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Cbfa1 Is a Positive Regulatory Factor in Chondrocyte Maturation*

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Cbfa1 is a transcription factor that belongs to the runt domain gene family. *Cbfa1*-deficient mice showed a complete lack of bone formation due to the maturational arrest of osteoblasts, demonstrating that Cbfa1 is an essential factor for osteoblast differentiation. Further, chondrocyte maturation was severely disturbed in *Cbfa1*-deficient mice. In this study, we examined the possibility that Cbfa1 is also involved in the regulation of chondrocyte differentiation. mRNAs for both Cbfa1 isotypes, type I Cbfa1 (Pebp2αA/Cbfa1) and type II Cbfa1 (Osf2/Cbfa1 or til-1), which are different in N-terminal domain, were expressed in terminal hypertrophic chondrocytes as well as osteoblasts. In addition, mRNA for type I Cbfa1 was expressed in other hypertrophic chondrocytes and prehypertrophic chondropcytes. In a chondrogenic cell line, ATDC5, the expression of type I Cbfa1 was elevated prior to differentiation to the hypertrophic phenotype, which is characterized by type X collagen expression. Treatment with antisense oligonucleotides for type I Cbfa1 severely reduced type X collagen expression in ATDC5 cells. Retrovirally forced expression of either type I or type II Cbfa1 in chick immature chondrocytes induced type X collagen and *MMP13* expression, alkaline phosphatase activity, and extensive cartilage-matrix mineralization. These results indicate that Cbfa1 is an important regulatory factor in chondrocyte maturation.

A transcription factor for osteoblast differentiation and osteogenesis has recently been revealed (1). This factor, Cbfa1 (core-binding factor), also called Pebp2αA (polyoma enhancer-binding protein), is a transcriptional regulatory factor that belongs to the runt domain gene family (2). It has a DNA-binding domain, *runt*, that is homologous with the *Drosophila* pair-rule gene *runt* (3). Targeted inactivation of *Cbfa1* resulted in a complete lack of bone formation due to the maturational arrest of osteoblasts (4, 5). Further, overexpression of *Cbfa1* induced markers for osteoblast differentiation, and the treatment of *Cbfa1* antisense oligonucleotides reduced the expression of these markers and mineralized nodule formation *in vitro* (6, 7). These observations revealed that Cbfa1 is an essential transcription factor for osteoblast differentiation (1). Mice heterozygously mutated in the *Cbfa1* locus showed a phenotype similar to the human inherited disease cleidocranial dysplasia, and mutations in the *Cbfa1* locus have been found in patients with this disease (4, 5, 8, 9).

Two isotypes of Cbfa1 with different N-terminal domains, one starting from exon 2, which we designated type I Cbfa1 (originally cloned as a T-cell-specific factor, Pebp2αA) (2), and the other starting from exon 1, designated type II Cbfa1 (originally cloned as an osteoblast-specific factor, Osf2/Cbfa1 or til-1) (6, 10), have been identified. Although two translational start sites were originally reported in the transcript starting from exon 1, the second ATG was extremely efficient for translation (11), and we designated it as type II Cbfa1. These isotypes showed different transcriptional activation in their target genes (12).

In endochondral ossification, skeletal formation begins with the condensation of mesenchymal cells and their differentiation into chondrocytes, forming a cartilage template. These chondrocytes then undergo a program of proliferation and maturation. The maturational process of chondrocytes can be defined by the expression of extracellular matrix genes as well as chondrocyte morphology. Prechondrogenic cells express type I collagen, and the induction of type II collagen occurs with the change of cellular phenotype from prechondrogenic cells to proliferating chondrocytes (13). The expression of type X collagen is restricted in hypertrophic chondrocytes, and the induction of type X collagen mRNA is regarded as signaling the change in cellular phenotype from proliferating chondrocytes to hypertrophic chondrocytes (14).

In *Cbfa1*-deficient mice, it has been shown that endochondral ossification is completely blocked and chondrocyte maturation is disturbed (4, 5, 15, 16), suggesting that Cbfa1 is related to chondrocyte maturation. In this study, we investigated *Cbfa1* expression in chondrocyte maturation and found that *Cbfa1* mRNA was detected in prehypertrophic and hypertrophic chondrocytes as well as in osteoblasts. To investigate the function of Cbfa1 in chondrocyte maturation, we used a mouse chondrogenic cell line, ATDC5 (17, 18) and chick primary chondrocytes (19, 20). We show that antisense oligonucleotides for *Cbfa1* suppressed the differentiation of ATDC5 cells into hypertrophic chondrocytes and that the introduction of either type I or type II Cbfa1 isotypes into immature primary chondrocytes markedly induced their differentiation to hypertrophic chondrocytes, indicating that Cbfa1 plays a crucial role in chondrocyte maturation.

MATERIALS AND METHODS

Hybridization Probes—Four *Cbfa1* cDNA probes (probes A, B, C, and D) were used for Northern blot analysis or *in situ* hybridization (see Fig.

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1). Probe A is a 0.6-kilobase pair *Pst*I–*Hind*III fragment of the 3′-untranslated region in exon 8 of mouse *Cbfa1* cDNA (4), and probe B is a 0.9-kilobase pair fragment including the coding region 3′ of the runt domain. Probe C is a 0.32-kilobase pair fragment of the 5′-untranslated region in exon 2 of mouse *Cbfa1* cDNA (2), and probe D is a 0.3-kilobase pair fragment of exon 1. Probes for type I collagen, type II collagen, type X collagen, and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)[1] were described previously (15).

In Situ Hybridization—Digoxigenin-11-UTP-labeled single-stranded RNA probes were prepared using a DIG RNA labeling kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. Hybridization was carried out as described previously (21).

Cell Culture—A chondrogenic cell line, ATDC5, was purchased from the RIKEN Cell Bank (Tsukuba Science City, Japan). ATDC5 cells were cultured in DMEM/Ham's F-12 (1:1) hybrid medium (Life Technologies, Inc.) supplemented with 5% fetal bovine serum (FBS) (Life Technologies), 10 μg/ml human transferrin (Roche Molecular Biochemicals), and 3×10^{-8} M sodium selenite (Sigma). To induce chondrogenesis, ATDC5 cells were plated at a density of 6×10^4 cells/well in six-well plates and cultured for 22 days in the above medium supplemented with 10 μg/ml of human recombinant insulin (Wako Pure Chemical, Osaka, Japan). RNA was extracted from cultured cells when ATDC5 cells became confluent (4 days after plating) and then extracted every 3 days after confluence.

Chicken embryo fibroblasts were obtained from the torso of virus-free White Leghorn 11-day-old embryos (line M) (Nisseiken, Yamanashi, Japan) and cultured in medium 199 containing 10% FBS. Chondrocytes were isolated from one-third of a caudal portion of 17-day-old embryo (line M) sternum and cultured in high glucose DMEM containing 10% FBS as described previously (19, 20). In primary cultures, these chondrocytes actively proliferate and display an immature phenotype characterized by lack of type X collagen and alkaline phosphatase (APase) (20).

