δEF1 Binds to a Far Upstream Sequence of the Mouse Pro-α1(I) Collagen Gene and Represses Its Expression in Osteoblasts*

Catherine Terraz‡§, Dave Toman¶, Madeleine Delauché‡, Pierre Ronco‡, and Jerome Rossert‡∥

From the ‡INSERM U489 and Université Paris VI, Paris, France and §Cohesion Technologies, Palo Alto, California 94303

The transcription of type I collagen genes is tightly regulated, but few cis-acting elements have been identified that can modulate the levels of expression of these genes. Generation of transgenic mice harboring various segments of the mouse pro-α1(I) collagen promoter led us to suspect that a repressor element was located between −10.5 and −17 kilobase pairs. Stable and transient transfection experiments in ROS17/2.8 osteoblastic cells confirmed the existence of such a repressor element at about −14 kilobase pairs and showed that it consisted in an almost perfect three-time repeat of a 41-base pair sequence. This element, which we named COIN-1, contains three E2-boxes, and a point mutation in at least two of them completely abolished its repressor effect. In gel shift assays, COIN-1 bound a DNA-binding protein named δEF1/ZEB-1, and mutations that abolished the repressor effect of COIN-1 also suppressed the binding of δEF1. We also showed that the repressor effect of COIN-1 was not mediated by chromatin compaction. Furthermore, overexpression of δEF1 in ROS17/2.8 osteoblastic cells enhanced the inhibitory effect of COIN-1 in a dose-dependent manner and repressed the expression of the pro-α1(I) collagen gene. Thus, δEF1 appears to repress the expression of the mouse pro-α1(I) collagen gene, through its binding to COIN-1.

Type I collagen is a fibrillar collagen composed of two α1 chains and one α2 chain coiled around each others in a triple helix. It is the most abundant protein of mammalian bodies, and a major component of most extracellular matrices. In the extracellular space, type I collagen molecules self-assemble into highly organized fibrils and then fibers, which largely contribute to the high tensile strength of the structural framework supporting body structures (reviewed in Ref. 1). Nevertheless, an abnormal accumulation of type I collagen, along with other components of the extracellular matrix, can greatly and irreversibly impair functions of various organs including lung, kidney, liver, or skin. Thus, the production of type I collagen needs to be tightly regulated, and this regulation appears to occur mostly at a transcriptional level (reviewed in Ref. 2). It involves a control of the levels of expression of type I collagen genes as well as a control of their coordinate expression and their cell-specific expression.

Different positive regulatory sequences have been identified in the pro-α1(I) collagen gene (reviewed in Ref. 2). The 220-bp pro-α1(I) proximal promoter is extremely active in transfection experiments and in vitro transcription assays, and it has been described as one of the most potent eukaryotic promoters (3, 4). It contains enhancers such as a CCAAT-box, Sp1-binding sites, and other GC-rich sequences (4, 5). The first intron of the pro-α1(I) collagen gene also contains positive regulatory elements such as an AP-1 binding site (6) or an Sp1-binding site that is involved in maintaining bone density (7). The role of this site in maintaining normal levels of expression of the pro-α1(I) collagen gene throughout life has been shown by knock-in experiments (8). A cis-acting element located in the 3′-flanking region of the pro-α1(I) collagen gene has been shown to drive high levels of reporter gene expression in transiently transfected fibroblastic cells (9). Contrasting with the existence of these enhancers, type I collagen turnover appears to be a slow process, which suggests that inhibitory factors are essential to control the overall level of expression of the pro-α1(I) collagen gene. Nevertheless, few repressor elements have been described in this gene. An inhibitory element located between −361 and −339 bp1 has been identified in the mouse pro-α1(I) collagen promoter, in transient transfection experiments (10). A GC-rich repressor element has also been described in the first intron of the human gene (11). It had a repressor effect in transient transfection experiments, but sequences adjacent to this element completely abolished its repressor effect (11). Moreover, deletion of most of the first intron did not increase the levels of expression of the pro-α1(I) collagen gene in vivo (8). A member of the Krüppel-like family of transcription factors named cKrox is the only transcription factor that has been reported as being able to down-regulate the level of expression of the pro-α1(I) collagen gene (12). Nevertheless, its role in modulating the transcription of this gene remains controversial (12, 13). In transient transfection experiments, overexpression of the mouse cKrox gene enhanced the transcription of a reporter gene cloned downstream of a promoter containing three copies of a cKrox binding site (13), while overexpression of a truncated form of the human cKrox gene had an inhibitory effect on the expression of the pro-α1(I) collagen gene (12).

Besides cis-acting elements able to modulate the level of transcription of the pro-α1(I) collagen gene, regulatory elements responsible for its cell-specific expression have been identified. Only a discrete subset of cells of mesenchymal origin synthesize type I collagen. These cells are mostly fibroblasts, osteoblasts, and odontoblasts. Studies performed using transgenic mice harboring various fragments of the mouse, rat, or

* This work was supported by grants from the Association pour la Recherche sur le Cancer (to J. R.) and from the University of Paris (to J. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡Recipient of a fellowship from the Ministère de l’Education, de la recherche, et de la Technologie.
¶To whom correspondence should be addressed: INSERM U489, Hôpital TENON, 4 rue de la Chine, 75020 Paris, France. Tel.: 33-1-56-01-69-99; Fax: 33-1-56-01-69-99; E-mail: jerome.rossert@tnm.hop-paris.fr.

