The Transcription Factor δEF1 Is Inversely Expressed with Type II Collagen mRNA and Can Repress Col2a1 Promoter Activity in Transfected Chondrocytes*

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The regulation of Col2a1, which encodes type II collagen, likely results from a balance of both positive and negative proteins. Here we present evidence that the transcription factor δEF1 participates in the negative regulation of Col2a1 transcription. A deletion analysis suggested that a region between −100 and −307 of the rat Col2a1 gene was required for activity in differentiating chick limb bud mesenchymal cells; however, mutation of a conserved E2 box site in this region actually increased promoter activity. Supershift analysis demonstrated that δEF1, a known transcriptional repressor, bound to the E2 box in a sequence-dependent manner. Chick limb bud mesenchymal cells, which do not express type II collagen, expressed abundant δEF1 mRNA, but, following differentiation in micromass culture, δEF1 mRNA expression was lost. Primary embryonic chick sternal chondrocytes, which express abundant type II collagen, displayed minimal levels of δEF1 mRNA. The inhibition of Col2a1 transcription following treatment of chick sternal chondrocytes with growth factors was accompanied by increased δEF1 expression. Overexpression of δEF1 in differentiated chondrocytes resulted in decreased expression of a reporter construct containing a collagen II promoter/enhancer insert; however, this negative regulation was not dependent on the proximal E2 box. This is the first report of a specific transcription factor involved in the negative regulation of Col2a1.

Type II collagen, a major structural protein of the cartilage extracellular matrix, is encoded by the Col2a1 gene (1, 2), which is transcribed at a high level in chondrocytes (3–6). In addition to the activation of Col2a1 transcription during chondrogenesis, a variety of factors have been reported to either stimulate or inhibit type II collagen synthesis in differentiated chondrocytes, such as specific growth factors (7, 8) and retinoic acid (9). The developmental pattern of expression of the Col2a1 gene and the fact that its transcription rate is subject to modulation by many factors suggest that the steady-state expression of this gene results from a balance between positive and negative regulatory proteins interacting with several different cis-acting DNA sequences.

The isolation of the Col2a1 gene has allowed investigators to identify and study the cis-acting elements believed to participate in transcriptional regulation of this gene. Previously, an enhancer element was discovered in the first intron of the rat gene that was required for chondrocyte-specific expression in vitro (10, 11). Other recent studies have identified additional cis-acting regulatory sequences in the first intron of the gene and identified specific transcription factors involved in regulating Col2a1 transcription (12–15). However, relatively little work has been reported on characterizing cis-acting DNA elements or regulatory proteins operating in the 5′-flanking region of the gene. Silencer elements are located in the regions −300 to −400 and −620 to −700 bp (1) that are believed to function in inhibiting synthesis of type II collagen in non-chondrocytes (16). In addition, Sp1 binding sites in the proximal promoter are believed to be important for efficient enhancer-mediated transcription (17) and Sp1 binding activity differs between differentiated and dedifferentiated chondrocytes (18). Although it has been reported that a fragment from the first intron enhancer can direct chondrocyte-specific expression through a heterologous promoter (12), it is likely that in vivo, the endogenous Col2a1 promoter also participates in gene regulation during certain developmental stages and in response to specific regulatory signals. In fact it has been reported recently that both positive and negative elements are located in the 5′-flanking region of the human COL2A1 gene and participate in developmental stage- and tissue-specific expression in transgenic mice (19).

In addition to the silencer elements and Sp1 sites, the proximal promoter of the rat Col2a1 gene also contains other putative regulatory sequences including E boxes (CANNTG) which bind basic helix-loop-helix transcription factors (20, 21). Also present in the promoter region is a subset of the E box motif known as the E2 box (CACCTG), which is an example of a cis-element containing both repressor and activator binding sites (20, 22). Specifically, the E2 box binds positive activator molecules from the basic helix-loop-helix family (23, 24), while δEF1 (22). δEF1 is a zinc finger homeodomain protein first identified as a protein that binds to an enhancer element in the third intron of the 61 crystalline gene (25, 26). Its ability to compete with bHLH activators for binding to the E2 box, and its function as a repressor molecule during development has...
been documented (22). A recent study determined that \( \Delta E F 1 \) was expressed at a high level in the early limb mesenchyme and that its expression was lost in these cells after condensation and the initiation of chondrogenesis (27). This pattern of expression suggests that \( \Delta E F 1 \) is involved in skeletal patterning during limb development and may function as a negative regulator of chondrocyte-specific genes. In the same report, it was shown that mice with a null mutation in \( \Delta E F 1 \) displayed a variety of limb and skeletal defects.

