

CREB-AP1 Protein Complexes Regulate Transcription of the Collagen XXIV Gene (*Col24a1*) in Osteoblasts*

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Collagen XXIV is a newly discovered and poorly characterized member of the fibril-forming family of collagen molecules, which displays unique structural features of invertebrate fibrillar collagens and is expressed predominantly in bone tissue. Here we report the characterization of the proximal promoter of the mouse gene (*Col24a1*) and its regulation in osteoblastic cells. Using well characterized murine models of osteoblast differentiation, we found that the *Col24a1* gene is activated sometime before onset of the late differentiation marker osteocalcin. Additional analyses revealed that *Col24a1* produces equal amounts of two alternatively spliced products with different 5'-untranslated sequences that originate from distinct transcriptional start sites. Cell transfection experiments in combination with DNA binding assays demonstrated that *Col24a1* promoter activity in ROS17/2.8 osteosarcoma cells is under the control of an upstream *cis*-acting element, which is shared by both transcripts and is recognized by specific combinations of c-Jun, CREB1, ATF1, and ATF2 dimers. Consistent with these results, overexpression of c-Jun, ATF1, ATF2, or CREB1 in transiently transfected osteoblastic cells stimulated transcription from reporter gene constructs driven by the *Col24a1* promoter to different degrees. Moreover, chromatin immunoprecipitation experiments showed that these nuclear factors bind the same upstream sequence of the endogenous *Col24a1* gene. Collectively these data provide new information about transcriptional control of collagen fibrillogenesis, in addition to implicating for the first time CREB-AP1 protein complexes in the regulation of collagen gene expression in osteoblasts.

Vertebrate collagens represent a very large superfamily of extracellular proteins that impart specific physical properties to the connective tissue of virtually every organ system (1–3). There are more than 42 collagen α -chains that form 27 distinct trimers or types, which in turn give rise to a large variety of specialized macroaggregates. The most abundant and ubiquitous collagen macroaggregates are the highly

ordered banded fibrils made of the so-called fibrillar collagens (types I–III, V, and XI) (1–3). All members of the fibrillar collagen family share a common structure that consists of a long triple helical domain, which is made of uninterrupted Gly-X-Y triplets and flanked at both ends by noncollagenous propeptides (1–3). These structural features also characterize collagen molecules that form fibrils in the extracellular matrices of primitive invertebrates, such as sponges, annelids, echinoderms, and mollusks (4, 5). Unlike the vertebrate counterparts, invertebrate fibrillar collagens display short interruptions in the triple helices and unique structural features in the amino- and carboxyl-terminal propeptides (4, 5). Vertebrate fibrillar collagens are either widely distributed in soft and hard tissues (types I, III, and V) or are restricted predominantly to cartilage (types II and XI) (1). Genetic evidence from animal and human studies has indicated that the quantitatively minor types V and XI collagen regulate the diameter of the major types I and II fibrils, respectively, by participating in fibril assembly (2, 6–9). These studies have also demonstrated the importance of heterotypic collagens I/V and II/XI fibrils in skeletal development and integrity.

As a result of the Human Genome effort, several new collagens have been recently identified which had escaped prior biochemical detection. Two among them (types XXIV and XXVII) bear the structural characteristics of fibrillar collagens and specifically, of invertebrate fibrillar collagens (10–12). Gene expression analyses in the mouse have revealed that *Col24a1* and *Col27a1* display mutually exclusive patterns in the developing and adult skeleton. These studies have in fact shown that whereas *Col24a1* transcripts accumulate at ossification centers of the craniofacial, axial, and appendicular skeleton, *Col27a1* activity is instead confined to the cartilaginous anlagen of skeletal elements (10–12). Additionally, structural considerations have suggested that collagens XXIV and XXVII are likely to form distinct homotrimers (11). Together these observations have been interpreted to indicate that these newly discovered fibrillar collagens may participate in the control of important physiological processes in bone and cartilage, such as collagen fibrillogenesis and/or matrix calcification and mineralization (10–12).

Bone formation is a complex and tightly regulated genetic program that involves two distinct pathways at different anatomical locations (13–15). In intramembranous ossification, mesenchymal cells condense and differentiate directly into collagen I-producing osteoblasts. In endochondral bone formation, cells at condensation sites differentiate into chondrocytes that form a cartilage (collagen II-rich) anlagen, which is replaced by a bony (collagen I-rich) matrix and bone marrow following chondrocyte hypertrophy, matrix calcification, and vascular invasion. At the same time, cells around the condensations form the perichondrial layer that gives rise to the osteoblast-forming periosteum and ulti-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) DQ157748.

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mately to cortical bone. Distinct transcriptional codes control osteoblastogenesis and chondrogenesis and thus, assembly of the collagen I-rich bone matrix and the collagen II-rich cartilage matrix (14, 15). The canonical Wnt signaling pathway has been shown recently to direct differentiation of mesenchymal cells toward either the osteoblast or chondrocyte lineages (16, 17). Previous investigations, on the other hand, have implicated the transcription factors Runx2 and Osterix in the progression of osteoblastogenesis during intramembranous and endochondral ossification, as well as the Sox5, 6, and 9 nuclear proteins in the regulation of chondrogenesis (18–22). A number of ubiquitous transcription factors have been also involved in osteoblast differentiation and function, including Msx proteins, Dlx5, Twist, and members of the AP1 complexes (23–28). Similarly, several studies have identified DNA *cis*-acting elements and nuclear *trans*-acting factors that regulate cartilage-specific expression of the collagen II and XI genes (29–32). By contrast, significantly less is known about the regulation of fibrillar collagen genes in osteoblasts.

One of our research interests is the study of the regulation and function of fibrillar collagen genes in normal and diseased conditions. As part of this ongoing effort, the present study was designed to characterize the proximal promoter of the mouse *Col24a1* gene using a combination of cell transfection and DNA binding assays. The results of these experiments suggest that *Col24a1* is activated during the mid to late phase of osteoblast differentiation mostly through the binding of CREB²-AP1 complexes to an upstream sequence, which is shared by two alternative transcription start sites. This study therefore extends our knowledge of the transcriptional regulation of collagen fibrillogenesis, in addition to implicating for the first time CREB-AP1 protein complexes in the expression of a fibrillar collagen gene in osteoblasts.