1 The abbreviations used are: bp, base pair(s); kb, kilobase pair(s); X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.
human pro-$\alpha$(I) collagen gene have shown that there is a modular arrangement of separate cell-specific cis-acting elements responsible for the expression of the pro-$\alpha$(I) collagen gene in different type I collagen-producing cells (14–16). So far, three cell-specific elements have been identified within the mouse gene: an element located within 900 bp of the proximal promoter induced reporter gene expression in some skin fibroblasts; a second element located between –1656 and –1570 bp conferred high levels of reporter gene expression in osteoblasts and odontoblasts; and a third element located between –2300 and –3200 bp conferred reporter gene expression in tendon and fascia fibroblasts (14, 17). The cis-acting element(s) responsible for the expression of the pro-$\alpha$(I) collagen gene in fibroblasts other than those present in fascia, tendons and skin remain(s) to be identified.

In order to identify new cis-regulatory elements within the mouse pro-$\alpha$(I) collagen gene, we have generated transgenic mice harboring segments of the corresponding promoter extending up to –17 kb and containing or not containing the first five introns of the gene. Analysis of these mice led to the identification of a repressor element that we named COIN-1 (for collagen-inhibitory element-1). COIN-1 is a three-time repeat of a 41-bp motif containing an E2-box and is located 14 kb upstream of the transcriptional start site. It binds a widely expressed transcription factor called $\Delta$EF1/ZEB-1, which appears to be responsible for the repressor effect of COIN-1, and is able to down-regulate the level of expression of the endogenous gene independently on chromatin compaction.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions**—We used two previously published plasmids containing segments of the mouse pro-$\alpha$(I) collagen promoter cloned upstream of the lacZ reporter gene in the placH expression vector (14). pJ251 contains a segment of the pro-$\alpha$(I) promoter extending from –2310 to +110 bp. pJ320 contains a sequence of this promoter extending from –3150 to +110 bp. Plasmids containing segments of the mouse pro-$\alpha$(I) promoter extending upstream of –3150 bp were constructed using placH and pDT816 (18). pDT816 is a cosmid clone that contains 110 bp. Plasmids containing segments of the mouse pro-$\alpha$(I) collagen gene have shown that there is a 5′ flanking region. pC15.56 was generated by cloning a SpeI/XbaI fragment extending from –15 kb to +110 bp in placH. pC3.112 was obtained by cloning an XbaI/EcoRI fragment extending from +192 to +3196 bp in pJ251, immediately upstream of the lacZ gene, and in frame with it. pC3.112 is thus coding for a fusion protein that contains the segment of the pro-$\alpha$(I) collagen chain encoded by the first six exons of the pro-$\alpha$(I) collagen gene (minus the signal peptide), and lacZ. PC6.267 and PC6.273 were generated by cloning an EcoRI/BamHI segment extending from –17 to –10.5 kb in pJ320 and in pC3.112, respectively. pC320.4 was obtained by cloning an EcoRI/SpeI fragment extending from –17 to –12.5 kb of the pro-$\alpha$(I) promoter in pJ320. pC320.1 was obtained by cloning an EcoRI/PstI fragment extending from –17 to –16 kb in pJ320. pC320.2XN was obtained by cloning a PstI/PstI fragment extending from –16 to –14 kb in pJ320, in the 5′–3′ orientation. pC320.312, pC320.400, and pC320.1.3 were obtained by cloning subfragments of this 2 kb segment: a SpeI/StuI fragment, a StuI/SpeI fragment, and a StuI/StyI fragment in pJ320, respectively. pC320.1.5 was obtained by cloning a PstI/SpeI fragment extending from –14 to –12.5 kb in pJ320. pC15.56.XN was obtained by cloning the PstI/PstI fragment extending from –16 to –14 kb in pC320.5 in the 5′–3′ orientation. pC123, pC123.2.1m, pC123.2m, pC123.3m, and pC123.3d were obtained by cloning double-stranded oligonucleotides in pJ320.

pDR583 contains a segment of the Hoxb-7 promoter, extending from –583 to +81 bp. It is cloned upstream of the firefly luciferase reporter gene, of an SV40 splice site and polyadenylation signal, and downstream of a polyadenylation cassette which prevents read-through transcription. In pDR6, the 6.5-krb pro-$\alpha$(I) promoter fragment extending from –17 to –10.5 kb was inserted upstream of the Hoxb-7 promoter in pDR583.

pGL3 control vector, pSV5-gal control vector, and pSVneo contain the luciferase reporter gene, the $\beta$-galactosidase reporter gene, and the neomycin resistance gene, respectively, cloned downstream of the SV40 promoter and enhancer (Promega).

pCMV-$\Delta$EF1 contains the cDNA encoding $\Delta$EF1, cloned in the pCMV- MVX expression vector (19).

**Generation and Analysis of Transgenic Mice**—Transgenes and transgenic embryos were generated using standard procedures (20, 21). $\beta$-Galactosidase activity was assessed on 15.5-day postconception embryos, previously described (14). For each embryo, immunostaining was scored semiquantitatively, using a 0–2 scale, by an investigator not aware of the construct harbored by the embryo. It was scored 0 for no expression, 1 for low levels of expression, and 2 for high levels of expression. To screen for transgenic mice, genomic DNA was extracted from the embryo’s yolk sacs with the DNeasy Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. A sequence of the lacZ gene was amplified by PCR, as previously described (22).

**Cell Lines**—ROS17/2.8 cells are rat osteoblastic cells, which produce type I collagen. They were cultured in 50% Dulbecco’s modified Eagle’s medium, 50% HAMF12 (Life Technologies, Inc.), supplemented with 10% fetal calf serum (Life Technologies). RC.VtSA58 cell line is a rabbit cortical collecting duct cell line that was obtained in our laboratory (23) and does not produce type I collagen. These cells were cultured in 50% Dulbecco’s modified Eagle’s medium, 50% HAMF12 supplemented with 2 mM glutamine, 5 mg/liter insulin, 50 mM dexamethasone, 5 mM transferrin, 30 mM selenium, 20 mM Hepes, and 2% fetal calf serum.