Here we present evidence that \( \Delta E F 1 \) binds to a CACCT sequence contained within an E2 box motif that is conserved in position and sequence in the proximal promoter region of the Col2a1 gene across several species. Mutation of this sequence actually increased transcriptional activity of the rat Col2a1 promoter in differentiating chick limb bud mesenchymal cells. In addition, the \( \Delta E F 1 \) pattern of expression inversely correlated with that of type II collagen in differentiating limb bud mesenchymal cells and in growth factor-modulated chick sternal chondrocytes. Finally, \( \Delta E F 1 \) expression inhibited the activity of a co-transfected Col2a1 reporter construct, although the conserved proximal E2 box was not required to mediate this suppression. Collectively, these results suggest that \( \Delta E F 1 \) either directly or indirectly participates in the negative regulation of Col2a1 transcription. This is the first report to identify a specific transcription factor involved in the negative regulation of type II collagen gene expression.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Nuclear Extract—** Chick limb bud mesenchymal cells were isolated from the limb buds of day 3 chicken embryos as described previously (10) and cultured in micromass culture at 0.5 x 10^6 cells/100-μl spot to induce chondrogenesis (10) where indicated. Primary chondrocytes were isolated from the sternum of day 16 chicken embryos as described previously (9) and cultured in Ham’s F-12 medium with 10% fetal calf serum. In order to down-regulate the expression of type II collagen (28), the cells were treated for 48 h with 10 ng/ml BFGF (R&D Systems) and 2 ng/ml TGF-β (R&D Systems).

Nuclear extracts were prepared as described previously (11, 29).

**Northern Blot Analysis—** Northern analysis of Col2a1 mRNA was performed as described previously (10). In brief, total RNA isolated from chick limb bud mesenchymal cells after various times in micromass culture was reverse transcribed and PCR-amplified with primers specific for Col2a1. The PCR-amplified products were fractionated on 1% agarose gel containing 2.2 M formamide and 20 mM MOPS and subsequently blotted onto nylon hybridization transfer membrane (GeneScreen, NEN Life Science Products). Hybridization was performed using a random prime-labeled cDNA probe for \( \Delta E F 1 \) (\( \Delta E F 1 \) cDNA probe was supplied courtesy of Dr. Hisato Kondoh, Institute of Molecular and Cell Biology, Osaka, Japan) or a cDNA probe for pro-α1(II) collagen (9) in 50% formamide, 0.1% SDS, 5 x SSC, 1 x Denhardt’s, 50 mM potassium phosphate buffer (pH 6.8), and 0.25 μg/ml denatured salmon sperm DNA at 42 °C for 16 h. The filter was then washed with 0.1% SDS and 2 x SSC for 20 min at 42 °C and exposed to Kodak XAR-5 film. The resulting autoradiograms were scanned and quantitated using the NIH densitometry program. The specific signal for either \( \Delta E F 1 \) or collagen II mRNA was normalized to the level of 28 S RNA for each sample to control for differences in the total amount of RNA loaded.

**Electromobility Shift Assays and Supershift Analysis—** To determine the presence of DNA-binding proteins in the nuclear extracts of chick limb bud mesenchymal cells, chick sternal chondrocytes, and growth factor-treated chick sternal chondrocytes, we used several different double-stranded oligonucleotide probes, which contained 6 \( \Delta E F 1 \) binding sites (E2 boxes) plus the unique flanking DNA from different regions of the Col2a1 promoter. These probes were used in electromobility shift assays (EMSA). In addition, we used oligonucleotide competitors with either an intact or mutated E2 box site in competition EMSA experiments. The oligonucleotide probes utilized in this study were synthesized on a DNA synthesizer (Integrated DNA Technologies). The sequences of the oligonucleotides used in all binding reactions are as follows (the \( \Delta E F 1 \) site is underlined in each case).

Probe 1 represents the proximal E2 box located at −117 in the rat promoter: WT 26-mer sense strand, 5'-TGGGACCTCTGCCAGGGTTTGCGCTG-3'; WT 22-mer antisense strand, 5'-CGAAGCCCAACCTGGCAAGGG-3'. For competition for probe 1: WT 1 sense strand, 5'-CTTGGCACGGTTTGCGCTG-3'; WT 1 antisense strand, 5'-AGCCCAAAGCTGGGAAAG-3'; mutant 1 sense strand, 5'-CTTGGACGGAGTTGGGCTG-3'; mutant 1 antisense strand, 5'-GGCCACCTGGGAAAG-3'.

Probe 2 represents the distal E2 box located at −560 in the rat promoter: sense strand, 5'-TGGGACCTCTGCCAGGGTTTGCGCTG-3'; antisense strand, 5'-AGCCCAAAGCTGGGAAAG-3'. For competition for probe 2: WT 1 (16) sense strand, 5'-AATCCCCATCCCCACCTGGGAAAG-3'; antisense strand, 5'-TTGAGGGTTAGGGGTTGG-3'; Probe 3 represents the silencer sequence located at −659 in the rat promoter (16): sense strand, 5'-CTTGGACAGCAGACCCTGGCAGCAGAGAGC-3'; antisense strand, 5'-GATCCGAGTCGTGTTGGAGGTG-3'.