RNA Extraction and Northern Blot Analysis—Total RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan), and poly(A) RNA was extracted from about 200 μg of total RNA using Dynabeads mRNA DIRECT kit (DYNAL A.S, Oslo, Norway) according to the manufacturer's protocol. The mouse cDNAs described above were labeled with [α-^{32}P]dCTP, and hybridization was performed as described previously (15). Each membrane was rehybridized with α-^{32}P-labeled GAPDH cDNA. The intensities of *Cbfa1*, type X collagen, and GAPDH bands were quantitated by densitometry using FMBIO Analysis software (Hitachi Software Engineering Co. Ltd., Japan).

Treatment with Antisense Oligonucleotide—For antisense studies, phosphorothioate oligonucleotides (S-oligos) were commercially synthesized (BEX Co. Ltd., Tokyo, Japan). ATDC5 cells were plated at a density of 1×10^5 cells/well in six-well plates, and S-oligos (10 μM) were added every day with fresh medium during the period from day 0 to 11 (with day 0 as confluence).The sequences of the antisense (AS) S-oligos, sense (SE) S-oligos, and control-scrambled (CS) S-oligos are as follows: AS for type I Cbfa1 (5′-CTACAGGAATACGCA-3′), SE for type I Cbfa1 (5′-TGCGTATTCCTGTAG-3′), CS for type I Cbfa1 (5′-CACGAACTAGACAGT-3′), AS for type II Cbfa1 (5′-CTGCGCTGAAGAGGCTGTTTGA-3′), SE for type II Cbfa1 (5′-TCAAACAGCCTCTTCAGCGCAG-3′), CS for type II Cbfa1 (5′-CGCGTATCGTGATGTAGACGTG-3′) (6).

Construction of Polymerase Chain Reaction (PCR) Competitors—Competitors for type I and type II Cbfa1 were generated according to the method previously described (Ref. 22; and see Fig. 4*A*). Competitors for type I and type II Cbfa1 cDNA were generated with Amp *Taq* DNA polymerase (Perkin-Elmer) and a Gene Amp PCR system 2400 (Perkin-Elmer) (30 s at 94 °C, 30 s at 63 °C, and 30 s at 72 °C) using the following primers: competitor for type I Cbfa1 (5′-GCACCGCCGAGATGGACTGCTGAAGGCCCCTACTGCAAGCTGTTA-3′ and 5′-GGCGGAAAGAGGATGGAGGTGACG-3′) and competitor for type II Cbfa1 (5′-GAGGGCACAAGTTCTATCTGGACAGCAGCCTGCAGCCCGGCAAGA-3′ and 5′-GGTGGTCCGCGATGATGTC-3′).

After gel purification by standard techniques, serial dilutions of the PCR products were added to the samples as competitors.

Competitive Reverse Transcriptase PCR (RT-PCR)—Total RNA was isolated from cultured cells, and first-strand cDNA was made as described previously (15). The cDNA and serial dilutions of the competitor were co-amplified by Amp *Taq* DNA polymerase using the following primers: cDNA for type I (5′-GCACCGCCGAGATGGACTGCTGAA-3′ and 5′-GGCGGAAAGAGGATGGAGGTGACG-3′) and cDNA for type II (5′-GAGGGCACAAGTTCTATCTGGA-3′ and 5′-GGTGGTCCGCGATGATGTC-3′) (6).

Thirty-five cycles of co-amplification were done for both type I and type II Cbfa1 and their competitors using a Gene Amp PCR system 2400 (30 s at 94 °C, 30 s at 70 or 63 °C, and 30 s at 72 °C). PCR products were electrophoresed and quantitated. If the amplified bands were faint or difficult to recognize, PCR cycling was increased to 45 cycles.

Construction of Recombinant Type I and Type II Cbfa1 Retroviruses—cDNAs including entire coding sequences of mouse type I or type II Cbfa1, in which the Kozak consensus sequence CCACC is attached to the 5′-end of ATG (23), were recently used for the functional analysis of Cbfa1 isotypes (12). Type I and type II cDNAs were each subcloned into the RCAS retroviral vector (24). Constructed vectors were transfected into chicken fibroblasts by the FuGENE6 transfection reagent according to the manufacturer's protocol (Roche Molecular Biochemicals). The recombinant virus in the medium was concentrated by centrifugal concentrators and used to infect freshly isolated chondrocytes (25).

Histochemical and Immunochemical Procedures—To detect APase activity, cultures were washed with saline twice, incubated with 50 mM Tris-HCl, pH 9.5, containing 0.5 mg/ml of naphtol AS-BI phosphate and 1 mg/ml of Fast Red trisodium salts (Sigma) for 15 min at 37 °C, and then fixed with 3.7% neutralized formaldehyde solution.

For determination of expression of the constructed genes, cultures were harvested by trypsinization and replated on poly-L-lysine-coated dishes at a density of 2.0×10^6 cells/ml. After 15 min, the cultures were washed with PBS twice, fixed with 3.7% neutralized formaldehyde solution, and permeabilized by incubation with PBS containing 0.5% Triton X-100 for 15 min. Cultures were then incubated with the polyclonal antibody (aA1c17) raised against a recombinant polypeptide fragment of mouse Cbfa1 (amino acids 359–513) (26). Localization of the antibody was visualized by ABC methods using Histofine kits (Nichirei, Tokyo, Japan).

To detect calcium accumulation, the cultures were washed with saline and fixed with 95% ethanol. Alizarine red staining (27) and von Kossa staining were then carried out.

To detect type X collagen, the cultures were washed with saline, fixed with 70% ethanol, and incubated with antibody to chicken type X collagen (28) and then with rhodamine-labeled anti-rabbit IgG.

Nuclear Extraction and Immunoblotting—Nuclear extracts were prepared from the virus-infected cultures (P1 cultures) according to the method previously described (29). 10 μg of nuclear extract was separated on a 10.0% gel by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore Japan, Tokyo, Japan). After blocking the membrane with 10% horse serum overnight at 22 °C, the membrane was incubated with polyclonal rabbit antibody (aA1c17) against Cbfa1 and then with peroxidase-conjugated anti-rabbit IgG goat antibody (Biomedical Technologies Inc., Stoughton, MA).

Measurement of DNA Content, Sulfated Glycosaminoglycan Content, and APase Activity—Cultures were harvested in saline solution containing 0.2% Triton X-100 and 0.02 N NaOH and sonicated. Cell lysates were then centrifuged, and the supernatant was used for the determination of DNA by the fluorometric procedure of Johnson-Wint and Wellis (30). To evaluate cartilage-specific proteoglycan synthesis, sulfated glycosaminoglycan, a major component of the proteoglycan, was measured by direct spectrophotometric microassay (31). APase activity of the cell layer was measured as described previously (4). 1 unit of APase activity corresponded to the hydrolysis of 1 nmol of *p*-nitrophenyl phosphate/30 min at pH 9.0.