**Transfection Experiments**—For transient transfection experiments, cells were plated at 400,000 cells/well in six-well plates (Nunc, Kamstrup, Denmark) and transfected using LipofectAMINE (Life Technologies) following the manufacturer’s instructions. In each well, 0.25 pmol of lacZ-containing plasmids were co-transfected with 0.10 pmol of pGL3 control vector. In some experiments, pCMVx and pCMVx-$\Delta$EF1 were also co-transfected in increasing concentrations (0.05, 0.1, and 0.2 pmol). Reporter gene expression was measured 72 h after the start of transfection. All transfection experiments were done in triplicate and repeated at least three times. Results are expressed as mean ± S.E.

For stable transfection experiments, linearized lacZ-containing plasmids were mixed with linearized pSVneo in a 10:1 molar ratio. They were transfected as described above in 10-cm diameter Petri dishes (Nunc). Seventy-two hours after the start of transfection, cells were incubated in medium supplemented with 100 μg/ml G418 (Life Technologies). Under these conditions, untransfected cells died within 10 days. Experiments were done in triplicate. In each triplicate, the transfected clones were pooled to eliminate an integration site effect.

**In Vitro $\beta$-Galactosidase Assay and Luciferase Assay**—Cell extracts were prepared as previously described (24). $\beta$-Galactosidase activity was determined with the transiently transfected $\beta$-galactosidase kit (Roche Molecular Biochemicals) following the manufacturer’s instructions in a luminometer (EG&G, Bad Wilsbad, Germany). Luciferase activity was also assayed by using a luminometer as previously described (24).

**DNase I Digestion and Southern Blotting**—Cells were plated at a density of 500,000 cells/10-cm Petri dish (Nunc) and grown to confluency. They were isolated as previously described (23). After isolation, 10^7 nuclei were resuspended in 90 μl of a buffer containing 15 mM Tris-HCl (pH 7.5), 15 mM NaCl, 60 mM KCl, 0.5 mM spermidine, 0.5 mM spermine, 0.34 mM sucrose, 1 mM dithiothreitol. Then 10 μl of assay buffer containing 0–60 IU/reaction of DNase I (Roche Molecular Biochemicals) were added to the nuclei. The mixture was incubated at 37 °C for 45 min, and the reaction was stopped by adding 200 μl of a buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM EDTA, 100 mM NaCl, 1% SDS, 800 μg of proteinase K. Proteins were then digested overnight at 55 °C. The DNA was purified and digested with appropriate restriction enzymes with standard techniques (20). It was then electrophoresed on a 0.8% agarose gel and blotted on a Zenota-Probe membrane (Bio-Rad). This membrane was then processed following the manufacturer’s recommendations and hybridized with a 205-bp lacZ probe.

**Eletrophoretic Mobility Shift Assay**—Nuclear extracts from ROS 17/2.8 cells were prepared as previously described (26). The probes were end-labeled by filling in with [α-32P]dCTP using the Klenow fragment of the E. coli DNA polymerase I. Then 0.3 ng of each probe was incubated with 10 μg of nuclear extracts at room temperature for 30 min, in 20 μl of 50 mM Tris-HCl (pH 8.0) containing buffer (27). Competition experiments were performed using a 30–100-fold molar excess of unlabeled competitor. Supershift experiments were performed by adding an anti-$\Delta$EF1 antibody in the binding reaction. The complexes were resolved by electrophoresis through 4% polyacrylamide gels containing 22 mM Tris borate (pH 8.0) and 0.5 mM EDTA.

**Northern Blot Analysis**—Northern analysis of pro-$\alpha$(I) collagen transcription was conducted with total RNA from ROS17/2.8 cells.
transfected with 0.1 pmol of pCMVX or 0.1 pmol of pCMVX-\(\text{EF1}\). Total RNAs were prepared with RNAwiz (Ambion, Austin, TX) following the manufacturer’s instructions. They were fractionated and blotted onto nylon membrane (Amersham Pharmacia Biotech) using standard techniques (20). Hybridization was performed using a random prime-labeled cDNA probe for the pro-\(\alpha_1(I)\) RNA (28). An 18 S RNA probe was also used as a probe to control for differences in the total amount of RNA loaded. The signals were quantitated using a STORM 860 PhosphorImager (Amersham Pharmacia Biotech) and the ImageQuant software.

**Statistical Analysis**—To compare different groups of transgenic embryos, we performed statistical analysis according to analysis of variance followed by the Fisher’s protected least significant difference test (Statview).

**RESULTS**

Analysis of Transgenic Mice Suggests the Existence of a Repressor Element in the Pro-\(\alpha_1(I)\) Promoter, and This Is Confirmed Using Stable Transfection Experiments in ROS17/2.8—In order to identify new cis-acting regulatory elements within the mouse pro-\(\alpha_1(I)\) collagen gene, we generated transgenic mice harboring segments of the promoter located upstream of \(-4\) kb and/or the first five introns. lacZ was used as a reporter gene, since X-gal staining allows us to easily detect tissues and cells expressing the transgene during embryonic development. Foster mothers were sacrificed at 15.5 days postconception, because at this time, the endogenous gene is expressed in most type I collagen-containing tissues as well as in ossification centers (28).