The single-stranded sense and antisense oligonucleotides were annealed in equimolar amounts. The double stranded WT probe was subsequently labeled with Klenow DNA polymerase and [α-32P]dATP (3,000 Ci/mmol, Amersham Pharmacia Biotech). The unincorporated label was separated from the labeled oligonucleotide using a Sephadex G-50 gravity flow column (Amersham Pharmacia Biotech). The DNA binding assay was performed as follows. Three μl of nuclear protein fraction from chick limb bud cells, chick sternal chondrocytes, or growth factor-treated chick sternal chondrocytes was preincubated for 10 min at room temperature in a final volume of 20 μl containing 50 mM Tris-HCl, pH 7.9, 12.5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, and 20% glycerol (TM buffer) and 5 μg of poly(dT)-dA. Subsequently, 1.0 μl of the probe (100,000 cpm) was added and incubated for 30 min at room temperature. For supershift studies, the anti-ΔEF1 antibody (supplied courtesy of Dr. Hisato Kondoh) or the anti-N-FAT antibody (Santa Cruz Biotechnology) was incubated with the nuclear extract for an additional 20 min prior to the addition of the labeled probe. The ΔEF1 antibody is a polyclonal antibody raised in a rabbits against recombinant chicken protein expressed in Escherichia coli (26). This antibody recognizes chicken, mouse, and human versions of ΔEF1, and has been successfully used for Western blotting, EMSA supershift studies, and immunohistochemistry (26). For competition assays, a 20–50-fold molar excess of the indicated unlabeled double-stranded competitor oligonucleotide was preincubated with the nuclear extracts in the binding reaction prior to the addition of the probe. In all cases, the final binding reaction mixture was spotted onto a 5.5% nondenaturing acrylamide gel in 1 x TBE buffer and electrophoresed at 120 V. Gels were dried and analyzed by autoradiography.

**UV Cross-linking—** To determine the approximate size and number of proteins binding to the proximal E2 box site, we used an 18-mer probe with the following sequence: sense strand, 5'-CTTGGACCGTTGTGGGCTGCT-3'; antisense strand, 5'-AGCCCAAAGCTGGGAAAG-3'. The probe was end-labeled using T4 polynucleotide kinase (Promega) and [γ-32P]dATP. Each binding reaction, containing 3 μg of a specific nuclear extract (total volume = 20 μl) was preincubated in TM buffer containing 5 μg of poly(dI-dC). Subsequently, 1.0 μl of the probe (100,000 cpm) was added and incubated for 30 min at room temperature. The binding reaction was then spotted on a paraffin membrane and exposed to short-wave UV light for 5 min at 4 °C. The resulting UV-cross-linked products were mixed with 2 x SDS loading buffer, boiled for 3 min, and resolved on a 10% SDS denaturing polyacrylamide gel.

**CAT Reporter Constructs—** The plasmid constructs pCI-1800, pCI-307, and pCI-100 refer to plasmids pCI1, pCI2, and pCI6, respectively, which have been previously described (10). Briefly, these constructs contain various length 5'-flanking sequences from the rat Col2a1 gene upstream of the CAT coding sequence along with a 1500-bp region from the first intron that has enhancer activity.

**Cell Transfection and CAT Assay—** Four spots, each containing 0.5 x 10^6 stage 24/25 chick limb bud mesenchymal cells/100 μl, were transfected with 10 μg of the indicated DNA by the calcium phosphate precipitation method (23) 2–5 h after plating. For ΔEF1 DNA overexpression, 3 x 10^6 cells per micromass culture studies, 3 x 10^6 cells for nuclear extract studies, or 10^5 cells for chick or the chick limb bud mesenchyme cells as described above were co-transfected with 5 μg of the indicated Col2a1 reporter construct along with 5 μg of the indicated Col2a1 reporter construct along with 5 μg of control vector pCMVX-pUC19 or 5 μg of the ΔEF1 cDNA expression vector pCMVX-ΔEF1 (both constructs were supplied courtesy of Dr. Hisato Kondoh). In both cases, the DNA-calciuim phosphate precipitate was left on the cells for 3 h, and the cells were harvested 48 h
Fig. 1. Col2a1 promoter activity in differentiating chick limb bud mesenchymal cells. A, the Col2a1 constructs used in the transfection studies. In all cases the constructs contained a 1500-bp region from the first intron of the rat Col2a1 gene that has chondrocyte-specific enhancer activity. B, activity of the indicated constructs following transfection into primary chick limb bud mesenchymal cells in micromass culture. The graph displays the mean and standard error from at least three separate experiments after transfection and cell extracts from equal cell numbers were assayed for CAT activity with [14C]chloramphenicol as described previously (10). All transfections were done in triplicate in at least three separate experiments using different preparations of plasmid DNA. The resulting autoradiographs were scanned and quantitated using the NIH Image densitometry program. Note that the normalization of CAT activity to a second reporter construct to control for transfection efficiency was not required because the relative level of activity of the various promoter constructs were only compared with each other within a given cell type or time point and replicate experiments were conducted to assess reproducibility of the results.