RT-PCR for Chick Chondrocyte Cultures—Total RNA from chick chondrocyte cultures was reverse transcribed and amplified for 30 cycles with a Gene Amp PCR system 2400 (10 s at 95 °C and 1 min at 60 °C) using the following primers: chick type IX collagen (5′-ATTCTGGGTGCTCGTCAAAGAAC-3′ and 5′-AGCACTGAGAAGCCATCAACACT-3′) (32), chick *MMP13* (5′-CTTCGTGGAGAAATGCTGGTATT-3′ and 5′-TCGCAGAACTCTGCTTTCCTCTA-3′) (33), chick osteocalcin (5′-ACGAGAGGTGTGTGAGCTGA-3′ and 5′-GCGCTCTGCCTTTATTTCTG-3′) (34), chick hypoxanthine guanine phosphoribosyl transferase (5′-GC(A/G)TCGTGATT(A/G)GCGATGATGA-3′ and 5′-GTC(A/G)AGGGC(A/G)TATCCAACAACA-3′) (GeneBank™ accession number AJ132697).

[1] The abbreviations used are: GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; APase, alkaline phosphatase; AS, SE, and CS, antisense, sense, and control-scrambled, respectively; Cbfa1, core-binding factor; FBS, fetal bovine serum; RT, reverse transcriptase; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; S-oligos, phosphorothioate oligonucleotides; bp, base pairs.

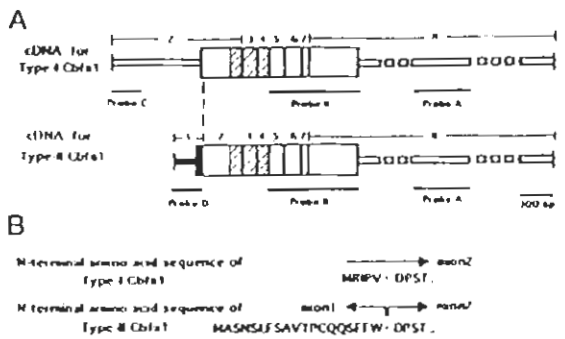

FIG. 1. **Organization of cDNAs for type I and II Cbfa1.** *A*, schematic representation of the organization of cDNAs for type I and II Cbfa1. Translated and untranslated regions are indicated as *tall boxes* and *short boxes*, respectively. The length of the untranslated region of exon 8 is arbitrary. Exons are numbered *above* the *boxes*, and the runt domain is *hatched*. A *vertical dotted line* shows the position where exon 1 (*closed box*) is spliced to the middle of exon 2 in the generation of type II Cbfa1. The upstream regions of the *vertical dotted line*, which include 5'-untranslated regions and N-terminal sequences, are different from each other, whereas the downstream regions of the *dotted line* have common sequences. Probes A and B recognize both mRNAs for type I and type II Cbfa1, probe C specifically recognizes mRNA for type I Cbfa1, and probe D specifically recognizes mRNA for type II Cbfa1. *B*, sequences of N-terminal amino acids of type I and II Cbfa1. Type I and type II Cbfa1 have different amino acid sequences in the N-terminal domains. Type I Cbfa1 has a unique 5-amino acid sequence, and type II Cbfa1 has a unique 19-amino acid sequence in the N terminus.

RESULTS

Cbfa1 Expression in Chondrocytes—Cbfa1 has two isotypes with different N-terminal domains, one starting from exon 2, which we designated type I Cbfa1 (originally cloned as Pebp2αA) (2), and the other starting from exon 1, designated type II Cbfa1 (originally cloned as Osf2/Cbfa1 or til-1) (6, 10) (Fig. 1). We first investigated the expression of these isotypes of Cbfa1 in growth plates of tibia at E16.5 mice by *in situ* hybridization. To distinguish the mRNAs for type I and type II Cbfa1, we used four probes (A, B, C, and D). Probe A, which hybridizes with an untranslated region in exon 8 and recognizes mRNAs for both type I and type II Cbfa1, detected *Cbfa1* transcripts strongly in osteoblasts and hypertrophic chondrocytes near the vascular invasion front (terminal hypertrophic chondrocytes) and weakly in other hypertrophic chondrocytes and prehypertrophic chondrocytes (Fig. 2, *A* and *B*). Similar results were obtained using probe B, which hybridizes with the translated region 3' of runt domain and also recognizes mRNAs for both type I and type II Cbfa1 (Fig. 2, *D* and *E*). When we used probe C, which hybridizes with an untranslated region in exon 2 and specifically recognizes the mRNA for type I Cbfa1, Chfa1 expression was similarly detected compared with that detected by probes A and B (Fig. 2, *G* and *H*). Probe D, which hybridizes with exon 1 and specifically recognizes the mRNA for type II Cbfa1, detected Cbfa1 transcripts strongly in osteoblasts and terminal hypertrophic chondrocytes, whereas the hybridization signals in other hypertrophic chondrocytes and prehypertrophic chondrocytes were faint (Fig. 2, *J* and *K*). Thus, the mRNAs for both Cbfa1 isotypes (types I and II) were expressed in osteoblasts, whereas the mRNA for type I Cbfa1 was expressed in chondrocytes with maturational stages ranging from prehypertrophic to terminal hypertrophic chondrocytes. The prominent expression of type II Cbfa1 in chondrocytes was restricted in terminal hypertrophic chondrocytes.

Cbfa1 Expression during ATDC5 Cell Differentiation to Hypertrophic Phenotype – The expression of *Cbfa1* in chondrocytes led us to the hypothesis that Cbfa1 plays an important role in chondrocyte differentiation. To examine this possibility, we