EXPERIMENTAL PROCEDURES

Cell Cultures—Rat osteosarcoma ROS17/2.8 and ROS25 cells, mouse fibroblast NIH-3T3, and mouse primary calvarial osteoblast cells (MCC) were used in this study. ROS17/2.8, ROS25 and NIH-3T3 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum at 37 °C in humidified 5% CO₂ and 95% air (33). MCC were prepared from 4-day-old mice and cultured in Minimum Essential Medium- α medium containing 10% heat-inactivated fetal bovine serum at 37 °C in humidified 8% CO₂ and 92% air (32).

RNA Analyses—Total RNAs from the mouse eye, brain, and whole embryo were purchased from BD Biosciences. Total RNA was isolated from neonatal calvarias and from cultured cells using commercial kits (Stratagene and Invitrogen) according to the manufacturers' recommendations. Reverse transcriptions were carried out using Omniscript reverse transcriptase (Qiagen GmbH, Germany) with oligo(dT) primer, and the resulting single-stranded cDNA molecules were PCR amplified using primers for *Col24a1* (forward primer, 5'-ATGCATTTAGGAGCCTACAG-3'; reverse primer, 5'-TCACGAGAGGTGACTCAATA-3'), *Col1a2* (forward primer, 5'-TGGTCTCTGGGCATCTCAGGC-3'; reverse primer, 5'-GGTGAACCTGCTGTTGCCCTCA-3'), osteocalcin (forward primer, 5'-ATGAGACCCTCTCTCTGCT-3; reverse primer, 5'-GGAGCTGCTGTGACATCCAT-3'), and *Gapdh* (forward primer, 5'-ACCACAGTCCATGCCATCAC-3'; reverse primer, 5'-TCCACCACCCTGTTGCTGTA-3'). Amplification conditions included a cycle at 94 °C for 2 min followed by 25 cycles at 94 °C for 30 s,

56 °C for 1 min, and 72 °C for 1.5 min, and a final elongation cycle at 72 °C for 5 min. To determine the transcriptional start sites, the oligonucleotide-capping RACE was performed using the GeneRacer kit according to the manufacturer's protocol (Invitrogen) and with amplification primers corresponding to the sequences of exon 3 (5'-TCACGAGAGGTGACTCAAT-3') and exon 1 (5'-TAGGCTCCTAAATGCAT-3') of *Col24a1*. Amplification products were subcloned into the pCR4-TOPO vector (Invitrogen) and sequenced.

Cell Transfection Assays—*Col24a1* promoter-luciferase (LUC) reporter gene constructs were derived from clone pBeroBAC RP23-205C6 using PCR amplification. Amplified products were cloned into the pGEM-T Easy vector (Promega, Madison, WI) and sequenced. Internal deletions and nucleotide substitutions were generated by site-directed mutagenesis as described previously (34). Transient transfections were performed using the Lipofectamine Plus reagent system (Invitrogen), and luciferase activity was assayed 48 h later using the Dual-luciferaseTM reporter assay system (Promega). The pRL-TK *Renilla reniformis* luciferase expression vector was used as an internal control for transfection efficiency. Results were expressed as the mean \pm S.E. of five to seven independent experiments and evaluated by Student's *t* test. Expression vectors for ATF1, ATF2, CREB1, and c-Jun expression vectors were kindly provided by Drs. Gerard Karsenty (Baylor College of Medicine, Houston, TX) and Lionel Ivashkiv (Hospital for Special Surgery, New York, NY).

DNA Binding Assays—Preparation of nuclear extracts and DNA binding assays were carried out according to the published protocols (34, 35). Wild-type and mutant oligonucleotide probes were generated by PCR amplification using HindIII site-linked primers. PCR products were subcloned into pGEM-T Easy vector, cleaved with HindIII, and radiolabeled with [α -³²P]dCTP using the Klenow enzyme (34, 35). DNA-nuclear protein binding was carried out at 25 °C for 30 min in 25 μ l of reaction buffer containing 3 μ g of poly(dI-dC). DNA-bound protein complexes were separated in a 4.5% nondenaturing polyacrylamide gel in 0.25% TBE buffer. For competition and antibody interference assays, unlabeled probes or antibodies were added to the reaction mixture for 1 h at 4 °C before the addition of labeled probe. The anti-CREB1 antibody was purchased from Upstate Biotechnology (Lake Placid, NY), and the other antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Chromatin immunoprecipitation (ChIP) assays were performed using a commercial kit (Upstate Biotechnology) (34). Quantitative PCR was carried out for 35 cycles using 5 μ l of sample DNA solution/50- μ l reaction, and amplification products were separated in 2.5% agarose gel in 1 \times TAE buffer.

RESULTS

***Col24a1* Contains Two Alternative Promoters**—Previous work established the entire coding sequence of the human α 1(XXIV) collagen (*COL24A1*) gene and of only part of the mouse *Col24a1* gene (11). We used this information to complete the primary structure of the mouse α 1(XXIV) collagen chain by identifying mouse expressed sequence tags in the GenBankTM and by generating PCR amplification products covering sequence gaps. As a result, we found a 19-amino acid insertion in the noncollagenous amino-terminal sequence of the mouse compared with the human chain (see GenBankTM accession numbers AY244357 and DQ157748). These experiments were also instrumental in identifying the foremost exon of *Col24a1* as consisting of a 5'-untranslated region (UTR) of undetermined length and a coding segment corresponding to the first 94 amino acid residues of the α -chain. The oligonucleotide-capping RACE approach was therefore employed to determine the *Col24a1* start site of transcription and implicitly, the

² The abbreviations used are: CREB, cyclic AMP-responsive element-binding protein; AP1, activator protein 1; ATF, activating transcription factor; ChIP, chromatin immunoprecipitation; CRE, cyclic AMP-responsive element; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LUC, luciferase; MCC, mouse primary calvarial osteoblast cells; RACE, rapid amplification of cDNA ends; UTR, untranslated region.