We first generated transgenic mice using pC3.112. This construct contains \(2.3\) kb of the pro-\(\alpha_1(I)\) proximal promoter, a sequence extending from the 3’-end of exon 1 (and thus lacking the sequence coding for the signal peptide) to the 5’-end of exon 6 and lacZ cloned in frame with the sixth exon (Fig. 1A). Out of 12 transgenic embryos, seven expressed lacZ in ossification centers at high levels, one at low levels and four showed no staining after overnight incubation with X-gal (Fig. 1B).

We then used pC15.56 to generate transgenic mice (Fig. 1A). Three out of seven transgenic embryos expressed lacZ in ossification and tendons at high levels, one expressed it at low levels, and three did not show any X-gal staining (Fig. 1B).

To test the role of sequences located upstream of \(-13\) kb, we generated transgenic embryos using pC6.267 (Fig. 1A). Only four out of 11 transgenic embryos harboring pC6.267 expressed the lacZ reporter gene, in both cases it was expressed in ossification centers at low levels (Fig. 1B). In these two cases, X-gal staining was restricted to ossification centers.
Taken together, these data showed that the percentage of founder embryos expressing the reporter gene at high levels was significantly lower with the two constructs containing the 6.5-kb pro-α1(I) promoter sequence, extending from −17 to −10.5 kb, than with the corresponding constructs lacking this upstream sequence (2 out of 17 versus 10 out of 19, respectively, \( p < 0.05 \)). Hence, these data suggested that the 6.5-kb fragment of the pro-α1(I) promoter, extending from −17 to −10.5 kb contains a repressor element. To confirm this, we stably transfected pC3.112 and pC6.273 in ROS17/2.8 osteoblastic cells. β-Galactosidase activity was 68% lower in ROS17/2.8 cells stably transfected with pC6.273 than in those stably transfected with pC3.112 (Fig. 1C). Besides, β-galactosidase activity was similar when ROS17/2.8 cells were stably transfected with pC6.273 and pC6.267, suggesting that the first five introns do not contribute to the inhibitory effect (data not shown).

Furthermore, we performed histological analysis of transgenic embryos sections to precisely map the pattern of expression of the transgenes out of ossification centers and tendons (data not shown). In particular, transgenic embryos, at 15.5 days postconception never disclosed X-gal staining in organ capsules, the lung, the trachea, the pericardial membranes, the aortic trunk, the cardiac valves, the digestive tract, the metaphyses, the muscles, and the soft connective tissues. These data suggested that neither the pro-α1(I) promoter sequence located between −17 kb and −3.2 kb nor the first five introns contain new tissue-specific cis-acting elements active during embryonic development.

The Repressor Activity of the 6.5-kb Fragment Is Not Mediated by Modification of Chromatin Conformation—Since chromatin structure appears to play an important role in regulating gene expression, and in particular the pro-α2(I) collagen gene expression (29), and since the DNase I-hypersensitive sites present within the mouse pro-α1(I) collagen gene have been precisely mapped (29), we studied whether DNase I-hypersensitive sites present within the endogenous gene were also present within constructs containing the repressor element and stably integrated in the genome of ROS17/2.8 cells. The pro-α1(I) collagen gene contains two DNase I-hypersensitive sites between −17 kb and −11 kb, another one immediately upstream of the transcription start site, one within the first intron, and one within the fifth intron (Fig. 2A).

First, nuclei isolated from cells stably transfected with pC6.267 were incubated with increasing amounts of DNase I, and the subsequently extracted DNA was digested with NarI (Fig. 2B). The DNA fragments were then separated on an agarose gel and blotted onto a nylon membrane, which was hybridized with a probe corresponding to lacZ. The probe hybridized with four fragments of different size (Fig. 2, B and D). The high molecular weight one was generated by NarI digestion of the DNA extracted from nuclei untreated with NarI. The 8- and 7-kb ones were generated by NarI digestion and DNase I digestion at two hypersensitive sites located in the proximal promoter (Fig. 2, B and D). Nuclei isolated from ROS17/2.8 cells stably transfected with pC6.273 were also used to identify DNase I-hypersensitive sites within the transgene. They were incubated with increasing amounts of DNase I, and the extracted DNA was digested with HindIII (Fig. 2C). The lacZ probe hybridized with a 10.5-kb HindIII restriction fragment when DNA was extracted from DNase I-untreated nuclei. In nuclei treated with increasing amounts of DNase I, the lacZ probe hybridized with additional DNA fragments migrating at about 7, 6.5, and 4 kb (Fig. 2, C and E). These fragments correspond to HindIII digestion and DNase I digestion at hypersensitive sites located in the proximal promoter, the first intron, and the fifth intron, respectively. The DNase I-hypersensitive sites in the two transgenes were identical to those present in the endogenous gene, in type I collagen-producing cells, suggesting that the repressor effect was not mediated by modifications of the chromatin structure. A 6.5-kb Fragment of the Pro-α1(I) Promoter Represses Reporter Gene Expression in Transiently Transfected ROS17/2.8 Cells—To confirm that the inhibitory effect of the fragment located between −17 and −10.5 kb was not mediated by modifications of chromatin conformation, we performed transient transfection experiments with constructs containing (pC6.267, pC6.273) or not containing (pJ320, pC3.112) the repressor element. Relative β-galactosidase activities in ROS17/2.8 cells transfected with pC6.267 and pC6.273 were 2 times lower than...
β-galactosidase activities in cells transfected with pJ320 and pC3.112, respectively (Fig. 3A).

To test the ability of the repressor element to inhibit the activity of an heterologous promoter, the pro-α1(I) promoter fragment extending from −17 to −10.5 kb was cloned upstream of a 664-bp segment of the Hoxb-7 proximal promoter and of the luciferase reporter gene (pDR6) and transiently transfected in RC.SVtsA58 renal collecting duct cells with the pSVβ-gal control vector to correct for transfection efficiency. The activity of a construct containing the −17 to −10.5 kb segment was compared with the activity of a similar construct lacking this fragment and was considered as 100%. Values represent the mean ± S.E. from at least three separated experiments.

