Cbfa1 is a critical regulator of cell differentiation expressed only in the osteochondrogenic lineage. To define the molecular basis of this cell-specific expression, we analyzed the murine Cbfa1 promoter. Here we show that the first 976 bp of this promoter are specifically active in osteoblastic cells. Within this region DNase I footprinting delineated a 40-bp area (CE1) protected differently by nuclear extracts from osteoblastic cells and from non-osteoblastic cells. When multimerized, CE1 conferred an osteoblast-specific activity to a heterologous promoter in DNA transfection experiments; this enhancing ability was conserved between mouse, rat, and human CE1 present in the respective Cbfa1 promoters. CE1 site-specific mutagenesis determined that it binds NF1- and AP1-like activities. Further analyses revealed that the NF1 site acts as a repressor in non-osteoblastic cells due to the binding of NF1-A, a NF1 isoform not expressed in osteoblastic cells. In contrast, the AP1 site mediates an osteoblast-specific activation caused by the preferential binding of FosB to CE1 in osteoblastic cells. In summary, this study identified an osteoblast-specific enhancer in the Cbfa1 promoter whose activity is achieved by the combination of an inhibitory and an activatory mechanism.

Osteoblasts are cells of mesenchymal origin required to form the skeleton during development and to maintain bone mass thereafter. Their unique ability to deposit a matrix that can eventually mineralize explains that abnormal osteoblast differentiation and/or osteoblast function have dramatic consequences. Indeed, arrest of osteoblast differentiation often leads to lethal skeletal dysplasia whereas impaired osteoblast activity causes bone fragility with risk of fracture, a hallmark of osteoporosis, the most common degenerative disease in the western hemisphere (1–3). This pivotal role of the osteoblasts in bone biology justifies the importance of identifying molecular regulators of osteoblast differentiation and function. However, as of today only a few factors playing such a role have been identified, and no genetic cascade can yet be drawn showing how the differentiation of a mesenchymal cell to a fully functional osteoblast is controlled. With the long-term goal to delineate such cascade we have embarked on studying the regulation of expression of Runx2/Cbfa1 (thereafter Cbfa1), which is to date the best characterized regulator of osteogenesis (4).

Cbfa1 is one of the three mammalian members of the runt family of transcription factors (5). Gene deletion experiments as well as overexpression studies have shown that Cbfa1 is necessary and sufficient for osteoblast differentiation (4). The finding that humans or mice heterozygous for Cbfa1 inactivation develop an identical phenotype termed Cleidocranial dysplasia (3, 4) established the remarkable conservation of Cbfa1 function during mammalian evolution. At the molecular level, Cbfa1 has been shown to directly regulate the expression of major osteoblastic marker genes such as the type I collagen genes, osteopontin, bone sialoprotein, and osteocalcin (6, 7). These findings, as well as the fact that impairing Cbfa1 function postnatally leads to osteopenia (8), indicate that it acts beyond development as a major regulator of bone mass via its control of the rate of bone matrix deposition by differentiated osteoblasts. This notion was recently extended to the resorption side of bone remodeling (9, 10). Indeed, overexpression of Cbfa1 in osteoblasts was shown to increase their ability to sustain osteoclastogenesis and therefore to regulate, although indirectly, bone resorption as well as bone formation.

The Cbfa1 gene potentially gives rise to two major transcripts, TypeII/Cbfa1p57/Osf2/MASNSL and TypeI/Cbfa1p56/ MRIPVD (11–13). These transcripts differ by the promoter they originate from (P1 or P2, respectively), by their 5’-untranslated region and N-terminal coding sequence and by their pattern of expression in cell lines (11, 12). However, by in situ hybridization on mouse embryos, the same pattern of expression is observed when using a probe specific to the Type II form and a probe common to the two Cbfa1 transcripts, indicating the existence of a predominant pattern of expression in vivo (6). Throughout development and in adult tissues, Cbfa1 expression appears to be strictly restricted to cells of the osteochondrogenic lineage, although its level of expression varies according their stage of differentiation (14). Cbfa1 is initially expressed in all cells of the mesenchymal condensations prefiguring each of the future skeletal elements, beginning at 10.5 days post-coitum in mouse (4). When chondrocyte differentiation proceeds in these structures, Cbfa1 expression becomes restricted to pre-hypertrophic chondrocytes and to the osteoblast progenitor cells present in the bone collar (14). Postnatally, osteoblasts will become the only cell type expressing Cbfa1 (6, 14).