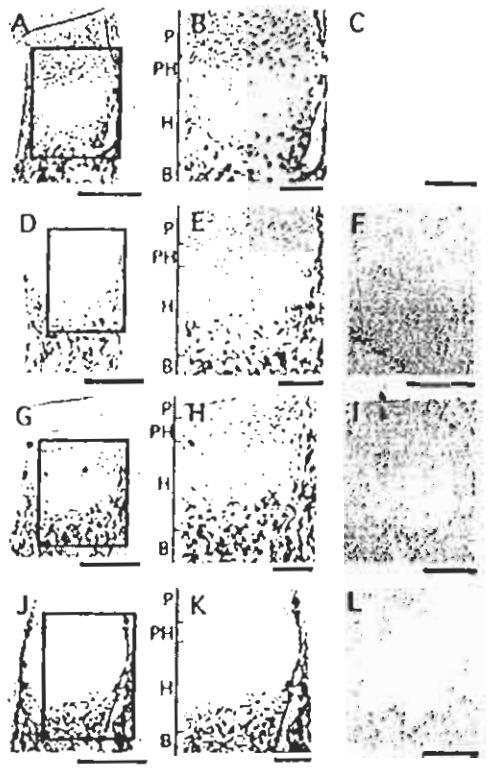

FIG. 2. ***Cbfa1* expression in E16.5 wild-type embryo.** *A* and *B*, *in situ* hybridization of tibia with antisense probe A (see Fig. 1). *Cbfa1* is strongly expressed in terminal hypertrophic chondrocytes as well as in osteoblasts. *Cbfa1* is weakly expressed in other hypertrophic chondrocytes and prehypertrophic chondrocytes. The *boxed region* in *A* is magnified in *B*. *C*, *in situ* hybridization of tibia with sense probe A. *D* and *E*, *in situ* hybridization of tibia with antisense probe B. The *Cbfa1* expression pattern is similar to that seen in *A* and *B*. The *boxed region* in *D* is magnified in *E*. *F*, *in situ* hybridization of tibia with sense Probe B. *G* and *H*, *in situ* hybridization of tibia with antisense Probe C. mRNA for type I Cbfa1 is detected strongly in terminal hypertrophic chondrocytes and osteoblasts and weakly in other hypertrophic chondrocytes and prehypertrophic chondrocytes, similar to the results with probes A and B. The *boxed region* in *G* is magnified in *H*. *I*, *in situ* hybridization of tibia with sense probe C. *J* and *K*, *in situ* hybridization of tibia with antisense probe D. mRNA for type II Cbfa1 is detected in terminal hypertrophic chondrocytes and in osteoblasts. Its expression in other hypertrophic chondrocytes and prehypertrophic chondrocytes is also detectable, but it is faint. The *boxed region* in *J* is magnified in *K*. *L*, *in situ* hybridization of tibia with sense probe D. *P*, layer of proliferating chondrocytes; *PH*, layer of prehypertrophic chondrocytes; *H*, layer of hypertrophic chondrocytes; *B*, bone marrow. *Bar*, 250 μm (in *A*, *C*, *D*, *F*, *G*, *I*, *J*, and *L*). *Bar*, 100 μm (in *B*, *E*, *H*, and K). See the legend to Fig 1 for the definitions of probes A, B, C, and D.

first investigated the relation between *Cbfa1* expression and chondrocyte differentiation using the chondrogenic cell line ATDC5, an *in vitro* model for chondrogenesis (17, 35). Undifferentiated ATDC5 cells began to form cartilage nodule 3 days after confluence. Type I collagen mRNA was detectable when the cells reached confluence (day 0) but was not detected at 3 days after confluence (day 3) (Fig. 3*A*). In contrast, type II collagen mRNA was detected at day 3 and increased during culture. Type X collagen mRNA began to be detected at day 9 and increased during culture. We examined *Cbfa1* expression during ATDC5 cell differentiation. Using probe A (see Fig. 1), two bands were detected until day 3, but after day 6 three bands were detected (Fig. 3*A*). To distinguish the two mRNAs for type I and II Cbfa1, we used probe C and probe D for Northern blot analysis (Fig. 3*B*). Among the three bands detected at day 6 using probe A, the two higher bands were detected and the lowest band was barely detectable using probe

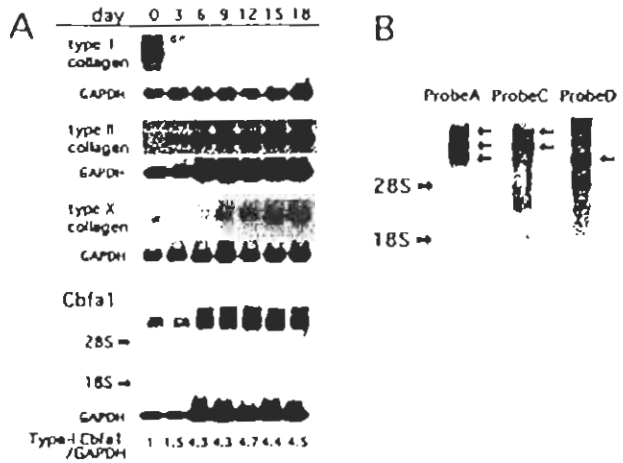

FIG. 3. **Northern blot analysis of ATDC5 cells.** *A*, expression of chondrocyte differentiation markers and *Cbfa1* during ATDC5 cell culture. Cells were cultured in the presence of insulin, and total RNA was extracted at the days indicated (with day 0 as confluence). 20 μg of total RNA was loaded and hybridized with mouse cDNA of type I collagen, type II collagen, type X collagen, and GAPDH. 2 μg of poly(A) RNA was loaded and hybridized with *Cbfa1* probe A and GAPDH. Hybridization with the GAPDH probe was used as an internal control. The ratios of the intensities of the two higher bands of *Cbfa1*, which represent mRNAs for type I Cbfa1, are shown against GAPDH (see Figs. 1 and 3*B*). *B*, Northern blot analysis of *Cbfa1* with various probes. ATDC5 cells were cultured in the presence of insulin, and RNA was extracted at day 6 (with day 0 as confluence). 2 μg of poly(A) RNA was loaded and hybridized with probes A, C, and D. The *higher two bands* are detected by probe C; the *lowest band* is detected by probe D. See the legend to Fig. 1 for the definitions of probes A, C, and D.

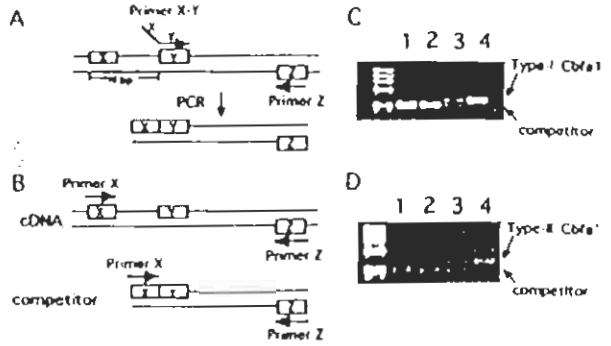

FIG. 4. **Schematic representation of the method of competitive PCR.** *A*, competitor construction. Primer X-Y, which matches sequence Y located *N* bp downstream from sequence X and contains sequence X at the 5′-end, is generated. Competitor is generated by PCR amplification using primers X-Y and Z using cDNA as a template. *B*, competitive PCR. After generation of the competitor by PCR using primers X-Y and Z, serially diluted PCR product is added to the samples as a competitor. Both the target sequence and competitor are competitively amplified by PCR using primers X and Z. The amplified product of competitor is *N* bp shorter than the target sequence. *C* and *D*, serial dilutions of the competitor are added to the fixed amount of sample cDNA and then co-amplified and examined by electrophoresis. The addition of a similar number of competitors to that of the target sequence results in two bands derived from the competitor and the target sequence, and the approximate number of molecules of the target sequence can be estimated from the number of molecules of competitor (*lane 3*). PCR products of type I Cbfa1 (356 bp) and competitor (286 bp) (*C*) and those of type II Cbfa1 (385 bp) and competitor (275 bp) (*D*) are shown.

C, whereas only the lowest band was detected using probe D. These results indicate that the higher two bands contain mainly mRNA for type I Cbfa1, while the lowest band contains mainly mRNA for type II Cbfa1. The intensities of the lowest bands were similar during culture, but those of the higher two bands increased 4-fold at day 6 and were maintained after day 6 (Fig. 3*A*). These data indicate that mRNA for type I Cbfa1 increased before the onset of type X collagen expression.

