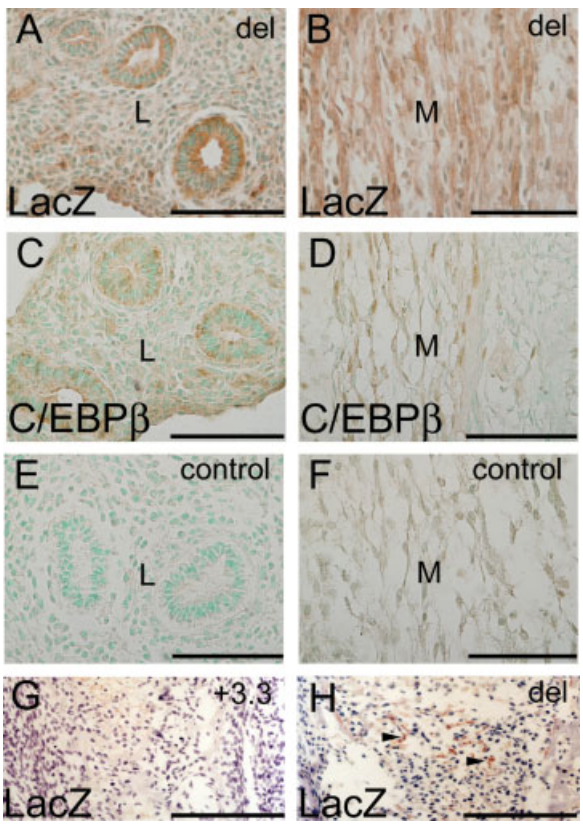

Fig. 6. Expression of the 3,345-bpΔ183 transgene and C/EBPβ. **A**: LacZ localization in 3,345-bpΔ183 (del) promoter mouse showing reactivity in the lung tissue, both in epithelial cells and surrounding tissue. **B**: LacZ localization in the 3,345-bpΔ183 promoter mouse showing reactivity in muscle. **C**, **D**: C/EBPβ localization in the nuclei of lung epithelium and surrounding cells (L), and in muscle (M). **E**, **F**: Negative control showing no staining of LacZ in wild type 3,345-promoter mice. **G**: Endochondral ossification of radius in 3,345-bp-*lacZ* mouse (embryonic day 15.5) immunostained for LacZ. **H**: Endochondral ossification of radius in 3,345-bpΔ183-*lacZ* mouse (embryonic day 15.5) immunostained for LacZ. In the endochondral ossification of 3,345-bpΔ183 promoter mouse, LacZ expression was observed in newly synthesized matrix around osteoblasts (H, arrowheads) whereas no LacZ staining is observed in the wild type 3,345-bp mice (G). Bars represent 100 μm.

Since we hypothesized that repression in non-cartilage tissue was due to functional C/EBP binding, the localization of C/EBPβ protein was also examined. C/EBPβ was detected in the nuclei of all cells where repression was abrogated by deletion of the 183 bp element: epithelial cells in lung (Fig. 6C), muscle cells, (Fig. 6D) and in liver cells (data not shown) in the same tissue distribution as that of β-galactosidase protein in the 3,345-bpΔ183 mice (Fig. 6A,B). In the cell cytoplasm center, newly synthesized osteoids were also positive for β-galactosidase in the 3,345-bpΔ183 mice (Fig. 6H) but not in the wild type 3,345 mice (Fig. 6G), suggesting that the 3,345-bpΔ183 promoter was no longer repressed in osteoblasts.

The C/EBP Site is Responsible for Repression of the *CD-RAP* Promoter in C2C12 Myoblastic Cells