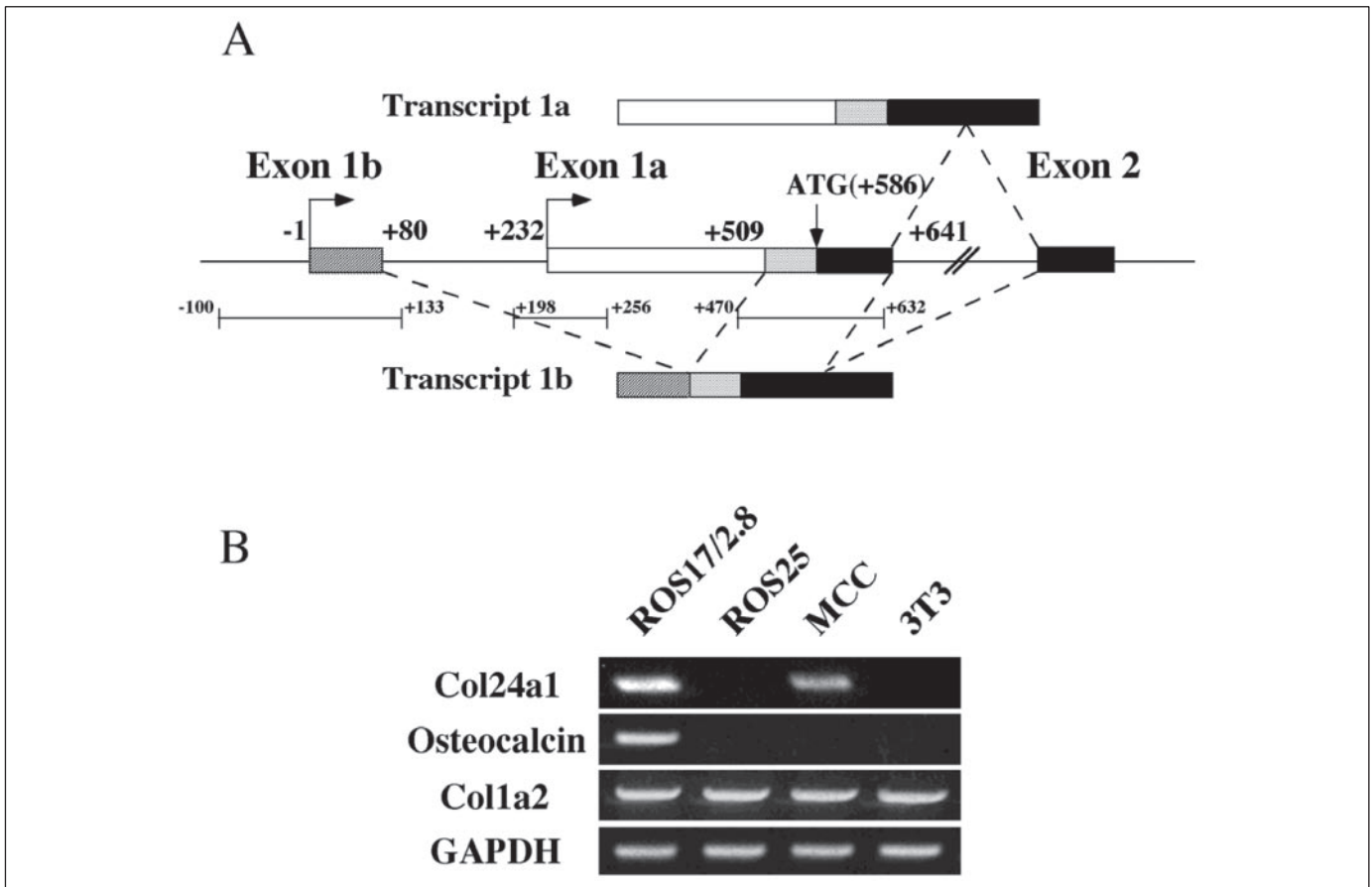


FIGURE 1. *Col24a1* transcription start sites and expression in osteoblastic cells. *A*, schematic representation of transcripts 1a and 1b (above and below the gene, respectively) with the indicated positions of the alternative spliced sequences of exons 1a and 1b, the ATG codon, and the common exon 2 sequence. Nucleotides are numbered beginning from the 5'-end of transcript 1b. The bars underneath the exon/intron scheme indicate the sequences that are conserved between the mouse and human genes. *B*, reverse transcription-PCR analysis of the relative expression of the indicated genes in ROS17/2.8 and ROS25 osteoblastic cell lines and in MCCs and NIH-3T3 fibroblasts (3T3).

5'-boundary of exon 1. As the source of template for the reaction, we utilized RNA purified from the eye and bone in which accumulation of *Col24a1* transcripts has been found to be the highest (11). Sequencing of nearly 40 independent cDNA clones from each set of RNA samples revealed the presence of two different 5'-UTRs that upon subsequent analysis of genomic clones, were accounted for by a combination of alternative splicing and transcriptional start sites. To be precise, half of the cDNA clones contained a 353-nucleotide long 5'-UTR that is continuous with the genomic sequence of the exon originally identified as the first of exon of the human gene and which was now renamed exon 1a (Fig. 1A) (11). The other half of the cDNA clones instead contained the 87 nucleotides immediately upstream of the ATG codon, in addition to an 80-nucleotide long 5'-UTR corresponding to an upstream exon (named exon 1b) that is separated from exon 1a by a 152-bp intervening sequence (Fig. 1A). Both transcripts 1a and 1b are spliced correctly into exon 2, leaving the open reading frame unaffected, and consequently, they are predicted to translate into identical $\alpha 1(\text{XXIV})$ chains (Fig. 1A). In summary, *Col24a1* contains two alternative start sites of transcription, thereby identified as -1 (exon 1a) and $+232$ (exon 1b), two alternatively spliced transcripts with different 5'-UTRs, the shortest of which (transcript 1b) splices into nucleotide $+509$ of exon 1a, and the same start site of translation, located at nucleotide $+586$ (Fig. 1A). The functional significance of *Col24a1* alternative promoters and 5'-UTR heterogeneity was not addressed in the present study. Comparison of the 5'-end sequences of the *COL24A1* and *Col24a1* genes revealed three

segments of high homology which span from nucleotides -100 to $+133$, from $+198$ to $+256$, and from $+470$ to $+632$ (Fig. 1A).