Effect of Cbfa1 Antisense Oligonucleotides on ATDC5 Cell Differentiation to Hypertrophic Phenotype—To investigate the role of Cbfa1 in chondrocyte differentiation, we examined whether the down-regulation of *Cbfa1* by treatment with two *Cbfa1* antisense oligonucleotides, one for type I Cbfa1 and the other for type II Cbfa1, affects the differentiation of ATDC5 cells. First, we compared the amounts of mRNA for type I and type II Cbfa1 using competitive RT-PCR analysis (Fig. 4) after the treatment of control-scrambled or sense oligonucleotides from day 0 to day 11 (with day 0 as confluence; see Fig. 3*A*). In the competitive RT-PCR analysis of mRNA for type I Cbfa1, the equivalent points, in which similar intensities of the bands of cDNA and competitor are detected (see Fig. 4, *B* and *C*), were observed between 1×10^{-1} and 3×10^{-2} (roughly 6×10^{-2}) dilutions of competitor when cells were treated with control-scrambled or sense oligonucleotides for type I Cbfa1 (Fig. 5*A*). In the competitive RT-PCR analysis of mRNA for type II Cbfa1, the equivalent points were observed at a 1×10^{-2} dilution of competitor in the treatment of control-scrambled or sense oligonucleotides for type II Cbfa1 (Fig. 6*A*). Therefore, the amount of mRNA for type I Cbfa1 was several times greater in ATDC5 cells at day 11 than that for type II Cbfa1, and a similar ratio was observed at day 12 by Northern blot analysis (Fig. 3*A*).

We next examined whether type I or type II Cbfa1 expression was reduced by treatment with their antisense oligonucleotides from day 0 to day 11. When the cells were treated with antisense oligonucleotides for type I Cbfa1, the equivalent point was observed at less than a 1×10^{-3} dilution of competitor in the competitive RT-PCR analysis of mRNA for type I Cbfa1 (Fig. 5*A*). Therefore, treatment with antisense oligonucleotides reduced mRNA for type I Cbfa1 to less than one-thirtieth, because the equivalent points were observed at more than a 3×10^{-2} dilution when cells were treated with control-scrambled or sense oligonucleotides for type I Cbfa1. The treatment of antisense oligonucleotides for type I Cbfa1 had no effect on mRNA for type II Cbfa1 (Fig. 5*B*). Northern blot analysis revealed that the treatment with antisense oligonucleotides for type I Cbfa1 in ATDC5 cells did not significantly affect type II collagen expression but decreased type X collagen expression to one-fifth of that after treatment with control-scrambled or sense oligonucleotides (Fig. 5*C*). The addition for 3 days (days 4–6) of antisense oligonucleotides for type I Cbfa1 into the culture before type X collagen expression also reduced expression to 60% of that after treatment with control-scrambled or sense oligonucleotides (data not shown). Thus, the treatment of antisense oligonucleotides for type I Cbfa1 inhibited the maturation of ATDC5 cells to hypertrophic phenotype.

In the competitive RT-PCR analysis of mRNA for type II Cbfa1, the equivalent point was observed at less than a 3×10^{-4} dilution of competitor in the treatment of antisense oligonucleotides for type II Cbfa1 (Fig. 6*A*). Therefore, treatment with antisense oligonucleotides reduced mRNA for type II Cbfa1 to less than one-thirtieth, because the equivalent points were observed at a 1×10^{-2} dilution when cells were treated with control-scrambled or sense oligonucleotides for type II Cbfa1. The treatment of antisense oligonucleotides for type II Cbfa1 had no effect on mRNA for type I Cbfa1 (Fig. 6*B*).

Type II collagen expression was similarly detected in ATDC5 cells following treatment with control-scrambled, sense, or antisense oligonucleotide for type II Cbfa1 by Northern blot analysis (Fig. 6*C*). However, the expression of type X collagen in ATDC5 cells following treatment with antisense oligonucleotides for type II Cbfa1 was about two-thirds of that after treatment with control scrambled or sense oligonucleotides. Thus, treatment with antisense oligonucleotide for type II Cbfa1 also

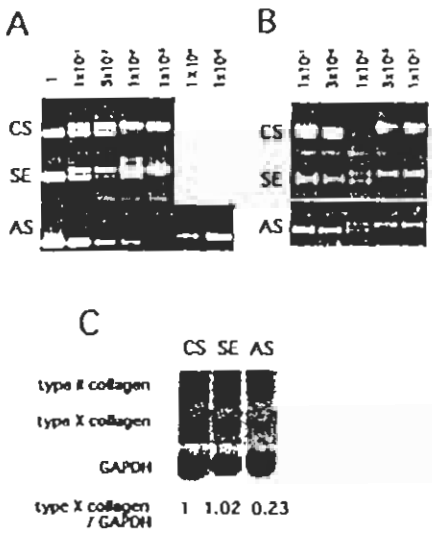

FIG. 5. **Effect of antisense oligonucleotides for type I Cbfa1 on ATDC5 cell differentiation to hypertrophic phenotype.** ATDC5 cells were treated with 10 μM CS, SE, and AS oligonucleotides for type I Cbfa1 from day 0 to 11. Cells were harvested 24 h after the final addition of oligonucleotides, and RNA was extracted for RT-PCR and Northern blot. Using the competitive RT-PCR for hypoxanthine guanine phosphoribosyl transferase, the amount of each cDNA sample was adjusted (data not shown). *A*, competitive RT-PCR analysis of mRNA for type I Cbfa1. The *number above* each *track* represents dilutions of the competitor for type I Cbfa1 (1×10^{-16} mol of competitor was used as a standard). The *upper bands* are derived from mRNA for type I Cbfa1; *lower bands* are derived from the competitor (see Fig. 4*C*). 35 cycles of co-amplification were carried out except in the PCRs in which the competitor was used at dilutions of 1×10^{-4} and 1×10^{-5}, for which PCR cycling was increased to 45 cycles. *B*, competitive RT-PCR analysis of mRNA for type II Cbfa1. The *number above* each *track* represents dilutions of the competitor for type II Cbfa1 (1×10^{-16} mol of competitor was used as a standard). The *upper bands* are derived from mRNA for type II Cbfa1; *lower bands* are derived from the competitor (see Fig. 4*D*). *C*, Northern blot analysis of type II and type X collagen expression after the treatment of oligonucleotides for type I Cbfa1. 30 μg of total RNA was loaded. GAPDH was used as an internal control, and the ratios of intensities of type X collagen expression to GAPDH expression are shown. Similar results were obtained in three independent experiments, and representative data are shown in *A–C*.