Site-directed Mutagenesis—The overlap-extension polymerase chain reaction method (31) was used to generate both the 307-bp site-directed mutant promoter constructs from the parent wild type pCII-307 promoter construct. The oligonucleotide primers used to generate this mutant are as follows: left arm, internal primer, 5'-CTGAGACCCCGGCCCCGGAGCAGGCTGGCCATTACATTTCCAAAGCCGAGCCTTCTTGGTGAAGCCCGCC-3'; left arm, end primer, 5'-CAGATCTGGGGCCGGAGGCCCTCTTCTCGCTGTGG-3'; right arm, internal primer, 5'-GGGCTGTCACCAAGGGGCGACTGCGCTTGGATGTATGGGCTCTGGTCCGACCTGGGACGGCGGCGG-3'; right arm, end primer, 5'-GGGCTGTCACCAAGGGGCGACTGCGCTTGGATGTATGGGCTCTGGTCCGACCTGGGACGGCGGCGG-3'.

The terminal NdeI and HindIII sites engineered into the left and right arm end primers were used to clone in the amplified fragment into the parental CAT vector. The specificity of the mutation was confirmed by sequence analysis.

RESULTS
Deletion Analysis of the Rat Col2a1 Promoter—Our initial goal was to identify the regions in the Col2a1 promoter required for enhancer-mediated transcription in differentiating chick limb bud (CLB) mesenchymal cells. We utilized this cell type in order to identify positive-acting transcription factors possibly present at high levels during the initial activation of type II collagen expression. The constructs tested contained successive 5' promoter deletions upstream from the CAT cDNA along with a 1500-bp first intron sequence from the rat Col2a1 gene (Fig. 1A) that has enhancer activity in differentiated CLB mesenchymal cells (10). The relative promoter activity of each construct was assessed by measuring the CAT activity in extracts from primary CLB mesenchymal cells placed in micromass culture for 48 h to induce differentiation (10) following transfection with the various constructs. A 1500-bp deletion from positions −1800 to −307 did not result in decreased CAT activity (Fig. 1B, compare pCII-307 with pCII-1800). In fact, the activity of the pCII-307 construct was consistently higher than the pCII-1800 construct. However, a construct containing only 100 bp of 5'-flanking sequence (pCII-100) averaged only 35% of the activity observed with the 307-bp construct (Fig. 1B). These results suggested that the information contained in the 307-bp Col2a1 promoter construct was sufficient to direct enhancer-mediated transcriptional activity in differentiating CLB mesenchymal cells and that specific sequences between position −100 and −307 were required for enhancer-mediated promoter activity in these cells.

A comparison of the proximal 307-bp promoter region of the Col2a1 gene from different species revealed a high degree of conservation in terms of putative regulatory sequences (Fig. 2). This region contains two E box motifs (CANNTG) in the rat, mouse and human genes. The rat and mouse genes each have a conserved distal E box element (CAAGTG), while the human gene has a more proximal E box element (CAGCTG) at approximately position −50 relative to the TATA box. Interestingly, all three genes share a common E box conserved in both location and sequence (CAGCTG) that is flanked by a conserved array of Sp1 sites. The complementary strand of this specific E box motif (CACCTG) was described previously as the E2 box (20, 22), which also has an overlapping ΔEF1 recognition site (CACCT).

Site-directed Mutagenesis of the Proximal E2 Box of the COL2A1 Promoter—Based on the above functional data and the high degree of conservation of the proximal E2 box, we determined the activity of a construct that contained 307 bp of 5'-flanking sequence with a site-directed mutation in this element (pCII-307M, Fig. 1A). We predicted that a mutation in this region might result in loss of promoter activity in the CLB mesenchymal cells. However, in several independent experi-
ments, there was a consistent increase in the activity of the pCH-307M construct compared with the wild type construct (Fig. 1B). Although the increase was only approximately 30% over the wild type, it was observed with several different preparations of DNA and was repeated with different primary cell isolations. This surprising result suggested that sequences within the E2 box were involved in negative regulation of the Col2a1 promoter activity in the differentiating CLB mesenchymal cells. Taken together, the results from the deletion analysis and the site-directed mutagenesis studies suggested that the region between −100 and −307 contain sequences that are involved in both the positive and negative regulation of Col2a1 promoter activity in differentiating limb bud mesenchymal cells.

Nuclear Proteins Interact with the Conserved E2 Box—The E2 box contains a sequence known to bind both positive and negative transcription factors in other cell types (22–24). These published studies and the fact that a mutation in proximal E2 box enhances promoter activity in differentiating limb bud mesenchymal cells. Taken together, the results from the deletion analysis and the site-directed mutagenesis studies suggested that the region between −100 and −307 contain sequences that are involved in both the positive and negative regulation of Col2a1 promoter activity in differentiating limb bud mesenchymal cells.