Considering that Cbfa1 expression is restricted to the skeletal cell lineage and that Cbfa1 is an inducer of osteoblast differentiation, it is likely that the factors controlling its expression would trigger the early steps of skeletal cell commitment and/or those of osteoblast differentiation. We therefore
embarked in the analysis of the mouse Cbfa1 promoter with the long-term goal to identify cis-acting elements and trans-acting factors that induce and regulate Cbfa1 expression specifically in the osteoblastic lineage. In this study we report that such a cis-acting element is present at \(-415/\sim 375\) in the mouse Cbfa1 P1 promoter. Despite the fact that this region binds AP1 and NF1 nuclear activities, two classes of transcription factors whose members are broadly expressed, this element acts as an enhancer only in osteoblastic cells. Our analysis therefore shows that an osteoblast-specific activity can be achieved through a specific interaction between broadly expressed factors.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and DNA Transfection—**UMR106 and ROS17/2.8 cells were cultured in Dulbecco’s modified Eagle’s medium/F12 (DMEM/F12; Invitrogen)/10% fetal bovine serum (Invitrogen). NIH3T3, C2C12, 3T3L1, and COS cells were cultured in DMEM/10% fetal bovine serum. F10 were cultured in Eagle minimal essential medium (MEM); Invitrogen)/10% fetal bovine serum. Transfections were performed using the calcium phosphate co-precipitation method as previously described (15) or using FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer’s instructions. An equal amount of pSVβ-galactosidase plasmid DNA was added to each DNA mix to account for the efficiency of transfection (15). Luciferase and β-galactosidase activities were assayed as previously described (15). All transfections were repeated at least 6 times with two different preparations of plasmid DNA.

**DNA Constructions—**All promoter fragments were cloned in the pGL3basic promoter-less luciferase expression vector (Clontech). The \(-2800/\sim 111\) mouse Cbfa1 promoter region was cloned as an EcoRI/PstI fragment excised from a mouse Sv/ev genomic clone (Clontech). This initial fragment was used to generate all deletion mutants using \(5’\)-ends internal NdeI (p1614-luc), XbaI (p1839-luc), BglII (p976-luc), SacI (p780-luc), SacI (p280-luc) restriction sites and as \(3’\)-end its terminal PstI site. Footprint analyses were performed using as templates the BglII/HindIII \((-976/\sim 734), \) HindIII/StuI (\(-733/\sim 342\), and StuI/SacI (\(-341/\sim 89\)) fragments cloned in pBluescript. Multimer (five copies) of the wild-type or mutant CE1 oligonucleotides (Table I) were cloned in front of the \(-46/10\) adenovirus type 2 major late promoter region itself fused to the luciferase reporter gene (pAMPL-luc). The FosB, Fra1, Fra2, JunB, and JunD expression vectors were a generous gift from Dr. J. Rosen (Baylor College of Medicine, Houston, Texas). The pCMV-ΔFosB was generated by cloning a PCR fragment amplified with the following primers: \(5’\)-CAGAGAGCCGCAACAAGCTGGCTGTCG-3’ and \(5’\)-GCTCTAGATGACCCGCAAGGCGG-3’ into pCMV5. NF1 expression vectors were a kind gift from Dr. J. Rosen (Baylor College of Medicine, Houston, TX).

**Northern Blot, RT-PCR, and Western Blot Analyses—**Extractions of total RNA and Northern blot experiments were performed using standard protocols (16). Membranes were successively hybridized with a Cbfa1 (6) and a glyceraldehyde-3-phosphate dehydrogenase probe used as a control for equivalence of RNA loading. For RT-PCR analysis, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, 8 mM MgCl2,2m M dithiothreitol, 4 mM spermidine, 20 mM g/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml of leupeptin and pepstatin. Poly(dI-dC)poly(dI-dC) (1 μg) and single-stranded DNA (500 fmoles) were used as nonspecific competitors. For supershift experiments antibodies (Santa Cruz Biotechnology) were incubated with 8 μg of nuclear extracts for 30 min at 4 °C prior to addition of the probe. Reactions were left for 20 min at room temperature then electrophoresed on a 5% polyacrylamide gel in 0.25% Tris borate/EDTA at 160 V for 2 h. Gels were dried and autoradiographed.