Analyses of the Pro-α1(I) Promoter—To delineate more precisely the boundaries of the inhibitory element, transient transfection experiments were performed using subsegments of this element, cloned upstream of 3.2 kb of the pro-α1(I) proximal promoter and of lacZ. Reporter gene activity in cells transfected with these constructs was compared with the activity of pJ320, which contains only 3.2 kb of the pro-α1(I) collagen promoter cloned upstream of lacZ. A 35–45% decrease in the levels of reporter gene expression was observed with pC6.267, pC3.112, or pC6.273 (Fig. 4). In contrast, no reporter effect was observed with pC320.400 and pC320.1.3, which contain a 400-bp and a 1.3-kb subsegment of the 2-kb repressor sequence, respectively (data not shown).

Sequencing of the 312-bp segment showed the existence of a 123-bp sequence that is an almost perfect three-time repeat of a 41 bp motif (Fig. 5A). This 123-bp decreased the activity of the reporter gene by 37%, suggesting that it corresponded to the repressor element (Fig. 5B). This sequence was named COIN-1 (for collagen-inhibitory element-1). Each of the three 41 bp motifs contains a CACCTG sequence, known as an E2-box. To test whether the E2-boxes were important in mediating the inhibitory effect of COIN-1, we compared β-galactosidase activities in ROS17/2.8 cells transiently transfected with pJ320, with pC123, and with plasmids harboring mutations or deletions in the three E2-boxes (Fig. 5B). Mutations or deletions of the three E2-boxes completely abolished the repressor effect of COIN-1, confirming that the E2-box played a key role in mediating the repressor effect of COIN-1. Mutations in the two most 3′ E2-boxes (pC123.2m) had the same effect. In contrast, a mutation in the five 5′ E2-box only (pC123.1m) did not modify the inhibitory effect of COIN-1 (Fig. 5B).

Repression Correlates with the Binding of δEF1—To study the proteins able to bind to COIN-1, we performed electrophoretic mobility shift assays using nuclear extracts from ROS17/2.8 cells and five probes. WT1 corresponds to the most 3′ 41 bp motif; DEL1 is similar to WT1, except for a deletion of the E2-box; WT2 corresponds to the two 3′ motifs; MUT2 is similar to WT2 except for a point mutation in the two E2-boxes (CACCCTG → CATCTG); and DEL2 is similar to WT2 except for deletions of the two E2-boxes.

Three retarded complexes (complexes B, C, E, in Fig. 6) were seen when WT1 was used as a probe. Complexes B and C, but not complex E, were competed by a 100-fold molar excess of the corresponding unlabeled probe (Fig. 6). One additional complex (complex A) was seen when WT2 was used as a probe (Fig. 6). In contrast, complex A was not seen when MUT2 or DEL2 were
**A**

\[ 5^-GC\overline{G}CG\overline{G}CCATGAGCTC\overline{G}GCGT\overline{A}C\overline{C}GTC\overline{T}G\overline{G}CCCT \]

\[ GG\overline{C}TGCCACATGAGCTCAGGCGTCACCTGTCTGTGC\overline{C}CT \]

\[ GCC\overline{G}\overline{G}CCATGAGCTCAGGCGTACCTGTCTGTGC\overline{C}CT 3^- \]

**B**

| Construct | Relative \( \beta \)-galactosidase activity |
|-----------|-------------------------------------------|
| pJ320 | ![Bar graph showing relative activity for pJ320] |
| pC123 | ![Bar graph showing relative activity for pC123] |
| pC123 30 | ![Bar graph showing relative activity for pC123 30] |
| pC123 2X | ![Bar graph showing relative activity for pC123 2X] |
| pC123 1X | ![Bar graph showing relative activity for pC123 1X] |
| pC123 3X | ![Bar graph showing relative activity for pC123 3X] |

**DISCUSSION**

Molecular mechanisms governing type I collagen gene expression are still quite elusive, and in particular the elements that down-regulate the highly active pro-\(\alpha\)-I(1) proximal promoter are unknown. By generating transgenic mice and performing transfection experiments, we have identified a 123-bp repressor element in the mouse pro-\(\alpha\)-I(1) promoter. When a segment of the promoter extending from \(-17\) to \(-10.5\) kb was cloned upstream of 2.3 kb of the pro-\(\alpha\)-I(1) proximal promoter, a decrease of expression of the lacZ reporter gene was observed in 15.5-day postconception transgenic embryos harboring various segments of the mouse pro-\(\alpha\)-I(1) promoter. When a segment of the promoter extending from \(-17\) to \(-10.5\) kb was cloned upstream of 2.3 kb of the pro-\(\alpha\)-I(1) proximal promoter and of the first five introns, the percentage of transgenic embryos expressing the reporter gene at high levels dropped from 58 to 0% (compare the results obtained with pC3.112 and pC6.273 in Fig. 1). Stable and transient transfection experiments confirmed the presence of this element, since the average levels of expression of the lacZ reporter gene were decreased by 66 and 60%, respectively, when the \(-17\) to \(-10.5\) kb segment was cloned upstream of 2.3 kb of the pro-\(\alpha\)-I(1) proximal promoter and of the first five introns. A similar inhibitory effect was observed in transient transfection experiments when the \(-17\) to \(-10.5\) kb segment was cloned upstream of an heterologous promoter. Using transient transfection experiments in ROS17/2.8 cells, the repressor element was progressively narrowed to a 123-bp sequence located 14 kb upstream of the transcription start site. Sequencing of this element, that we named COIN-1, showed that it consists of an almost perfect three-time repeat of a 41-bp motif containing an E2-box. The nonidentical nucleotides are underlined. The E2-boxes are in boldface type.