The results from transgenic experiments suggest that the repressive activity of the 183 bp element is responsible for the lack of expression in non-cartilaginous tissues. We hypothesized that this repressive activity was due to the binding of C/EBP. Because the 3,345-bpΔ183 promoter induced LacZ gene expression in muscle cells, in vivo, the function of the 183-bp element and the C/EBP binding site were examined in C2C12 myoblasts, a non-cartilaginous cell line. C2C12 cells were transfected with wild type 3,345 CD-RAP promoter, the 3,345Δ183 promoter, and a promoter in which the C/EBP site was inactivated (Fig. 7A). The 3,345-bp promoter construct was not active in C2C12 cells (Fig. 7B). However, the 3,345-bpΔ183 promoter demonstrated approximately 3.7-fold activation. Moreover, a 2-bp inactivating mutation at the C/EBP site (C/EBP mu3345-pGL3b) [Okazaki et al., 2002] yielded a promoter with even higher activity, about sevenfold higher than the wild type 3,345-bp. These results show that within the context of the 3,345-bp long promoter, the elimination of C/EBP binding at a single site in the DNA, increased gene expression in muscle cells by almost an order of magnitude compared to the wild type DNA. To confirm the inhibitory effect of the isolated element, the 183-bp module was also cloned into the PGL3 promoter vector and transfected into C2C12 cells. Inclusion of the 183 bp-module in pGL3pr reduced activity of the vector by 75%. Therefore, the 183-bp element acted as a repressor in C2C12 myoblasts and was

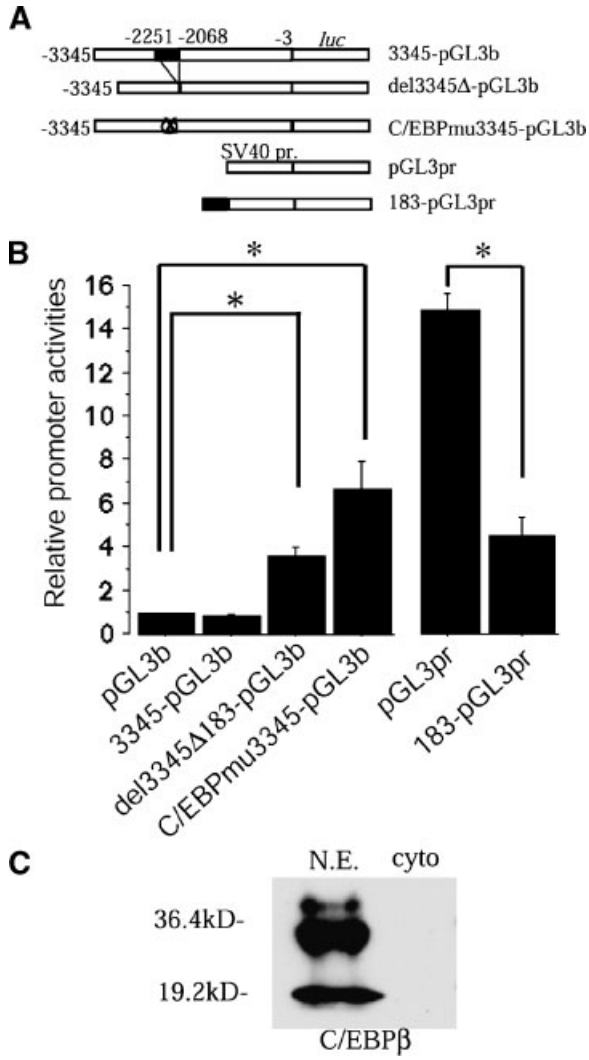

Fig. 7. Mutation of C/EBP DNA binding site relieves repression in C2C12 Cells. **A**: Diagram of promoter constructs used for transfection. C/EBPmu3,345-bp-pGL3b has a two-bp mutation in the C/EBP site at −2,078 and −2,079-bp within the 3,345-bp promoter that abrogates C/EBP binding in vitro [Okazaki et al., 2002]. **B**: Transient transfection of various constructs into C2C12 myoblasts. The activity of the promoterless pGL3b is set as 1. Each bar represents the mean ± SD ($P < 0.0001$, $^*P < 0.05$). **C**: Western blotting for C/EBPβ protein in C2C12 nuclear and cytoplasmic extracts. Three isoforms of C/EBPβ (full length 38 kDa, LAP 36 kDa, and LIP 20 kDa) were observed in the nuclear extract.

dependent upon the C/EBP binding site for this activity. The presence of endogenous C/EBPβ isoforms was confirmed in C2C12 nuclear extracts by Western blot analysis (Fig. 7C).