Fig. 2. The proximal region of the Col2a1/COL2A1 gene is highly conserved. Note the E2 box that is conserved both in location relative to the TATA box and in specific sequence. The dashed line within the proximal E2 box delineates the binding site for δEF1.

To analyze the specific sequences within the 26-mer probe responsible for the formation of the observed complexes, we performed a competition EMSA. We used two different unlabeled 18-mer oligonucleotides at 50-fold molar excess to the probe as competitors. We utilized this level of competitor DNA in order to clearly show competition while avoiding the likelihood of nonspecific effects as indicated by additional studies varying the molar ratio of the competitor oligonucleotides (data not shown). The competitors were preincubated with nuclear extract from CLB mesenchymal cells, which produced a binding pattern that included all four complexes (Fig. 4, lane 1, control). The 18-mer oligonucleotide competitor with sequences identical to the labeled probe competed for all four complexes (Fig. 4, lane 2, WT competitor 1). In contrast, the 18-mer oligonucleotide competitor with a single base pair change (CACCTG to CATCTG) that abolished the E2 box (specifically the δEF1 recognition motif, see below) but maintained an E box motif (CANNTG), competed for the formation of bands C, D, and to a lesser extent, band B, but did not compete for the formation of band A (Fig. 4, lane 3, competitor M-1).

Characterization of E2 Box-binding Proteins by UV Crosslinking Studies and Supershift Analysis—The EMSA results suggested that one or more proteins from CLB mesenchymal cells formed a complex with the E2 box. We next concentrated on determining the approximate molecular mass and number of DNA-binding proteins that recognized this element. We first employed UV cross-linking experiments using an 18-mer probe that has the E2 box as the core sequence. A protein-DNA complex of −130 kDa (based on comparison to the co-electrophoresed size markers) formed with a nuclear extract preparation from the undifferentiated CLB cells and GF-CSC cells, both of which express very low levels of type II collagen (Fig. 5, lanes 1 and 2, band A). A second major complex of −65 kDa formed with extracts from GF-CSC and CSC cells only (Fig. 5, lanes 2 and 3, band C), and a minor complex was also observed migrating just faster than band A in the CLB and CSC cells (Fig. 5, lanes 1 and 3, band B). This result demonstrated that multiple nuclear proteins interact with the E2 box probe under these binding conditions and that one of the protein complexes (band A) was present at high levels only in cells that displayed...
negative regulation of Col2a1 by $\delta$EF1

A major DNA-binding protein (band A) is present in nuclear extracts from primary CLB mesenchymal cells (lane 1) and growth factor-treated chick sternal chondrocytes (lane 2). This binding activity is minimal in extracts from primary differentiated chick sternal chondrocytes (lane 3). A second major DNA-binding protein (band C) is present in chick sternal chondrocyte nuclear extract, either primary or growth factor-treated, but is absent from the primary CLB mesenchymal cells.

Minimal expression of type II collagen. The size of the primary DNA-binding protein that formed band A was in general agreement with the size reported for $\delta$EF1 (26). However, in order to directly determine if $\delta$EF1 was present in the complex identified as band A in the EMSA, we next performed a supershift analysis. The 26-mer probe containing the E2 box element was incubated with nuclear extracts from primary CLB mesenchymal and GF-CSC cells. An antibody specific for the $\delta$EF1 protein was added to the binding mixture containing these two nuclear extracts. The addition of the $\delta$EF1 antibody to the binding reaction with both CLB and GF-CSC nuclear extracts resulted in the loss of band A and the appearance of a new, slower migrating band (Fig. 6, lanes 2 and 5). This result was identical to the pattern observed using the same antibody with brain extracts that contain $\delta$EF1 (26). The addition of an equal amount of an antibody specific for the unrelated N-FAT protein was used as a control for nonspecific protein interactions and did not produce a supershift (Fig. 6, lanes 3 and 6). This result identified $\delta$EF1 as a protein present in the band A complex.