Collagen XXIV has been estimated to represent about 4% of the amount of collagen I in bone, thus slightly less than collagen V, the regulator of collagen I fibrillogenesis (11). The estimate was based on gene expression analyses that in addition documented coexpression of collagens I and XXIV genes at ossification centers in the mouse embryo (11). Osteoblasts were therefore chosen as the experimental system in which to study the transcriptional regulation of *Col24a1*. Reverse transcription-PCR amplifications were used to assess the levels of *Col24a1* expression in ROS17/2.8 and ROS25 osteosarcoma cell lines, which represent late and early stages of osteoblast differentiation, respectively (33). As positive and negative controls, PCR amplifications were also performed with RNAs purified from MCC and NIH-3T3 fibroblasts. Osteoblast-specific genes included *Col1a2* (early osteoblast differentiation marker) and osteocalcin (late osteoblast differentiation marker), whereas the ubiquitous GAPDH gene served as the normalizing control. The results of these experiments suggested that the onset of *Col24a1* expression occurs sometime after *Col2a1* and prior to osteocalcin gene activation (Fig. 1B). Implicitly, they also identified ROS17/2.8 cells as the most suitable model in which to study the anatomy of the minimal *Col24a1* promoter.

A Short Upstream Sequence Promotes Col24a1 Transcription in Osteoblasts—Cell transfection experiments were initially employed to delineate the shortest promoter sequence of *Col24a1* capable of direct-

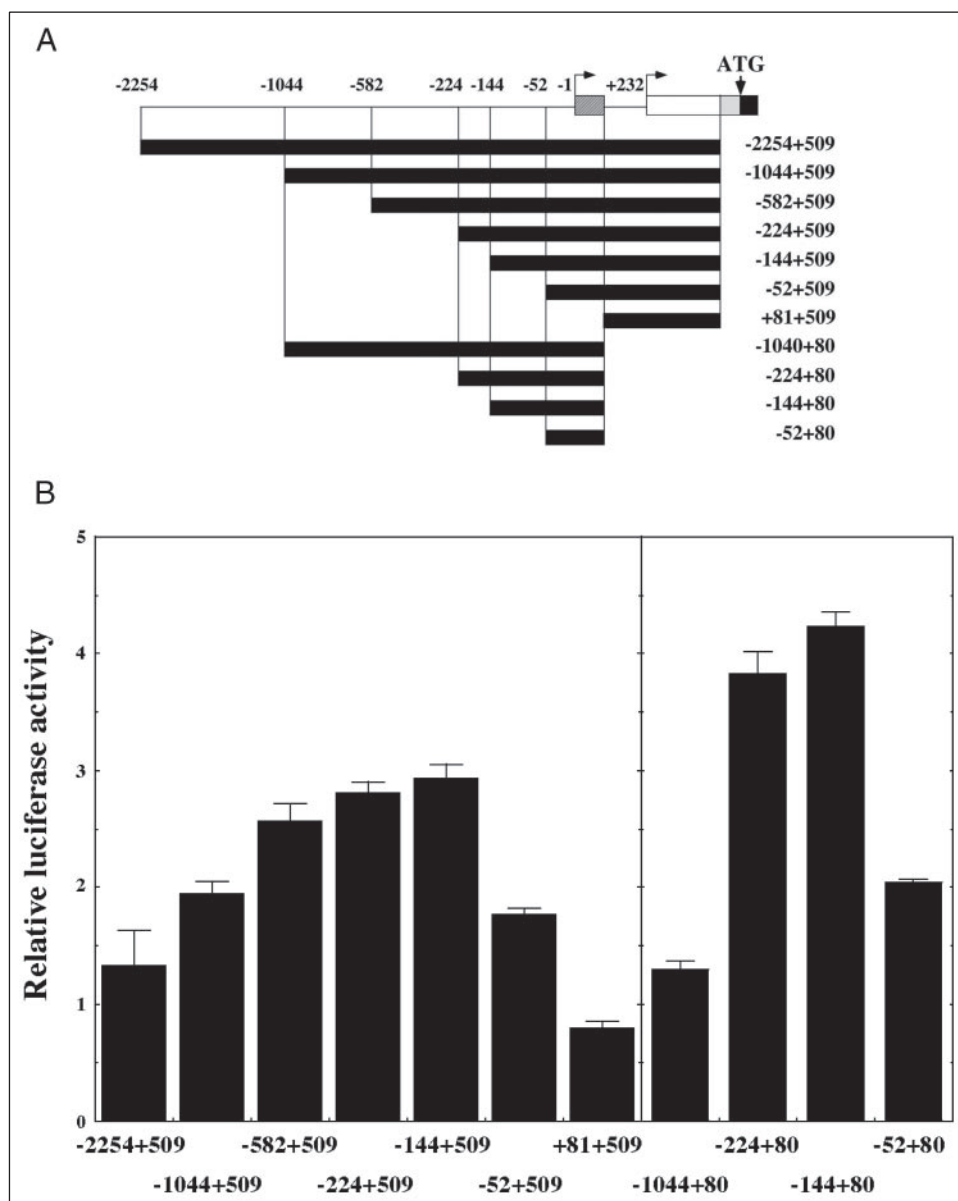


FIGURE 2. Determination of the minimal promoter sequence of *Col24a1*. *A*, luciferase reporter gene constructs harboring progressive 5'-deletions of the *Col24a1* promoter sequences that share either +509 or the +80 3'-ends. The locations of exons 1b and 1a are noted along with the ATG codon common to both transcripts 1a and 1b. *B*, relative luciferase activities of the plasmids shown in *A* assessed in transiently transfected ROS17/2.8 cells. Bars indicate the S.E. of the means.

ing transcription in ROS17/2.8 cells. To this end, we engineered two distinct sets of LUC reporter gene constructs representative of the alternative promoters of *Col24a1*. The first set of *Col24a1* promoter-LUC constructs shared the same 3'-end at position +509 and included both start sites of transcription, whereas the 3'-ends of the second set of *Col24a1* promoter-LUC constructs was located at +80 and excluded the start site of transcript 1b (Fig. 2A). Both sets of promoter-LUC constructs included progressive 5'-deletions of the upstream *Col24a1* sequence (Fig. 2A). Irrespective of the 3'-end of the promoter-LUC construct, cell transfection assays assigned maximal transcriptional activity to the region between nucleotides -144 and +80 (Fig. 2B). This promoter segment contains one of the three homology sequences of the *COL24A1* and *Col24a1* genes (Fig. 1A).