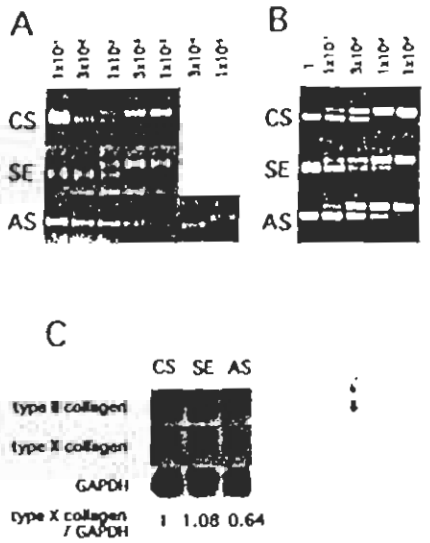

FIG. 6. **Effect of antisense oligonucleotides for type II Cbfa1 in ATDC5 cell differentiation to hypertrophic phenotype.** ATDC5 cells were treated with 10 μM of CS, SE, and AS oligonucleotides for type II Cbfa1 from day 0 to 11. Cells were harvested 24 h after the final addition of oligonucleotides, and RNA was extracted for RT-PCR analysis and Northern blot analysis. Using the competitive RT-PCR for hypoxanthine guanine phosphoribosyl transferase, the amount of each cDNA sample was adjusted (data not shown). *A*, competitive RT-PCR analysis of mRNA for type II Cbfa1. The *number above* each *track* represents dilutions of the competitor for type II Cbfa1 (1×10^{-16} mol of competitor was used as a standard). The *upper bands* are derived from mRNA for type II Cbfa1; *lower bands* are derived from the competitor (see Fig. 4*D*). 35 cycles of co-amplification were done except in the PCRs using the competitor at dilutions of 3×10^{-4} and 1×10^{-5}, in which PCR cycling was increased to 45 cycles. *B*, competitive RT-PCR analysis of mRNA for type I Cbfa1. The *number above* each *track* represents dilutions of the competitor for type I Cbfa1 (1×10^{-16} mol of competitor was used as a standard). The *upper bands* are derived from mRNA for type I Cbfa1; *lower bands* are derived from the competitor (see Fig. 4*C*). *C*, Northern blot analysis of type II and type X collagen expression after the treatment of oligonucleotides for type II Cbfa1. 30 μg of total RNA was loaded. GAPDH was used as an internal control, and the ratios of intensities of type X collagen expression to GAPDH expression are shown. Similar results were obtained in three independent experiments, and representative data are shown in *A–C*.

inhibited the hypertrophy of ATDC5 cells, but the effect was not as strong as that of antisense oligonucleotide for type I Cbfa1.

Both Type I and Type II Cbfa1-Induced Expression of Hypertrophic Phenotype in Immature Chondrocytes—To investigate whether Cbfa1 affects the expression of hypertrophic phenotype in chondrocytes and whether type I and type II Cbfa1 have different actions on chondrocytes, we retrovirally introduced type I or type II Cbfa1 cDNA into primary cultured chondrocytes. Immature chondrocytes that do not express the hypertrophic phenotype were isolated from the lower portion of chick sterna and infected with viruses encoding type I Cbfa1, type II Cbfa1, or vector alone. We first determined whether the infected cultures produced Cbfa1 protein. 6 days after infection, cultures were stained with an antibody to the carboxyl-terminal fragment encoded by exon 8 of *Cbfa1*, which is common to both isotypes (26) (Fig. 7*A*, *e* and *f*). Most cells infected with viruses encoding type I Cbfa1 (Fig. 7*A*, *e*) or type II Cbfa1 (Fig. 7*A*, *f*) showed a strong signal in the nuclei, whereas control virus-infected cells (Fig. 7*A*, *d*) were negative. We also examined the production of Cbfa1 protein in infected chondrocytes by immunoblotting. As shown in Fig. 7*B*, the bands from the nuclear extracts of type I and type II Cbfa1 virus-infected cells corresponded well to the expected sizes of the two products: 56 kDa for type I Cbfa1 and 57 kDa for type II Cbfa1, indicating that both type I and type II Cbfa1 virus-infected cells expressed the constructed gene products.

Chondrocytes expressing either type I Cbfa1 or type II Cbfa1 became large and round and seemed to grow very slowly (Fig. 7*A*, *b* and *c*, respectively), whereas those infected with the control virus proliferated actively and showed a fibroblastic shape or small polygonal shape (Fig. 7*A*, *a*). The cultures infected with type I Cbfa1 virus (Fig. 7*A*, *b*) were morphologically indistinguishable from the type II Cbfa1 virus-infected cultures (Fig. 7*A*, *c*). To obtain quantitative data on the effects of type I/II Cbfa1 on chondrocyte proliferation, a DNA content analysis was performed in the secondary cultures. The increase in DNA content in both the type I and type II Cbfa1 virus-infected cultures was much slower than in the control cultures (Fig. 8*A*). The control cultures had accumulated about 3-fold more DNA than type I or type II Cbfa1-expressing cultures by day 8 (Fig. 8*A*). The biochemical quantification of sulfated glycosaminoglycan content revealed that either type I or type II Cbfa1-expressing cells synthesized cartilage-specific proteoglycan and accumulated more proteoglycan from early periods of cultures as compared with the control cells. (Fig. 8*B*). The effects of type I and type II Cbfa1 on proliferation and proteoglycan accumulation were indistinguishable, like the effects of the two isotypes on cell morphology.

Type I/type II Cbfa1-expressing cells increased in size and

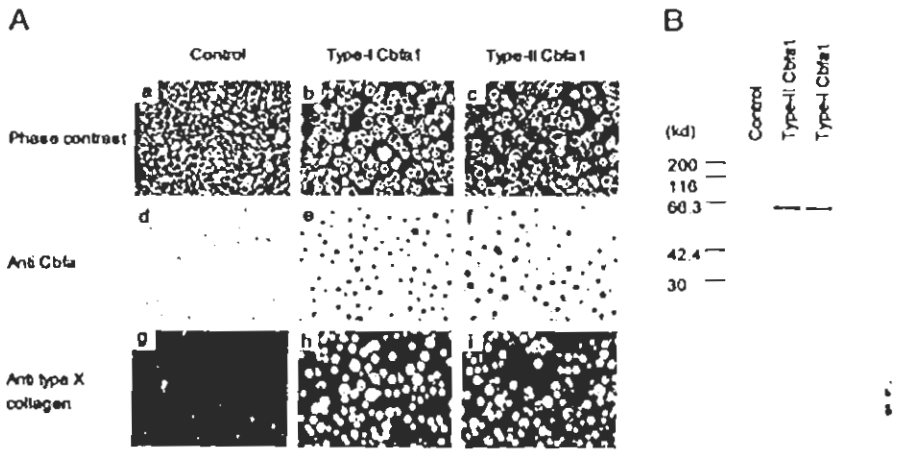

FIG. 7. **Effects of forced expression of either type I or type II Cbfa1 encoding viruses on chondrocyte phenotype.** *A*, freshly isolated immature chondrocytes were infected with RCAS retroviral vector encoding type I Cbfa1 (*b*, *e*, *h*) or type II Cbfa1 (*c*, *f*, *i*). Companion cells were infected with insertless virus and served as control (*a*, *d*, *g*). 6 days after infection, phase-contrast microphotographs (*a–c*) were taken. Cells were then harvested by enzymatic treatment and replated on poly-L-lysine-coated dishes. Protein expressions of type I/type II Cbfa1 (*d–f*) and type X collagen (*g–i*) in chondrocytes were analyzed by immunohistochemical and immunofluorescence techniques as described under "Materials and Methods." *B*, production of Cbfa1 protein in infected chondrocytes. Nuclear extracts were prepared from the virus-infected cultures, and 10 μg of nuclear extract from each culture was separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with polyclonal Cbfa1 antibody (aA1c17).