The existence of such a repressor element was first suggested by comparing the levels of expression of the lacZ reporter gene in 15.5-day postconception transgenic embryos harboring various segments of the mouse pro-\(\alpha\)-I(1) promoter. When a segment of the promoter extending from \(-17\) to \(-10.5\) kb was cloned upstream of 2.3 kb of the pro-\(\alpha\)-I(1) proximal promoter and of the first five introns, the percentage of transgenic embryos expressing the reporter gene at high levels dropped from 58 to 0% (compare the results obtained with pC3.112 and pC6.273 in Fig. 1). Stable and transient transfection experiments confirmed the presence of this element, since the average levels of expression of the lacZ reporter gene were decreased by 66 and 60%, respectively, when the \(-17\) to \(-10.5\) kb segment was cloned upstream of 2.3 kb of the pro-\(\alpha\)-I(1) proximal promoter and of the first five introns. A similar inhibitory effect was observed in transient transfection experiments when the \(-17\) to \(-10.5\) kb segment was cloned upstream of an heterologous promoter. Using transient transfection experiments in ROS17/2.8 cells, the repressor element was progressively narrowed to a 123-bp sequence located 14 kb upstream of the transcription start site. Sequencing of this element, that we named COIN-1, showed that it consists of an almost perfect three-time repeat of a 41-bp motif containing a CACCTG E2-box. These E2-boxes play a key role in mediating the inhibitory

**Fig. 5. Analysis of COIN-1.** A, sequence of COIN-1. This sequence is an almost perfect three-time repeat of a 41-bp motif containing an E2-box. The nonidentical nucleotides are underlined. The E2-boxes are in boldface type. B, schematic representation of constructs used to perform transient transfection experiments and results of these assays. A star indicates a point mutation in an E2-box (CACCTG → CATCT). An open box indicates a deleted E2-box. Each construct was co-transfected in ROS17/2.8 cells with PGL3 control vector to correct for transfection efficiency. Values are expressed as relative \(\beta\)-galactosidase activity, the activity of pJ320 being considered as 100%. All values represent the mean ± S.E. from at least three separate experiments.
EF1 Represses Pro-α1(I) Collagen Gene Expression

EF1 binds to COIN-1, which regulates the expression of the endogenous pro-α1(I) collagen gene. Thus, it is very likely that EF1 binds to COIN-1 and mediates its inhibitory effect.

EF1 is a DNA-binding protein that belongs to an emerging family of two-handed zinc finger transcription factors. It is expressed in lens, central nervous system, neural crest derivatives, and various mesodermal tissues (33). It contains two widely separated clusters of C2H2 Krüppel-like zinc fingers and a homeodomain-like segment, but only the two clusters of zinc fingers seem to be involved in DNA binding (34). In vitro studies have shown that EF1 binds ctg-acting elements containing two E2-boxes (34), which is in complete agreement with our results. Furthermore, the affinity of a zinc finger cluster for its binding site appears to be largely increased when a guanine residue is located immediately downstream of the CACCTG motif (34), which is the case for all three CACCTG motifs in COIN-1. In previously described EF1-binding sites, only one E2-box out of two contained such a guanine residue (34). COIN-1 is thus the first EF1-binding element that contains a high affinity binding site for each cluster of zinc finger, and it might bind EF1 with a greater affinity than the other EF1-binding sites. Moreover, since two E2-boxes are sufficient to allow the binding of EF1, it would be of interest to examine in greater detail the respective role of each of the three E2-boxes contained in COIN-1 and the number of EF1 molecules able to bind to COIN-1. The inhibitory effect of COIN-1 is in agreement with results reported by other groups, who showed that EF1 had a repressor effect and was able to decrease the activity of the α1-crystallin promoter, of the pro-α1(I) collagen promoter, and of the α1-integrin promoter (27, 31, 32). The ability of EF1 to down-regulate the pro-α1(I) collagen gene in osteoblastic cells may explain that EF1-null mice display a variety of defects in bones, where large a amount of type I

Fig. 6. Electrophoretic mobility shift analysis of the proteins binding to COIN-1. Lanes 1 shows the proteins binding to WT1. Lanes 4 and 11 show the proteins binding to WT2. Complex A was only seen with the WT2 probe, which contains two E2-boxes. Lanes 2, 5, and 6 show the proteins binding to DEL1, MUT2, and DEL2. Complex A was not seen with MUT2 and DEL2 that contain mutated or deleted E2-boxes, respectively. In lane 3, a competition assay was performed using a 100-fold molar excess of the WT1 unlabeled probe. In lanes 7–10, competition assays were done using a 30–100-fold molar excess of the WT1 and the MUT2 unlabeled probes. Complex A was not eliminated in competition experiments with MUT2, which harbors a point mutation in the E2-boxes, while it was eliminated in competition experiments with WT2. In lane 12, incubation of the nuclear extracts with an anti-δEF1 antibody eliminated the formation of complex A and produced a slower migrating complex (star).

Fig. 7. Effects of δEF1 overexpression. A, transient transfection experiments in ROS17/2.8 cells were performed using pC123, together with increasing amounts of pCMVX and pCMVX-δEF1. PGL3 control vector was used to correct for transfection efficiency. EF1 de-
collagen is produced during embryonic development (35). The repression of the pro-α(I) promoter activity mediated by COIN-1 may not appear dramatic, but it is likely to be physiologically relevant for at least two reasons. First, the pro-α(I) proximal promoter being very active, transcriptional repression is probably a key phenomenon in controlling the level of expression of the corresponding gene. Second, since type I collagen protein turnover is very slow (36), only a modest increase in pro-α(I) mRNA participate in the onset of fibrosis (37).