$\delta$EF1 expression inversely correlated with the differentiated chondrocyte phenotype. Previous studies have demonstrated that $\delta$EF1 functions as a repressor of transcription (22). In addition, our EMSA and supershift studies established that the band A complex containing $\delta$EF1 was present at high levels in cells with minimal expression of type II collagen compared with the more differentiated cells. Therefore, we predicted that the expression of $\delta$EF1 mRNA would inversely correlate with the differentiated phenotype of chondrocytes. We conducted Northern blot analysis to monitor the levels of $\delta$EF1 mRNA in primary CLB mesenchymal cells and CLB mesenchymal cells differentiating in micromass culture for 12, 18, and 24 h. The $\delta$EF1 mRNA was highly expressed in undifferentiated primary CLB mesenchymal cells and decreased by 50% after 18–24 h in micromass culture (Fig. 7A). In contrast, the steady-state level of type II collagen mRNA was minimal in the primary mesenchymal cells and showed a substantial increase over the 24-h time period (Fig. 7B). This increase in type II collagen was consistent with several other studies using primary CLB mesenchymal cells in micromass culture (32, 33). In addition, we observed minimal expression of $\delta$EF1 mRNA in differentiated chick sternal chondrocytes cultured for 48 h in control medium (Fig. 7C, CSC). However, the steady-state level of $\delta$EF1 mRNA increased dramatically when the chondrocytes were treated for 48 h with TGF-β and bFGF (Fig. 7C, GF-CSC), which suppressed the transcription of the Col2a1 gene and results in decreased type II collagen mRNA (Fig. 7D). This result demonstrated a clear inverse relationship between the expression of $\delta$EF1 and that of type II collagen and further supported a role for $\delta$EF1 in the negative regulation of Col2a1 expression.

Overexpression of $\delta$EF1 suppresses Col2a1 gene promoter activity. Taken together, the previous results demonstrated that $\delta$EF1 was present in nuclear extracts from prechondrogenic mesenchyme cells and growth factor modulated chondrocytes and bound to the E2 box in the proximal promoter of the Col2a1 gene. Further, that mutation of this sequence actually resulted in an increase in promoter activity in differentiating CLB mesenchyme cells. In addition, the pattern of expression of $\delta$EF1 mRNA supported the hypothesis that this factor was involved in the negative regulation of Col2a1 expression. However, it was not clear if the $\delta$EF1 site in the conserved proximal E2 box was uniquely responsible for mediating this negative
regulation. This was an important question since there are additional E2 boxes located both upstream of the Col2a1 promoter, as well as in the first intron enhancer region, which could contribute to the negative regulation of this gene by δEF1. Specifically, there is an E2 box located at −560 and also there is an E2 box associated with each of the silencer elements located at −439 and −659. In each case, the core E2 box sequence (CACCTG) is surrounded by different flanking DNA. We first determined if the upstream E2 box as well as the two E2 boxes associated with the silencer regions of the Col2a1 promoter (16) would interact with DNA-binding proteins from differentiated and undifferentiated chondrocytes in a manner similar to that for the proximal E2 box. As shown in Fig. 8, probes representing the E2 boxes with flanking DNA located in the two silencer elements produced a band A complex with extracts from the undifferentiated limb bud mesenchymal cells (T0) as well as the growth factor-treated chondrocytes (GF-CSC). A probe containing the E2 box 2 sequence plus flanking DNA also produced a band A complex with the primary mesenchyme extracts (Fig. 8), and the intensity of the band A complex was much less for all three probes when extracts from the differentiated sternal chondrocytes was used (Fig. 8, CSC). Further, the antibody against δEF1 blocked the formation of the band A complex when pre-incubated with the extract from the growth factor-treated chondrocytes prior to the addition of the probe (data not shown). These data clearly demonstrate that the additional E2 boxes in the promoter region can bind δEF1 in the context of the different flanking sequences. We next tested whether δEF1 was involved in regulating the activity of the Col2a1 promoter either directly or indirectly, by examining the effects of overexpression of δEF1 on the activity of a Col2a1 promoter/enhancer CAT reporter construct in differentiated chick sternal chondrocytes and chick limb bud mesenchymal cells. As shown in Fig. 9A, cotransfection of a δEF1 cDNA expression vector with the pCII-1800 reporter construct resulted in a clear suppression of promoter activity in both cell types as compared with cotransfection with the parental expression vector that did not contain the δEF1 cDNA insert. Overexpression of δ EF1 did not down-regulate the activity of the pCDNA3 reporter construct in CLB mesenchymal cells, suggesting that the effects of δ EF1 were promoter-specific. We next examined the effect of expressing δEF1 on the activity of the shorter 307-bp promoter construct in fully differentiated chick sternal chondrocytes. In contrast to the situation with the differentiating CLB mesenchymal cells, the activity of the pCII-307 construct was less than that observed for the pCII-1800 construct in chondrocytes (compare Figs. 1B and 9B) and the overexpression of δEF1 produced a small but consistent suppression of this activity. Similar to the result obtained with the differentiating CLB mesenchyme cells (Fig. 1B), a mutation in the E2 box of the 307-bp construct resulted in increased activity compared with the intact promoter (Fig. 9B). Most important was the finding that overexpression of δEF1 still down-regulated the activity of the pCII-307M construct in chondrocytes, suggesting that the inhibitory activity of δEF1 was not mediated exclusively through the conserved proximal E2 element.