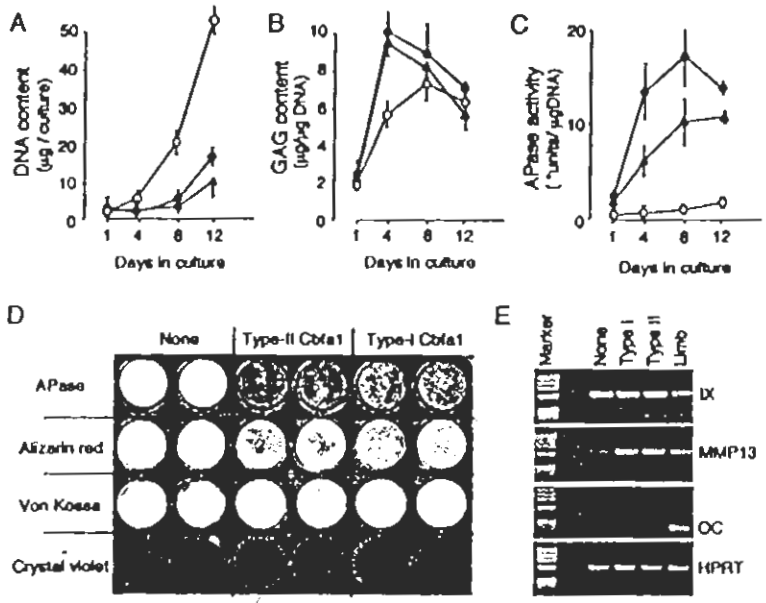

FIG. 8. **Chondrocyte maturation by type I and type II Cbfa1-encoding viruses.** *A–C*, effects of forced expression of type I and type II Cbfa1 on proliferation, proteoglycan synthesis, and APase activity in chondrocytes. Freshly isolated immature chondrocytes were infected with RCAS retroviral vector encoding type I or type II Cbfa1. Companion cells were infected with insertless virus and served as control. 6 days after infection, cells were inoculated onto 35-mm diameter six-well plates at a density of 1×10^5 cells/well and maintained in high glucose DMEM containing 10% FBS. Values were averages of three wells ± S.D. *A*, DNA contents in control (○), type I Cbfa1 (▲), and type II Cbfa1 (●) cultures. *B*, sulfated glycosaminoglycan contents in control (○), type I Cbfa1 (▲), and type II Cbfa1 (●) cultures. *C*, APase activity in control (○), type I Cbfa1 (▲), and type II Cbfa1 (●) cultures. Similar results were obtained in two independent experiments. *D*, effects of forced expression of type I and type II Cbfa1 on APase activity and cartilage-matrix mineralization. Freshly isolated immature chondrocytes were infected with RCAS retroviral vector encoding type I or type II Cbfa1. Companion cells were infected with insertless virus and served as control. 6 days after infection, cells were inoculated onto 16-mm diameter type I collagen-coated multiwell plates at a density of 2×10^5 cells/well. The cultures were maintained in high glucose DMEM containing 10% FBS and 10 μg/ml ascorbic acid for 8 days and then treated with 1 mM β-glycerophosphate for one more day. The cultures were stained with alizarin red or von Kossa on day 10 to visualize the mineralized area. Wells stained by crystal violet are also presented to show the confluence of cells. *E*, expression of phenotype markers for chondrocytes and osteoblasts. Freshly isolated immature chondrocytes were infected with RCAS retroviral vector encoding type I or type II Cbfa1. Companion cells were infected with insertless virus and served as control. 6 days after infection, cells were inoculated onto 16-mm diameter type I collagen-coated multiwell plates at a density of 2×10^5 cells/well. The cultures were maintained in high glucose DMEM containing 10% FBS and 10 μg/ml ascorbic acid for an additional 7 days. Total RNAs were then extracted from the cultures infected with RCAS retroviral vector encoding type I (*Type I*) or type II (*Type II*) Cbfa1 or vector alone (*None*). Total RNAs were also extracted from the whole hind limb of 11-day-old chick embryos (*Limb*). Expressions of type IX collagen (*IX*), *MMP13*, osteocalcin (*OC*), and hypoxanthine guanine phosphoribosyl transferase were analyzed by RT-PCR. HPRT, hypoxanthine guanine phosphoribosyl transferase

attenuated their proliferating activities, suggesting that the cells shifted to a hypertrophic state. We next examined whether type I/type II Cbfa1-expressing cells expressed the hypertrophic phenotype characterized by type X collagen expression, high APase activity, and matrix calcification. As shown in Fig. 7*A*, *h* and *i*, most of the type I and type II

Cbfa1-expressing cells were positive for type X collagen, whereas the control cells were almost entirely negative. Further, type I or type II Cbfa1 expression strongly stimulated APase activity in chondrocytes (Fig. 8, *B* and *D*) and induced matrix calcification (Fig. 8*D*; alizarine red and von Kossa), while the control cultures showed very low levels of APase activity (Fig. 8, *C* and *D*) and no matrix calcification (Fig. 8*D*; alizarine red and von Kossa). Again, the stimulatory activities of type I and type II Cbfa1 on the induction of hypertrophic phenotype in chondrocytes were comparable, as determined by type X collagen expression, APase activity, and matrix calcification.

To examine the possibility that Cbfa1 induced the osteoblastic phenotype in hypertrophic chondrocytes at the late stage cultures, we analyzed expression of the phenotype markers for chondrocytes and osteoblasts by RT-PCR. Either type I or type II Cbfa1-expressing chondrocytes expressed type IX collagen, which is a characteristic of chondrocytes (36), but did not express osteocalcin, a marker of mature osteoblasts, whereas a positive signal for osteocalcin was observed using RNAs extracted from the whole limbs of 11-day-old chick embryos (Fig. 8*E*). Interestingly, expression of *MMP13*, which is a marker of the late stage of chondrocyte hypertrophy (15) and also a direct target gene for Cbfa1 (37), like osteocalcin, was strongly increased in both types of Cbfa1-expressing chondrocytes (Fig. 8*E*). These findings suggest that forced expression of either type I or type II Cbfa1 strongly promoted chondrocyte maturation from the immature stage to the terminal stage but did not induce osteoblastic phenotype.

DISCUSSION

We have shown that mRNAs for type I and type II Cbfa1 are expressed in terminal hypertrophic chondrocytes as well as osteoblasts and that mRNA for type I Cbfa1 is also expressed in other hypertrophic chondrocytes and prehypertrophic chondrocytes. mRNA for type I Cbfa1 was increased during ATDC5 cell differentiation to hypertrophic phenotype, and antisense oligonucleotides for type I Cbfa1 strongly suppressed the maturation of ATDC5 cells. Further, forced expression of either type I or type II Cbfa1 induced maturation of chick immature chondrocytes. These data demonstrate that Cbfa1 is a maturational promoter for chondrocytes, implying a crucial role of Cbfa1 in chondrocyte as well as osteoblast differentiation.