**RESULTS**

A -\(976/\sim 111\) Cbfa1 Promoter Fragment Is Active Only in Osteoblastic Cells—Cbfa1 is encoded by a single gene whose expression is restricted to cells of the osteochondrogenic lineage in vivo (4). To analyze the mechanisms controlling Cbfa1 promoter cell-specificity we therefore relied on comparing its activity in osteoblastic and in non-osteoblastic cells. ROS17/2.8 (18) and UMR106 cells (19) were initially chosen as osteoblastic references because they express high levels of Cbfa1 (Fig. 1A). Both cell lines derive from rat osteosarcomas and represent a mature osteoblastic phenotype, expressing the PTH/PTHrP receptor and Osteocalcin (15, 18, 19). UMR106 is however expressing Cbfa1 at a higher level than ROS17/2.8 (Fig. 1A). As non-osteoblastic references we used cell lines that are, like osteoblasts, of mesenchymal origin such as fibroblasts (NIH3T3), preadipocytes (3T3L1), and myoblasts (C2C12), and cells of different embryonic origin such as F9 teratocarcinoma and COS monkey kidney cells. Cbfa1 expression was not detectable in any of these cell lines (Fig. 1A).

Using the first Cbfa1 exon as a probe we screened a mouse genomic library and isolated a clone containing 2.8 kb lying upstream of the Cbfa1 start site of transcription (20). We first performed DNA transfection experiments using a reporter construct harboring this region \((-2800/\sim 111)\) fused to the luciferase gene (p2800-luc). This construct was significantly more

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1 The abbreviation used is: EMSA, electromobility shift assay.

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**FIG. 1. Osteoblast-specific activity of the \(-976/\sim 111\) mouse Cbfa1 promoter region in DNA transfection experiments. A, Northern blot analysis (15 μg of total RNA per lane) of Cbfa1 expression in cell lines (upper panel). The blot was re-probed with a glyceraldehyde-3-phosphate dehydrogenase probe to account for RNA loading and transfer efficiency (lower panel). B, osteoblast-enriched activity of the 2.8-kb proximal region of the mouse Cbfa1 promoter (p2800-luc). C, activity of \(5’\)-deletion mutants of the p2800-luc construct transfected in UMR106 osteoblastic cells. A schematic representation of each reporter construct is shown on the left. D, osteoblast-specific activity of the 976-bp proximal region of the mouse Cbfa1 promoter (p976-luc). Data represent ratios of luciferase to β-galactosidase activities (Rlu) and values are means of 8 to 12 independent transfection experiments, with error bars representing the S.E. of the mean.
active in osteoblastic cells than in any non-osteoblastic mesenchyme-derived cells, and it was virtually inactive in F9 and COS cells (Fig. 1B). This result suggested that the −2800/+111 region contained regulatory element(s) favoring its expression in osteoblastic cells. To localize them more precisely, activities of 5′-deletion mutants of this 2.8-kb promoter fragment were compared in UMR106 osteoblastic cells. Deletions from −2800 to −976 did not alter significantly the level of activity (Fig. 1C). In contrast, deletion from −976 to −89 decreased by 6-fold the level of luciferase expression, indicating that critical cis-acting elements are present in this region (Fig. 1C). To determine whether such elements could be cell-specific we compared the activity of p976-luc in osteoblastic and non-osteoblastic cell lines. As shown in Fig. 1D, p976-luc displayed a tight osteoblast-specific activity. Consistent with the difference of Cbfa1 expression in these cells (Fig. 1A), p976-luc was more active in UMR106 than in ROS17/2.8 cells (Fig. 1D). We therefore chose the UMR106 cell line as reference in all subsequent experiments. These results thus established the osteoblast-specificity of the −976/+111 Cbfa1 promoter fragment in DNA transfection and indicated that at least one osteoblast-specific regulatory element lied between −976 and −89.

An Osteoblast-specific Binding Site, CE1, Is Present in the Cbfa1 Promoter between −415 and −375—DNase I footprint assays were performed to define the sites of binding of osteoblast-specific nuclear proteins within the −976/−89 area, searching for areas differentially protected by nuclear extracts from UMR106 osteoblastic cells compared with non-osteoblastic mesenchyme-derived NIH3T3 and 3T3L1 cells or F9 cells. Analysis of the −976/−734 region did not show any protected area (data not shown). In agreement with such absence of cis-acting elements, a reporter construct missing this −976/−734 region displayed a similar activity that p976-luc in UMR106 cells (p976-luc, 58260 Rlu; p780-luc, 106752 Rlu).