DISCUSSION

Collective evidence shows that the control of type II collagen expression in chondrocytes involves transcription factors that operate through cis-acting elements found in both the promoter and first intron enhancer (10, 11, 13–17, 19, 34). In this study, we present evidence demonstrating that the transcription factor δ EF1 is involved in the negative regulation of Col2a1 transcription. Northern blot analysis demonstrated an inverse correlation between δEF1 expression and the expression of type II collagen mRNA. In addition δEF1 bound to a highly conserved site known as an E2 box in the proximal promoter region, and mutation of this site actually increased promoter activity in differentiating CLB mesenchymal cells. δEF1 also bound to additional E2 boxes located at different regions in the promoter region. Mutation of the proximal conserved E2 box did not eliminate the down-regulation of promoter activity resulting from overexpression δEF1, suggesting that additional δEF1 binding sites in the promoter or first intron region are able to mediate the suppression.

Promoter deletion data from this investigation revealed that a relatively small (307 bp) proximal region of the Col2a1 promoter along with an intron enhancer sequence was active during chondrogenesis of CLB mesenchymal cells in micromass culture. A shorter promoter construct containing 100 bp of 5′-flanking sequence showed greatly diminished promoter activity. This analysis suggests that the region between −100 and −307 contains regulatory information that is important for expression in differentiating CLB mesenchymal cells. Previous work has demonstrated that specific sequences in the first intron of the Col2a1 gene can function as a chondrocyte enhancer with a very short region of the homologous promoter or with a heterologous promoter (12). Our work seems contradictory to this finding; however, we are suggesting that the sequences in the proximal promoter may be operating at very specific times in development, for example during the earlier stages of chondrocyte differentiation. This is consistent with a recent study, in which it was reported that sequences required for the negative regulation of human COL2A1 in neuroepithe-
Panel A demonstrates that the proximal E2 box, is still down-regulated by co-transfection with the promoter/enhancer element, there may be different requirements for minimal promoter sequences. In addition to this general conservation, the position and sequence of an E box regulatory motif within this region was identical in the rat, mouse, and human Col2a1/Col2a1 gene. This E box located at position −147 through −152 (CAGGTG) is actually a composite site, in that it also contains the recognition sequence for the binding of the transcription factor δEF1. This combination of an E box and a δEF1 recognition site is referred to as an E2 box (20, 22). We initially hypothesized that this E2 box was involved in the positive regulation of the Col2a1 promoter in the differentiating CLB cells. However, mutation of the conserved E2 box did not eliminate promoter activity in these cells; instead, it resulted in a modest increase of promoter activity. We have not yet identified the specific sequence within the −100 to −307 that is required for promoter activity in the CLB mesenchymal cells. However, our preliminary analysis suggests that deletion of the region between −210 and −307 eliminates promoter activity in this cell type (data not shown). We are now in the process of further defining the positive regulatory sequences within this region.