Cbfa1 Isotypes with Different N-terminal Domains and Their Expression in Chondrocytes—Cbfa1 is known to be an essential factor for osteoblast differentiation and to be strongly expressed in osteoblasts (1). However, we and another group found that *Cbfa1* is also expressed in chondrocytes in mouse embryos (15, 16). In this study, we attempted to distinguish the localization of transcripts for two Cbfa1 isotypes by using probes that were either specific to an individual isotype or common to both isotypes. We showed by *in situ* hybridization that mRNAs for both type I and type II Cbfa1 were expressed in terminal hypertrophic chondrocytes as well as in osteoblasts. In other hypertrophic chondrocytes and prehypertrophic chondrocytes, the signal of mRNA for type I Cbfa1 was weakly but significantly detected, while that of mRNA for type II Cbfa1 was less prominent. It is suggested that the expression of type I Cbfa1 begins to be up-regulated in chondrocytes from the early stage of differentiation, *i.e.* the prehypertrophic stage, whereas the expression of type II Cbfa1 is only up-regulated at the terminal stage of chondrocyte maturation.

The two isotypes of Cbfa1, type I and type II, have a difference of only 19 amino acids in their N-terminal domains (Fig. 1). Recently, type I and type II Cbfa1 have been proved to have different transactivational capacities in the regulation of the osteocalcin gene (12), and in nonosteoblastic cell lines functional difference between type I and type II Cbfa1 has also been suggested (38). Moreover, type II Cbfa1 has a unique activation domain in the N-terminal portion (11). Therefore, it is suggested that the two isotypes of Cbfa1, type I and type II, are functionally different.

Cbfa1 Promotes Chondrocyte Maturation—Immature chondrocytes infected with retrovirus encoding either type I or type II Cbfa1 became large and round, and they showed decreased proliferation and increased APase activity (Figs. 7*A* and 8). Their maturation was also demonstrated by the expression of type X collagen and *MMP13* and matrix mineralization. Either type I or type II Cbfa1-expressing chondrocytes kept synthesizing proteoglycan and type IX collagen, characteristics of chondrocytes, but not osteocalcin, a marker of mature osteoblasts. These data indicate that both isotypes of Cbfa1 can induce maturation from immature chondrocytes to terminal hypertrophic chondrocytes but not induce osteoblastic phenotype. When we introduced DNA-binding protein c-Myc in chondrocytes using the same retrovirus system, the infected chondrocytes proliferated actively and failed to express hypertrophic phenotype (25), which is in contrast to the behavior of *Cbfa1*-expressing chondrocytes. Thus, the phenotype of chondrocytes induced by forced expression of *Cbfa1* is specific to the introduced gene.

Cultures of ATDC5 cells have been shown to be a useful *in vitro* model for examining the multistep differentiation of chondrocytes. Cultured in the presence of insulin, ATDC5 cells form many spots of cartilage-like cellular condensation, and they are able to differentiate to hypertrophic chondrocytes (18, 35). During ATDC5 cell differentiation, type I, but not type II, Cbfa1 expression was increased before the expression of type X collagen. In accordance with these findings, antisense oligonucleotide for type I Cbfa1 suppressed type X collagen expression more efficiently than that for type II Cbfa1. Therefore, the difference that was observed in the treatment of antisense oligonucleotides for type I and type II Cbfa1 may have resulted from the different levels of expression of the two isotypes in ATDC5 cells, because the level of type I Cbfa1 expression was several times greater than that of type II Cbfa1 (Figs. 5*A* and 6*A*). In addition, it may be that the type II isoform is more stable than the type I isoform, and treatment with antisense oligonucleotide for the type II isoform would decrease the level of type II protein only partially. Another possibility is that the mRNA for type II Cbfa1 may not be efficiently translated in ATDC5 cells, and type II protein levels normally may be low. In this case, also, treatment with antisense oligonucleotide would have had a minor effect on chondrocyte phenotype, because type II protein normally is of less importance than type I Cbfa1 in ATDC5 cells.

The possibility exists that Cbfa1 directly regulates the activation of the type X collagen gene, like that of the osteocalcin gene. Once ATDC5 cells acquire the hypertrophic phenotype, however, the treatment of antisense oligonucleotides for type I Cbfa1 produced no significant suppression of type X collagen expression, irrespective of the marked reduction of *Cbfa1* expression (data not shown). Further, type X collagen was expressed in restricted regions of *Cbfa1*-deficient mice, tibia, fibula, ulna, and radius, where hypertrophic chondrocytes were observed. Indeed, there is no Cbfa1 binding site in the 5′-region of the type X collagen gene (39). Further, the treatment of antisense oligonucleotides for either Cbfa1 isotype did not affect type II collagen expression in ATDC5 cells, indicating that the chondrogenic activity was maintained in ATDC5 cells during culture. Therefore, these results suggest that Cbfa1 is not a direct activating factor for type X collagen gene and that the inhibition of type X collagen expression is a result of the sup-

pression of ATDC5 cell differentiation to the hypertrophic phenotype caused by the treatment with antisense oligonucleotides for Cbfa1.

Function of Cbfa1 in Chondrocytes—In E18.5 Cbfa1-deficient mice, whose entire skeleton was composed of cartilage, hypertrophic chondrocytes were not observed, and the expression of parathyroid hormone/parathyroid hormone-related peptide receptor, Indian hedgehog, and type X collagen was not detected in most parts of the skeleton except for tibia, fibula, ulna, and radius, indicating that chondrocyte differentiation was blocked before the stage of prehypertrophic chondrocytes in most parts of the skeleton (15, 16). These findings, together with the findings in the present study, indicate that Cbfa1 is an important factor for the differentiation of immature chondrocytes to hypertrophic chondrocytes.

In *Cbfa1*-deficient mice, calcification of cartilage was observed in restricted parts of skeleton including tibia, fibula, radius, and ulna. However, osteopontin, bone sialoprotein, and *MMP13* were not expressed at all in the terminal hypertrophic chondrocytes of these mice (15, 16). Cbfa1 appears to regulate the transcription of osteocalcin, bone sialoprotein, osteopontin, and type I collagen in osteoblasts and the transcription of bone sialoprotein, osteopontin, and *MMP13* in terminal hypertrophic chondrocytes (4, 6, 37, 40). mRNA for type I and type II Cbfa1 was strongly detected in terminal hypertrophic chondrocytes as well as in osteoblasts. Thus, high levels of *Cbfa1* expression in mature osteoblasts and terminal hypertrophic chondrocytes seem to be important for mineralization and the production of mineralization-related matrix proteins.

In conclusion, we have demonstrated that *Cbfa1* is expressed in prehypertrophic and hypertrophic chondrocytes and induces maturation from immature chondrocytes to terminal hypertrophic chondrocytes. These findings, coupled with the observations in *Cbfa1*-deficient mice, indicate that Cbfa1 is a major positive regulatory factor in chondrocyte maturation.

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