Next, an electrophoretic mobility shift assay (EMSA) was employed to identify potential DNA-nuclear protein interactions within the -144 to +81 segment of the *Col24a1* promoter. To this end, four overlapping probes (p1-p4) spanning from nucleotide -163 to nucleotide +116 were each incubated with ROS17/2.8 nuclear extracts (Fig. 3A). Specific band shifts were only obtained with overlapping probes p2 and p3,

which cover together the sequence between nucleotides -98 and +51 (Fig. 3A). To be precise, p2 yielded four retarded bands (b1-b4) of which p3 appeared to migrate as band b2 of probe p3 (Fig. 3A). In support of this postulate, band b2 disappeared from the p2 EMSA pattern when competed with a molar excess of unlabeled probe p3; conversely, formation of the p3 retarded band was eliminated by competition with a molar excess of the p2 sequence (Fig. 3B). Taken together, these results mapped the b1, b3, and b4 binding sites between nucleotides -98 and -33 and the binding site of b2 between nucleotides -33 and -15 (Fig. 4).

The functional contribution of the segment encompassing the b1-b4 binding sites was evaluated by cell transfection experiments using the -144 + 509/LUC plasmid bearing internal deletions of the -98 to -33 sequence (p2D; b1,3,4 binding sites), the overlapping -33 to -15 sequence (p2/3D; b2 binding site), or both of them (p2,3D; b1-b4 binding sites) (Fig. 4A). Unlike elimination of the b2 binding site in the p2/3D construct, deletion of the upstream sequence that gives rise to retarded bands b1, b3, and b4 led to a drop in luciferase activity of construct p2D nearly equal to the -144 + 509/LUC construct with the internal dele-

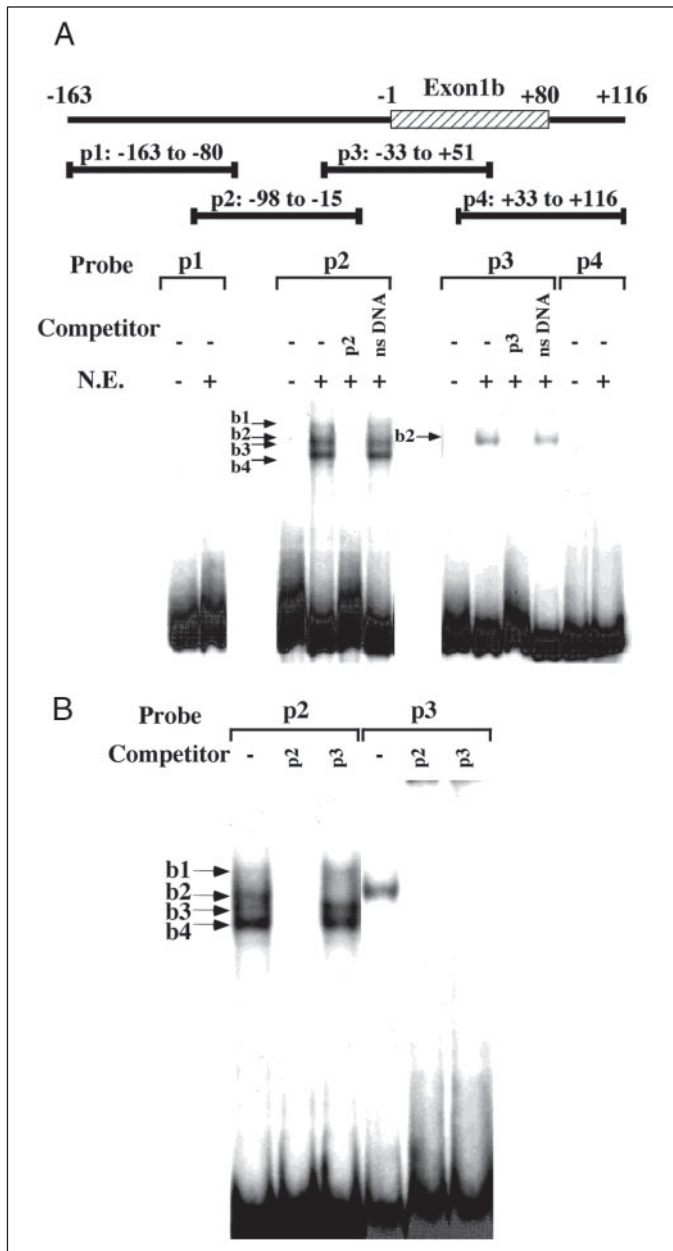


FIGURE 3. Identification of nuclear protein binding sites in the *Col24a1* proximal promoter. A, EMSAs showing the retarded complexes that are formed with the probes shown at the top (p1–p4) in the absence (–) and in the presence (+) of ROS17/2.8 nuclear extracts (N.E.) and in the absence (–) or presence (+) of a 100-fold molar excess of the indicated p competitors or an unrelated DNA (ns DNA). The relative positions of retarded complexes b1–b4 are shown on the left of the p2 and p3 autoradiographs. B, EMSAs demonstrating that binding of the b2 retarded complex is in the sequence in common between probes p2 and p3.

tion of all binding sites (p2,3D) or the shorter $-52 + 509$ /LUC plasmid (Fig. 4B). We therefore focused on the characterization of the factors binding to the sequence between -98 and -33 because this *cis*-acting element appears to drive most of *Col24a1* promoter expression in ROS17/2.8 cells.