Considering the previous studies suggesting that the E2 box mediated the negative regulation of promoter activity by δEF1 and the results of our deletion analysis, we next determined the pattern and partial identity of proteins that bind to this sequence. Although we did observe several complexes that expressed higher binding activity in more differentiated chondrocytes, most impressive was one band that was inversely correlated with the expression of type II collagen. Based on the approximate size of the protein observed with UV cross-linking, as well as the recognition sequence required for binding as determined by competition EMSA analysis, and the supershift observed with a specific antibody, we identified δEF1 as a protein present in the band A protein-DNA complex. It is noteworthy that the EMSA analysis also resolved two additional complexes that bound to this region of the promoter (bands B and C in the EMSA). These bands showed a relative increase in intensity that coincided with differentiation of the CLB mesenchymal cells, suggesting that these proteins may participate in the positive regulation of type II collagen expression. Competition EMSA showed that the binding of one of these proteins was, at least partially, competed for with the competitor oligonucleotide that lacked a δEF1 binding site but maintained an E box sequence (CAAGTG). This results suggests that these proteins may be members of the bHLH family of trans-activators (20). The binding of both δEF1 and an, as yet unidentified bHLH protein, to this proximal E2 box (or other E2 boxes) in the Col2a1 gene would be consistent with the previously published role of δEF1 as a repressor of E2 box-mediated gene activation. Specifically, it has been shown that the δEF1 human homologue ZEB binds to a CACCT sequence in the IgH gene enhancer element μE5, which overlaps an E box (35). This binding led to gene suppression only in the absence of B cell-specific bHLH proteins, which bound to the E2 box site (CACCGT) as well. Binding of B cell-specific bHLH proteins relieved repression of transcription by displacing ZEB from its overlapping CACCT recognition sequence. Our DNA binding results suggest that an E2 box-mediated repression mechanism may also act to negatively regulate Col2a1. Interestingly, our binding data using CLB and GF-CSC nuclear extracts showed that both δEF1 (band A) and bands B and C were present together. Since neither of these cell types express high levels of type II collagen, we postulated that the regulation of the Col2a1 promoter may be dependent on a certain ratio of the positive and negative regulatory proteins. To test this hypothesis, we conducted cotransfection experiments overexpressing the δEF1 cDNA along with the pCII-1800 reporter construct in CLB mesenchymal cells and embryonic chick sternal chondrocytes (GF-CSC) and/or primary chick limb bud mesenchymal cells (CLB-T0) reacted with all three probes containing the δEF1 binding site plus the unique flanking DNA to produce a Band A complex. Nuclear extracts from differentiated chick sternal chondrocytes (CSC) did not produce a prominent band A complex with either of the three probes.
cytes. Overexpression of δEF1 suppressed Col2a1 promoter activity in both cell types but not the activity of the ubiquitous cytomegalovirus promoter present in the control construct, pCDNA3-CAT. We also examined the response of the shorter promoter construct (pCII-307) to δEF1 in sternal chondrocytes since these cells had minimal endogenous δEF1 binding activity. The inhibition of this construct, although present, was not as dramatic as with the longer promoter. This may have been due to the fact that the activity of the shorter promoter was actually lower than the longer construct in the chondrocytes, which was different from what was observed in the limb bud mesenchymal cells. Another likely possibility is that additional E2 boxes that are present in the longer promoter construct may function in an additive way to mediate suppression by δEF1. In fact, the 1800-bp promoter construct contains four putative δEF1 recognition sites (CACCT), including the most proximal E2 box. Two of these δEF1 recognition sites are part of common sequences located in two separate regions of the promoter previously described as silencer elements that were reported to negatively regulate Col2a1 expression in fibroblasts (16). The other two putative δEF1 recognition sequences in the promoter are overlapping E2 box motifs (CACCTG). As reported here, we have demonstrated that δEF1 binds to all of these. In addition, there are at least two known δEF1 sites in the enhancer region of the first intron of the Col2a1 gene. This is important since we observed that a 307-bp promoter construct with a mutation that eliminated the proximal δEF1 binding site was still down-regulated by overexpression of δEF1. This result suggests that δEF1 may be working through E2 boxes present in the intron or possible on genes upstream of Col2a1. These results are consistent with a mechanism involving a role for δEF1 in the negative regulation of Col2a1 gene expression but suggest that the proximal E2 box is not uniquely required for mediating the action of δEF1. It will be important in future studies to fully evaluate the contribution of these other sequences to the regulation of Col2a1 expression. It is also important to point out that, while the major mechanism proposed for the action of δEF1 as a transcriptional repressor is competition for the binding of positive factors to a shared site, there are additional possibilities. For example it has been shown that δEF1 will actively repress the activation of the DC5 enhancer by δEF3 even when the two proteins bind to non-overlapping sites (36). This mechanism is dependent on a specific NR domain close to the N terminus of the δEF1 protein (36). In future studies, it should be possible to further define the mechanism by which δEF1 is acting as a transcriptional repressor by co-expressing various domains of δEF1 along with Col2a1 reporter constructs.

There is very clear relevance of our in vitro studies to the regulation of chondrogenesis especially during skeletal development in vivo. During embryogenesis the primary site of δEF1 expression is the mesoderm and previous studies have described a pattern of δEF1 expression that suggests that this transcription factor may participate in the suppression of chondrocyte-specific gene expression (27). Specifically, the in vitro expression of δEF1 was high in the limb bud of the embryonic day 9.5 mouse embryo and this expression was lost during the condensation of mesenchymal cells, which gives rise to the cartilaginous skeleton. Mesenchymal cell condensation has been described as the first overt morphological change to take place before chondrogenesis (37). This pattern of expression suggests that δEF1 may be involved in the suppression of chondrocyte-specific genes in limb bud mesenchyme before the onset of chondrogenesis, although a molecular mechanism responsible for this suppression has not been described. Our experiments with primary limb bud mesenchymal cells grown in micromass culture established that δEF1 expression is also significantly down-regulated within a similar time frame in vitro. Based on the description of the expression pattern of δEF1 during embryogenesis and our current work presented here, we suggest a model whereby the transcription factor δEF1 has a role in suppressing Col2a1 gene activation in prechondrogenic mesenchyme. Presumably, during limb development negative regulation of this gene would be crucial in preventing its premature activation in prechondrogenic cells or the inappropriate expression in non-chondrocytes. Since type II collagen has been shown to be the first chondrocyte-specific protein observed during limb chondrogenesis, the precise timing of Col2a1 activation could be crucial in the initiation of the full chondrocyte-specific program of gene expression. A recent report describing a specific pattern of skeletal defects in δEF1 null mutant mice is consistent with this hypothesis (27). Among others, the homozygous animals showed limb defects that included broadening of long bones and fusion of bones and joints, which could result from inappropriate and mistimed expression of chondrocyte-specific genes.

This report describes evidence for a role of δEF1 in the negative regulation of Col2a1 gene expression. It is the first report of a specific transcription factor that acts to repress expression of a chondrocyte-specific gene. Our future work will be directed toward determining if δEF1 regulates other chondrocyte genes such as aggrecan and studies modulating the endogenous levels of δEF1 in various cell models by overexpressing sense and antisense transcripts to assess its role in differentiation.

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