The fact that COIN-1 is located far upstream of the transcriptional start site and that its inhibitory effect is orientation-dependent raises the question of its mode of action. In eukaryotes, transcription can be repressed through different mechanisms, including modifications of chromatin structure, interference with the binding of activators, and interactions with components of the general transcription machinery (reviewed in Ref. 38). The binding of δEF1 to COIN-1 does not seem to modify chromatin structure, since the DNase I-hypersensitive sites identified within the mouse pro-α(I) collagen promoter or within the first and the fifth introns, in type I collagen-producing cells (29), were still present in constructs containing COIN-1 stably integrated into the genome of ROS17/2.8 cells. Furthermore, COIN-1 was active not only in stable transfection experiments but also in transient transfection experiments. Thus, δEF1 is likely to repress the pro-α(I) promoter activity by interacting with other transcription factors, as suggested for the δI-crystallin gene (27). The interactions between δEF1 and other components of the transcription machinery may be direct, involving the repression domain of δEF1, but they may also be indirect, since recent studies reported that δEF1 was able to recruit co-repressors named C-terminal binding proteins (39). Quite surprisingly, the inhibitory effect of COIN-1 was orientation-dependent. Other cis-repressor elements, such as a potent repressor located in the myelin basic protein gene, have been reported to be active only in one orientation (40). This led to the assumption that the regulation of gene transcription can involve the formation of DNA-multiprotein complexes through distant regions of DNA and that higher order formations may require a correct three-dimensional structure given by the binding of transcription factors in precise orientation (41).

Analysis of the transgenic embryos confirmed that an osteoblast-specific element is located within 2.3 kb of the pro-α(I) proximal promoter (compare results obtained with pc3.112 and pc15.56) and that a tendon- and fascia-specific element is located between −3.2 and −2.3 kb (compare results obtained with pc6.267 and pc6.273). In contrast, it did not disclose the existence of a new tissue-specific element, either upstream of −3.2 kb or within the first five introns. The absence of tissue-specific elements within the first intron extend results obtained by Hormuzdi et al. (8). They also showed the absence of tissue-specific elements in this intron by generating knock-in mice lacking most of it (12). Analysis of heterozygous mice showed that it was important for maintaining normal levels of expression of the pro-α(I) collagen gene in lung and muscle during adult life. Nevertheless, study of homozygous mice showed that this intron was not necessary for inducing the expression of the pro-α(I) collagen gene in type I collagen-producing cells. The absence of tissue-specific elements within a fragment of promoter extending from −17 to −3.2 kb active during embryonic development extends results obtained by Krempen et al. (43). They generated transgenic mice harboring segments of the mouse pro-α(I) promoter extending up to −19.5 kb that were cloned upstream of the green fluorescent protein reporter gene (42). Analysis of these mice did not show new tissue-specific elements, with the exception of an element located at −8 to −7 kb that enhanced the expression of the reporter gene in endometrial cells and in muscle cells of the uterus during the oestrous cycle in adult females.

In conclusion, we have identified a 123-bp repressor element located at about 14 kb upstream of the transcription start site in the mouse pro-α(I) collagen gene. This element, which we named COIN-1, is a three-time repeat of a 41-bp motif. Each repeat contains an E2-box, and the repressor effect of COIN-1 appears to occur through the binding of δEF1 to these E2-boxes. COIN-1 was able to decrease the activity of the pro-α(I) promoter not only in transient transfection experiments but also in stable transfection experiments and in transgenic mice. Furthermore, overexpression of δEF1 enhanced the inhibitory effect of COIN-1 and down-regulated the expression of the endogenous gene. These data suggest that this inhibitory sequence could be an important player in the regulation of the overall levels of expression of type I collagen genes.

Acknowledgments—We thank B. de Crombrugghe for the generous gift of ROS 17/2.8 cells and H. Kondoh for the generous gift of the pCMV-δEF1 expression vector and the anti-δEF1 antibody. We are also grateful to J. Chambard and C. Lasne for welcoming and helping us in the IFR 58 translgenic facility. We thank A. Calmont and G. Bou-Gharios for carefully reading the manuscript.