DISCUSSION

We have identified a regulatory module that can direct gene expression in chondrocytes and repress expression in muscle, bone and other tissues. Studies in vitro demonstrate that the activity of the regulatory module is dependent on the presence of specific transcription factors, specifically members of the Sox and C/EBP protein families. Removal of this chondrocyte regulatory module from a 3,345-bp promoter abrogates tissue specific expression in vitro, and, in transgenic mice, permits expression in muscle, bone, and other tissues where it would not normally be expressed. Other transcription factors that bind to this DNA domain may also contribute to redundant or additional regulation, such as the negative factor αA-crystallin binding protein 1 (CRYBP1) [Tanaka et al., 2000] located close to the C/EBP site in Col2al and CD-RAP genes. The function of this putative chondrocyte-regulatory module supports an emerging paradigm where gene repression is mediated by lack of gene activation coupled with active repression [Barolo and Posakony, 2002].

Many studies have demonstrated the ability of the enhancers of *Col2a1* and *Col11a2* to direct cartilage-specific gene expression. In *Col2a1*, a 48-bp core enhancer was defined [Lefebvre et al., 1996]. Eight copies of this 48-bp core enhancer directed gene expression in cartilage in vivo, suggesting this element is sufficient to generate cartilage-specific expression [Zhou et al., 1998]. Positive expression was attributed to Sox protein binding. However, unrecognized at that time was the mechanism of negative regulation in non-cartilage tissues. In *Col11a2*, two different elements have been identified by different laboratories: a 60-bp element in the first intron [Liu et al., 2000] and multiple HMG sites between −620-bp to −489-bp in the 5′ region [Bridgewater et al., 1998]. Multiple repeats of the 60-bp intronic element directs cartilage-specific gene expression [Liu et al., 2000], similar to the 48-bp enhancer of *Col2a1*. Mutation of the HMG site at −502-bp in the 5′ region of *Col11a2* eliminates the Sox9 binding resulting in loss of gene expression in cartilage [Bridgewater et al., 1998]. These previous studies were designed to identify positive regulators, and showed that all three cartilage-specific elements are positively regulated by Sox proteins: Sox9, L-Sox5, or Sox6. The role of a repressor element in non-cartilaginous tissues was not appreciated in these studies, although it was most probably active. Indeed, the lack of expression in non-cartilage tissues when the *Col2a1* or *Col11a2* elements were used

to drive tissue-specific expression, strongly suggests that repressive sites were functional in non-cartilaginous cells. All of these modules that direct cartilage specific expression also contain potential C/EBP binding motifs.

We hypothesized that the negative motif responsible for repression is the C/EBP binding site. This hypothesis arises from our recent findings that (1) the negative response induced by IL-1 functions through a C/EBP site in the CD-RAP 183-bp domain [Okazaki et al., 2002]; (2) the current data showing that mutation of the CEBP site in the context of the 3,345 bp promoter activates gene expression in myoblasts; and (3) the expression pattern of C/EBP proteins in vitro and in vivo. The core sequence for binding of C/EBP family members is T(T/G)NNGNAA [Akira et al., 1990]. We found that the 48-bp enhancer of *Col2a1* also contains a C/EBP motif-like sequence (TTGAGAAA) next to the multiple HMG-like sites, although the function of C/EBP at this specific site has not been investigated. Moreover, in *Col11a2*, both of the cartilage-specific regulatory elements in the first intron [Liu et al., 2000] and 5′ promoter region [Bridgewater et al., 1998] contain TTTCCTCA and TTTTCAAA, respectively, that are also similar to the C/EBP motif, located in a position overlapping or close to functional HMG sites. In the 530-bp promoter of *Col11a2*, deletion of the cartilage-specific regulatory element (453-bp promoter) resulted in random tissue expression in transgenic mice [Tsumaki et al., 1998], but no expression in cartilage. The similarity of cartilage-specific regulatory elements among these genes and the lack of specificity generated by the deleted promoter in the current study imply the importance of repressor function coincident with HMG-binding sites as a common role of cartilage-specific gene regulation. Two other functional negative regulatory elements; CRYBP1 [Tanaka et al., 2000] and δEF-1 [Murray et al., 2000] also demonstrate inverse correlations with cartilage Col2a1 expression. These elements have not been tested in vivo.