Binding of CREB-ATF and AP1 Complexes Is Required for *Col24a1* Transcription—A series of shorter oligonucleotides encompassing the -98 to -33 sequence was used in the EMSA to compete *in vitro* binding to the p2 probe to narrow down the site(s) of nuclear protein interaction within this *cis*-acting element. The results clearly demonstrated that all p2 retarded complexes (b1, b3, and b4) were competed by the p2b oli-

gonucleotide that extends from nucleotides -55 to -38 (Fig. 5A). Inspection of the -55 to -38 sequence identified within it the TGACGTCA sequence, which represents a perfect match of the cyclic AMP-responsive element (CRE) protein/ATF binding site (Fig. 5B) (36). Indeed competition experiments using a molar excess of a mutated CRE oligonucleotide failed to interfere with formation of the b1, b3, and b4 complexes (Fig. 5B). The significance of the binding assays was corroborated further by cell transfection experiments that showed a loss of promoter activity in the $-144 + 509$ /LUC plasmids bearing the same p2b mutation (Fig. 4).

Members of the CREB-ATF family of nuclear factors bind with high affinity to CRE, but AP1 dimers can also interact with CREB-ATF sites, depending on the composition of their flanking sequences (36). Antibodies specific for AP1 and CREB family members were therefore used in a screen to identify which of the possible protein combinations bind the p2b element of *Col24a1*. The results showed supershifts or binding interferences only with c-Jun, CREB1, ATF1, and ATF2 antisera (Fig. 6). Importantly, each of the antibodies was noted to affect formation of different retarded bands, implying that a distinct combination of AP1 and CREB-ATF proteins binds to the p2b element that directs *Col24a1* transcription in ROS17 cells (Fig. 6, A and B). This last conclusion was corroborated by EMSAs in which different combinations of antibodies suggested that band b1 corresponds to c-Jun/ATF2 heterodimers, band b3 to CREB1 homodimers, and band b4 to CREB1/ATF1 heterodimers (Fig. 6, C and D).

Expression vectors for c-Jun, ATF1, ATF2, or CREB1 were each cotransfected into ROS17/2.8 cells together with the wild-type or p2a mutant $-144 + 509$ /LUC plasmid to correlate DNA-protein binding with promoter function. These functional assays demonstrated that each of the four recombinantly expressed nuclear factors was capable of stimulating *Col24a1* promoter activity in a dose-dependent manner and only when the integrity of the p2b sequence was preserved (Fig. 7A). Independent confirmation of the functional assays was obtained by a ChIP assay that documented *in vivo* occupancy by CREB1, ATF1, c-Jun, and ATF2 of the p2b element in the endogenous *Col24a1* promoter (Fig. 7B). Consistent with the EMSA data, this *in vivo* assay also provided evidence for CREB-AP1 specificity by showing lack of JunD and ATF4 binding to the collagen but not the TIMP1 or osteocalcin promoters (Fig. 7B) (37, 38).

DISCUSSION

The present study demonstrated that *Col24a1* is a marker of late osteoblast differentiation which is positively regulated by the binding of specific combinations of CREB-AP1 proteins to an upstream *cis*-acting element, which is shared by the two alternative promoters of the gene. These findings advance knowledge of the transcriptional pathways that regulate formation of fibrillar collagen assemblies in the skeleton. They will also inform the characterization of genetic programs that may be negatively affected by the loss of collagen XXIV in the developing and adult mouse bone.

The role of collagen I fibrils in bone physiology is well established as is the contribution of the minor collagen V to guiding nucleation of collagen I fibrils in the skin and the eyes and by extrapolation, in bone tissue (1–3, 6, 8, 9). The expression pattern of *Col24a1* in the developing bone and the eye is at least consistent with a similar role of this macromolecule in collagen I fibrillogenesis, even though the lack of suitable antibodies has hampered experimental confirmation of this hypothesis. Our preliminary findings indicate that *Col24a1* is inactive in ROS25 cells, which correspond to early differentiating osteoblasts, but not in ROS17/2.8 cells, which represent late differentiating osteoblasts, or in calvarial

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FIGURE 4. Functional contribution of the b1,3,4 and b2 binding sites to minimal promoter activity. Luciferase activity of various mutant promoter sequences was evaluated in transiently transfected ROS17/2.8 cells in relationship to that of the wild-type -144 to +509 promoter construct in which the relative positions of the b1,3,4 and b2 binding sites are shown. Mutations include deletions of the sequence encompassing probes p2 and p3 (p2,3D plasmid), only probe p2 (p2D plasmid), only probe p3 (p3D plasmid), or the overlap between probes p2 and p3 (p2/3D plasmid), as well as single nucleotide substitutions in the nuclear protein binding sites of probe p2 (plasmid p2bmt, see also *top* of Fig. 5B). Bars indicate the S.E. of the means.