REFERENCES

1. van der Rest, M., and Garrone, R. (1991) FASEB J. 5, 2514–23
2. Rossert, J., and de Crombrugghe, B. (2001) Principles of Bone Biology, Academic Press, Inc., San Diego. In press
3. Maity, S. N., Golombek, P. T., Karsenty, G., and de Crombrugghe, B. (1988) Science 241, 582–585
4. Karsenty, G., and de Crombrugghe, B. (1990) J. Biol. Chem. 265, 9934–9942
5. Nehils, M. C., Rippe, R. A., Veloz, L., and Brenner, D. A. (1991) Mol. Cell. Biol. 11, 4065–4073
6. Luu, D. J., Slack, J. L., and Bournstein, P. (1990) Cell Regul. 1, 487–489
7. Grant, S. F., Reid, D. M., Blake, G., Herd, R., Fogelman, I., and Ralston, S. H. (1996) Nat. Genet. 14, 203–205
8. Hormuzdi, S. G., Pientrins, R., Jaemisch, R., and Brenner, D. P. (1998) Mol. Cell. Biol. 18, 3368–3375
9. Rippe, R. A., Umezawa, A., Kimball, J. P., Breindl, M., and Brenner, D. A. (1997) J. Biol. Chem. 272, 1753–1760
10. Ravazzolo, R., Karsenty, G., and de Crombrugghe, B. (1991) J. Biol. Chem. 266, 7382–7387
11. Bournstein, P., and McKay, J. (1988) J. Biol. Chem. 263, 1603–1606
12. Widom, R. L., Culic, I., Lee, J. Y., and Korn, J. H. (1997) Gene (Amst.) 198, 397–420
13. Galera, P., Musso, M., Duy, C., and Karsenty, G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9372–9376
14. Rossert, J., Eberspaecher, H., and de Crombrugghe, B. (1995) J. Cell Biol. 129, 1421–1432
15. Bogdanovic, Z., Bedalov, A., Krebsbach, P. H., Pavlin, D., Woody, C. O., Clark, S. H., Thomas, H. F., Rowe, D. W., Kream, B. E., and Lichter, A. C. (1994) J. Bone Miner. Res. 9, 285–292
16. Liska, D. J., Reed, M. J., Sage, E. H., and Bournstein, P. (1994) J. Cell Biol. 125, 695–704
17. Rossert, J. A., Chen, S. S., Eberspaecher, H., Smith, C. N., and de Crombrugghe, B. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1027–1031
18. Toman, D., and de Crombrugghe, B. (1995) Matrix: A Practical Approach, pp. 353–391, Oxford University Press, Great Britain
19. Kamachi, Y., and Kondoh, H. (1995) Mol. Cell. Biol. 15, 5206–5215
20. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
21. Hogan, B., Constantini, F., and Lacy, E. (1986) Manipulating the Mouse Embryo: A Laboratory Manual, pp. 115–252, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
22. Wassarman, P. M., and DePa ngànis, M. L. (1993) Guides to Techniques in Mouse Development, pp. 451–469, Academic Press, Inc., San Diego
23. Prie, D., Ronco, P. M., Baudouin, B., Genieau-Legendre, M., Antoine, M., Piedagnel, R., Estrade, S., Lelongt, B., Verroust, P. J., Cassingena, R., and Vandewalle, A. (1991) J. Cell Biol. 113, 951–962
24. Calmont, A., Reichwald, K., Ronco, P., and Rossert, J. (2000) Mol. Endocrinol. 14, 1692–1695
25. Bou-Gharios, G., Garrett, L. A., Rossert, J., Niederreither, K., Eberspaecher, H., Smith, C., Black, C., and Crombrugghe, B. (1996) J. Cell Biol. 134, 1333–1344
26. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1475–1489
27. Sekido, R., Murai, K., Funahashi, J., Kamachi, Y., Fujisawa-Sehara, A., Nabetaka, S., and Kondoh, H. (1994) Mol. Cell. Biol. 14, 5692–5700
28. Niederreither, K., D’Ou爽a, R., Metersan, M., Eberspaecher, H., Toman,
P. D., Vuorio, E., and De Crombrugghe, B. (1995) Matrix Biol. 14, 705–713
29. Salimi-Tari, P., Cheung, M., Safar, C. A., Tracy, J. T., Tran, I., Harbers, K., and Breindl, M. (1997) Gene (Amst.) 196, 61–72
30. Quandt, K., Fresh, K., Karas, H., Wingerdner, E., and Werner, T. (1995) Nucleic Acids Res. 23, 4878–4884
31. Murray, D., Precht, P., Balakir, R., and Horton, W. E., Jr. (2000) J. Biol. Chem. 275, 3610–3618
32. Postigo, A. A., and Dean, D. C. (1997) EMBO J. 16, 3935–3943
33. Funahashi, J., Sekido, R., Murai, K., Kamashi, Y., and Kondoh, H. (1993) Development 119, 433–446
34. Remacle, J. E., Kraft, H., Lerchner, W., Wuytens, G., Collart, C., Verschaeren, K., Smith, J. C., and Huylebroeck, D. (1999) EMBO J. 18, 5073–5084
35. Takagi, T., Moribe, H., Kondoh, H., and Higashi, Y. (1998) Development 125, 21–31
36. LeRoy, E. C. (1974) J. Clin. Invest. 54, 880–889
37. Jimenez, S. A., Feldman, G., Bashey, R. I., Bienkowski, R., and Rosenbloom, J. (1986) Biochem. J. 237, 837–843
38. Stefanovic, B., Hellebrand, C., Holck, M., Breindl, M., Liebhaber, S. A., and Brenner, D. A. (1997) Mol. Cell. Biol. 17, 5201–5209
39. Maldonado, E., Hampsey, M., and Reinberg, D. (1999) Cell 99, 455–458
40. Furusawa, T., Moribe, H., Kondoh, H., and Higashi, Y. (1999) Mol. Cell. Biol. 19, 8581–8590
41. Givogri, M. I., Kampf, K., Schonmann, V., and Campagnoni, A. T. (2000) Gene (Amst.) 252, 183–193
42. Dhar, M., Mascareno, E. M., and Siddiqui, M. A. (1997) J. Biol. Chem. 272, 18490–18497
43. Krempen, K., Gratkopp, D., Hall, K., Bache, A., Gillan, A., Rippe, R., Brenner, D., and Breindl, M. (1999) Gene Expr. 8, 151–163
δEF1 Binds to a Far Upstream Sequence of the Mouse Pro-α1(I) Collagen Gene and Represses Its Expression in Osteoblasts
Catherine Terraz, Dave Toman, Madeleine Delauche, Pierre Ronco and Jerome Rossert

J. Biol. Chem. 2001, 276:37011-37019.
doi: 10.1074/jbc.M104185200 originally published online July 25, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M104185200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 38 references, 23 of which can be accessed free at
http://www.jbc.org/content/276/40/37011.full.html#ref-list-1