The 183-bp element was necessary in the 2,251-bp CD-RAP promoter to produce expression in cartilage since the 2,068-bp promoter did not exhibit gene expression in the previous transgenic mice study [Xie et al., 2000]. In the longer 3,345-bp promoter used in this study, deletion of the 183-bp element resulted in a high level of non-specific gene expression. Therefore, the 183-bp element participates in both positive and negative tissue-specific expression. A specific inactivating mutation of the C/EBP binding site increased the promoter activity of −3,345-bp promoter in C2C12 myoblasts, suggesting that the C/EBP site within the 183-bp element is necessary for repression in myoblasts. We have recently reported that IL-1β down-regulates both CD-RAP and *Col2a1* through induction of C/EBPβ and δ [Okazaki et al., 2002], whereas Murakami et al. [2000] have shown that IL-1β represses *Col2a1* activity through repression of *Sox9*. This reciprocal switch in transcription factor expression is consistent with our hypothesis that the balance of transcription factors capable of binding at the chondrocyte regulatory module will determine the level of gene expression. This hypothesis is also supported by our RT-PCR results showing that neither C5.18 nor C2C12 cells express CD-RAP although these cells do express Sox9 and C518 cells synthesize Sox5 and Sox6. We believe this is due to the presence of C/EBPs in both cell types (Fig. 2).

Recently, we [Imamura et al., 2005] and others [Tsuda et al., 2003] have shown that the nuclear co-regulators, p300/CBP can alter the availability of Sox9 and C/EBP. In COL2A1 [Tsuda et al., 2003] and CD-RAP [Imamura et al., 2005], p300/CBP acts to increase binding of Sox9 to DNA, while inhibiting binding of C/EBP to DNA. Consequently, p300/CBP helps to increase cartilage gene expression by enhancing the positive transcription factor (Sox9) and inhibits the negative factor (C/EBP). C/EBPβ and δ were detected in osteoblasts and C2C12 myoblasts. C/EBPβ and δ may contribute to the repression of chondrocyte matrix genes, such as *Col2a1* and CD-RAP, as well as activation or maintenance of the osteoblastic or myoblastic phenotype. For example, in osteoblasts, C/EBPβ and δ act synergistically to activate osteocalcin by specific interaction with runt-related gene/core-binding factor α1 [Gutierrez et al., 2002]. The mechanism of this interaction is thought to be through the C/EBP binding site and not through the runt-related gene/core-binding factor α1 site. This regulatory mechanism may be important in osteoblast differentiation as C/EBPβ and δ are expressed in skeletal tissues and are developmentally regulated during osteoblast maturation. In addition, C/EBPβ and δ are responsive to vitamin D_3, a positive regulator of osteoblast differentiation and

osteocalcin gene expression [Gutierrez et al., 2002]. The addition of 183-bp element upstream of a SV-40 promoter resulted in repression of promoter activity in C2C12 cells. The 3,345-bpΔ183 promoter was expressed in osteoblasts at the endochondral ossification center (Fig. 6H) Consequently, in bone, the presence of C/EBP proteins favors the expression of bone genes, while repressing cartilage genes.

In summary, we show that the 183-bp element of the CD-RAP promoter is essential for both positive and negative gene expression in cartilage. Additionally, the 183-bp element repressed the SV-40 promoter in myoblasts and mutation of the C/EBP site abrogated repression of the CD-RAP promoter in myoblasts. These results strongly suggest that the 183-bp element, in particular the C/EBP binding site, acts as a repressor to insure that specific genes are not expressed in C/EBP-containing, i.e., non-cartilaginous cells. Lastly, the arrangement of positive and negative *cis*-regulatory motifs in the 183 bp element is similar to other cartilage-specific enhancer elements, and, consequently, these elements may be considered a chondrocyte-regulatory module, enhancing gene expression in chondrocytes and repressing in non-chondrocytes.

ACKNOWLEDGMENTS

We thank Dr. Richard Pierce for critical reading of this manuscript. We are grateful to Mr. Carl Franz for help in preparing this manuscript.

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