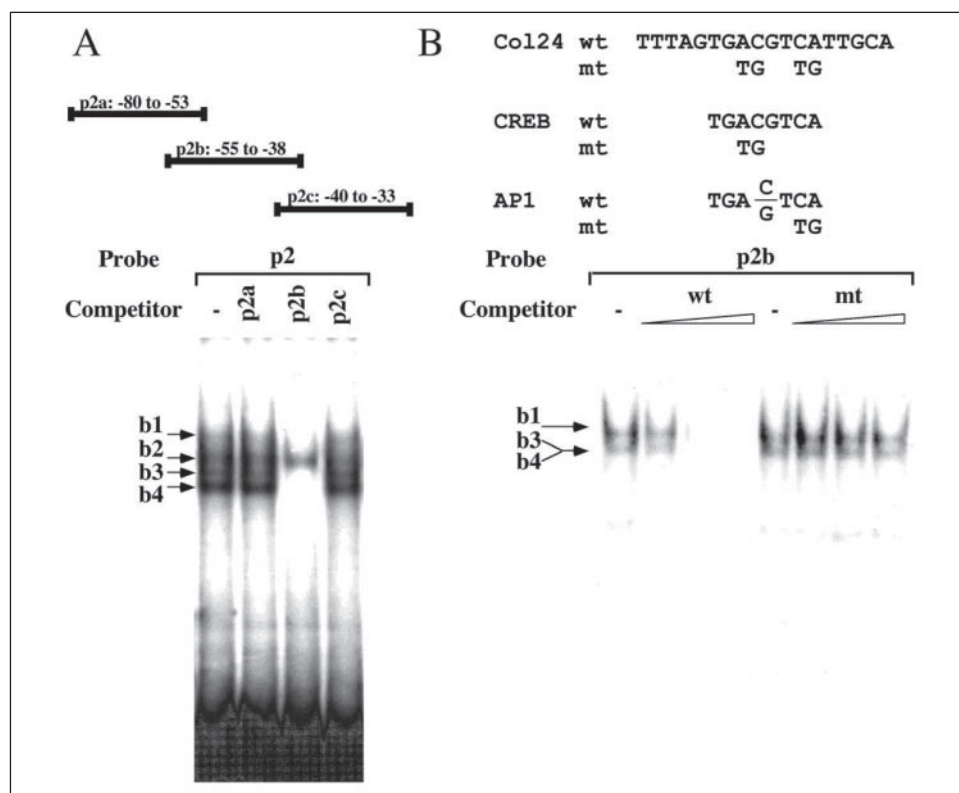
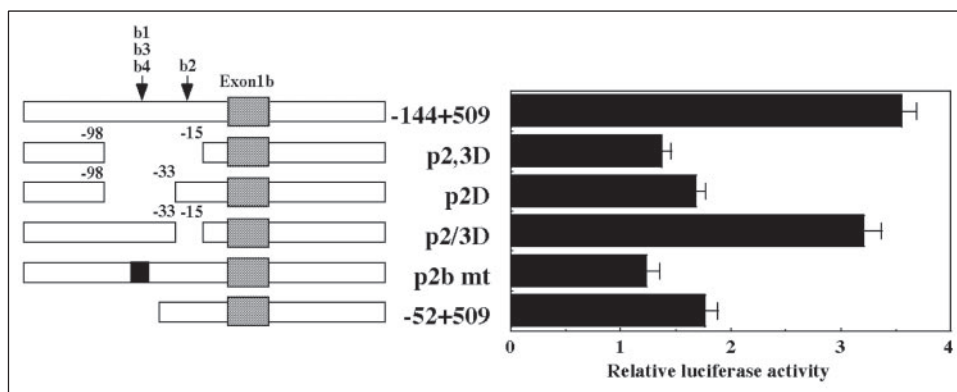


FIGURE 5. Mapping of the nuclear protein binding sites within the probe p2 sequence. A, EMSA using the p2 probe in the absence (-) or in the presence (+) of a 100-fold molar excess of unlabeled oligonucleotides p2a-c that cover the p2 sequence (nucleotide positions shown above the autoradiographs). B, *top*, the CREB-ATF binding site within the p2b sequence is highlighted, and the nucleotide substitutions (*mt*) are indicated (p2bmt, see also relevant functional assay of Fig. 4); *bottom*, EMSAs showing formation of retarded complexes between the p2b probe and ROS17/2.8 nuclear extracts in the absence (-) or in the presence (+) of increasing (10-100-fold) molar excess of unlabeled wild-type (*wt*) or mutant (*mt*) p2b oligonucleotides.

osteoblasts, albeit at seemingly lower levels than in ROS17/2.8 cells. Within the limitations of this experimental system, these results nonetheless suggest that collagen XXIV is an integral part of the genetic program of osteoblast terminal differentiation (39). That collagen XXIV is expressed at a lower level in nonskeletal tissues, such as the brain and the eye, also suggests a potentially broader role in organogenesis (11).

Our study adds AP1 and CREB-ATF proteins to the list of transcription factors that are involved in the regulation of fibrillar collagen genes, particularly in osteoblastic cells. A large body of work has demonstrated the critical contributions of these two families of basic leucine zipper proteins to bone formation and remodeling (40-42). For example, genetic alterations in functions of AP1 and related proteins have been shown to affect negatively osteoblast differentiation and function as well as bone development (40). Similarly, transgenic interference of CREB protein activity greatly impairs the normal process of endochondral bone formation (43). Members of the AP1 or CREB-ATF family of nuclear factors can form homodimeric or heterodimeric protein complexes, which transduce distinct signals and exert discrete transcriptional responses on various promoter targets (36). The heterogeneity in

dimer composition is the main determinant of the functional diversification of AP1 and CREB-ATF complexes, which include dimers within and between selected members of each family of transcription factors (36, 40, 41). Obligatory combinations of CREB-ATF proteins recognize the octameric TGACGTCA element, as do heterodimers between ATFs and specific Jun and/or Fos proteins (36). In line with this last consideration, our antibody interference experiments indicate that CREB1, CREB1/ATF1 and c-Jun/ATF2 dimers can specifically bind *in vitro* to the evolutionarily retained TGACGTCA sequence (p2b element) of the *Col24a1* and *COL24A1* promoters. Further confirmation of this *in vitro* finding was provided by the ChIP assay, which showed that the endogenous p2b site of the rat gene is occupied in ROS17/2.8 cells by the CREB1, ATF1, ATF2, and c-Jun proteins. Consistent with this result, we showed that overexpression of each of these four nuclear proteins in osteoblasts stimulates transcription from the wild-type *Col24a1* promoter, but not from the same promoter harboring four nucleotide substitutions in the CREB-ATF binding site.

Although our experiments left unresolved whether and how the various CREB-AP1 complexes may compete for 2b binding, they clearly

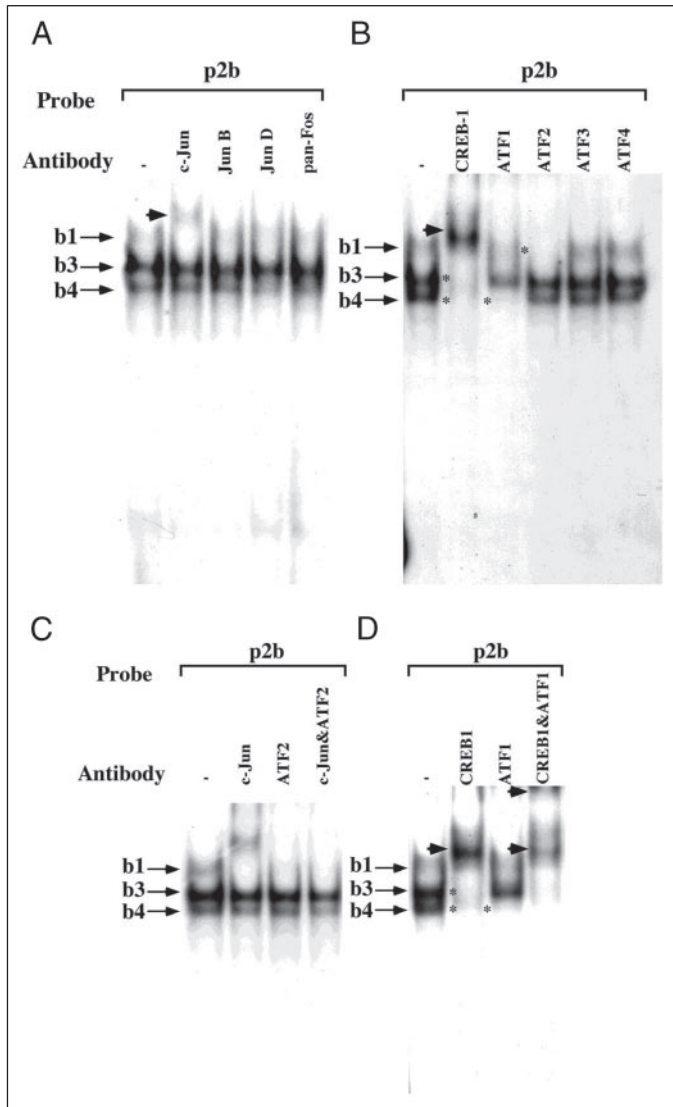


FIGURE 6. **Composition of the b1,3,4 retarded complexes.** EMSAs show the effects of the indicated antibodies (supershift (arrowhead) or binding interference (asterisk)) on protein complex formation between probe p2b and ROS17/2.8 nuclear extracts. Specifically, antibodies included those against Jun/Fos family members (A) or CREB-ATF family members (B), as well as those against individual components or combinations of c-Jun/ATF2 (C) or of CREB1/ATF1 (D).

indicate that binding of these nuclear proteins stimulates transcription from the minimal *Col24a1* promoter in osteoblasts. This conclusion is based on the absolute requirement of element 2b integrity for promoter activity and on the positive effect on promoter activity of each of the four nuclear proteins overexpressed in ROS17/2.8 cells. The expression profiles of the proteins that bind element 2b during osteoblast differentiation *in vitro* are also consistent with the time of *Col24a1* onset estimated by the present study. Jun proteins are in fact highly expressed in differentiating osteoblasts prior to matrix production and mineralization, and phosphorylated CREB reaches its highest level in the early mineralization stage (44, 45). Irrespective of whether or not these findings are functionally correlated, the characterization of the *Col24a1* promoter further support the emerging notion that distinct regulatory pathways coordinate expression of different fibrillar collagen genes in bone tissue. Transgenic studies have in fact indicated that cooperation between an Sp1 binding site in the proximal promoter and uncharacterized complex(es) interacting with a far upstream enhancer directs bone-specific expression of the human *COL1A2* gene (46). The same

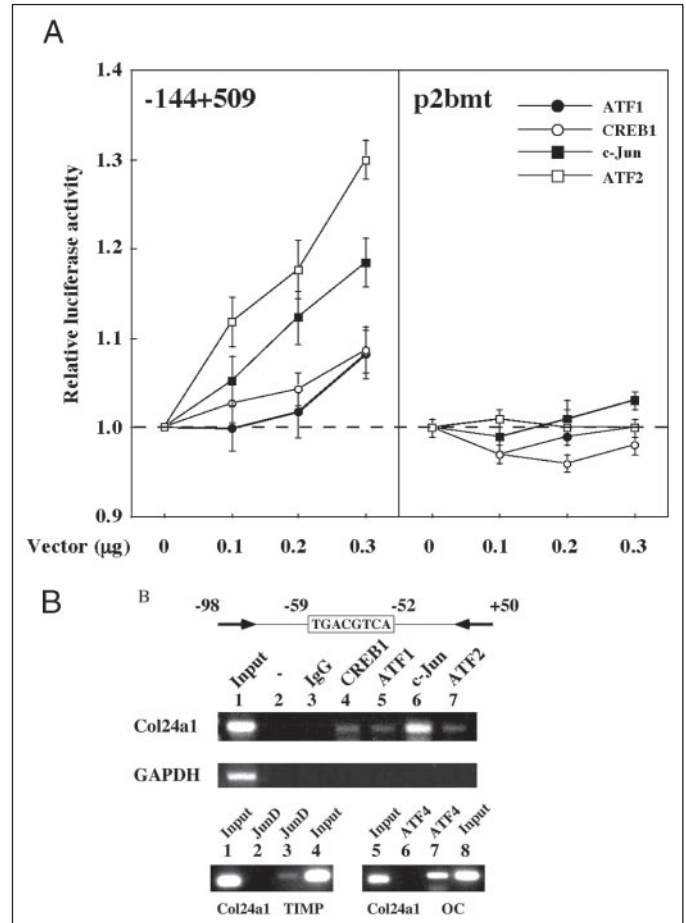


FIGURE 7. **Specific CREB and AP1 family members bind and stimulate transcription of the minimal *Col24a1* promoter.** A, cotransfection experiments showing that increasing amounts of the indicated expression vectors stimulate the activity of the wild-type (-144 + 509) but not of the mutant (p2bmt) minimal *Col24a1* promoter. B, ChIP analysis of the relevant *Col24a1* proximal promoter sequence using antibodies against CREB1, ATF1, c-Jun, or ATF2. Controls include input DNA, no antibodies, and preimmune serum (IgG). At the bottom of the figure are other control ChIP assays that confirm lack of JunD and ATF4 binding to the collagen but not to the TIMP1 or osteocalcin (OC) promoters.

kind of approach has identified positive osteoblast-specific elements in the upstream promoter of the mouse and rat *Col1a1* genes, as well as a δ EF1/ZEB1 binding site further upstream which represses transcription of the mouse promoter in osteoblasts (47–49). Work in progress is extending the present work to the characterization of possible interactions of the CREB-AP1 complexes with other nuclear proteins, in addition to evaluating these *in vitro* findings within the physiological context of the transgenic mouse model.

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