Analysis of the −733/−342 region delineated three protected regions. First, on its lower DNA strand nuclear extracts from NIH3T3 fibroblasts protected an area between −470 and −415 (Fig. 2, A, white box). This area was not protected by any other non-osteoblastic nuclear extracts and more importantly it was not protected by any extracts on the opposite strand (Fig. 2, A–C). For these two reasons this region was not further analyzed. Two other regions, we named Cbfa1 element 1 (CE1) and Cbfa1 element 2 (CE2), were also found footprinted (Fig. 2, A, gray boxes). Independently of the DNA strand analyzed CE1 laid between −415 and −375 and was protected by all three mesenchymal cell-derived but not by F9 cells nuclear extracts (Fig. 2, A–C). However, on both strands CE1 pattern of protection was different between UMR106 extracts and NIH3T3 or 3T3L1 extracts, suggesting that this region bound different nuclear proteins in osteoblastic and non-osteoblastic cells. CE2, which lies between −558 and −470 on the lower DNA strand,
was protected in a similar fashion by nuclear extracts from the three mesenchymal cell-derived but was not protected by F9 cells nuclear extracts (Fig. 2A). This suggested that this region was binding a factor widely expressed in mesenchymal cells rather than an osteoblast-specific factor. For this reason and because no protected area could be observed when we analyzed the opposite strand (Fig. 2C) this region was thus not further analyzed.

Lastly, when the −341/−89 region was analyzed no cell-specific protection could be observed (data not shown). Taken together, the results of the footprinting analysis of the −976/−89 region of the mouse Cbfa1 promoter indicate that CE1, a 40-bp area lying between −415 and −375 (Fig. 2D), is the only region binding distinct factors in osteoblastic and non-osteoblastic cells.

**CE1 Binds NF1-like and AP1-like Nuclear Activities**—A double-stranded oligonucleotide covering CE1 (Fig. 3A and Table I) was used as a probe in EMSA using UMR106 nuclear extracts as a source of proteins. Four DNA-protein complexes were observed (Fig. 3B, lane 1) and mutant (lanes 2–4) CE1 oligonucleotides were used as probes in presence of UMR106 nuclear extracts. The four complexes formed are marked as *1, *2, *3, and *4. C, competition experiments in EMSA. Labeled double-stranded wild type CE1 oligonucleotide was used as probe in presence of UMR106 nuclear extracts along with indicated molar excess of cold oligonucleotide competitors containing wild type (lanes 2, 3, 6, 7) or mutant (lanes 4, 5, 8, 9) consensus binding sites for NF1 (lanes 2–5) or AP1 factors (lanes 6–9). D, EMSA. Equivalent amounts (3 μg) of nuclear extracts from osteoblastic cells (UMR106: lanes 1, 4, 7) or non-osteoblastic mesenchymal cells (NIH3T3: lanes 2, 5, 8 and 3T3L1: lanes 3, 6, 9) were compared for binding to labeled wild type CE1 (lanes 1–3), mutant Insa (lanes 4–6), and mutant mb (lanes 7–9) probes. The four osteoblastic complexes are indicated as in B, the two non-osteoblastic AP1-related complexes are indicated by black dots and marked as *2/3.
NF1 binding (Insa) (21), a mutation abolishing AP1 binding (mb) (22) or both mutations (Insa/mb) (Fig. 3A and Table I), and tested them in EMSA. The Insa mutation dramatically reduced (although did not abolish) formation of complexes *1 and *4 while it did not affect formation of complexes *2 and *3 (Fig. 3B, lane 2). This result suggested that the factors forming *2 and *3 do not bind to the NF1 site. In contrast, the mb mutation of the AP1 site led to the sole formation of complex 4 (Fig. 3B, lane 3), suggesting that this complex does not bind to the AP1 element. Interestingly, both single mutations abolished the presence of complex *1 (Fig. 3B, lanes 2 and 3), suggesting that it is formed by simultaneous binding of NF1 and AP1 factors. No factors bound to CE1 independently of the NF1 and AP1 sites since simultaneous mutation of these two sites abolished the binding of all complexes (Fig. 3B, lane 4).

We next performed competition experiments in EMSA. Labeled wild type CE1 oligonucleotide was used as probe in the presence of 0-, 10-, or 100-fold molar excess of cold oligonucleotides containing consensus wild type or mutant NF1 and AP1 binding sites (Table I). NF1 wild type consensus oligonucleotide competed away both *1 and *4 complexes (Fig. 3C, lanes 2 and 3). When mutated using the Insa insertion (Table I) this core sequence did not compete binding at 10-fold molar excess, but did reduce formation of complexes *1 and *4 at higher concentrations (Fig. 3C, lanes 4 and 5). This indicates that the Insa mutant site can still bind NF1, although with a lower affinity than the wild type site. This result is consistent with the direct binding experiment presented in Fig. 3B showing that formation of complexes *1 and *4 is reduced but not abolished by the Insa mutation (Fig. 3B, lane 2). When we used as cold competitor an AP1 consensus site, only complex *4 formed (Fig. 3C, lanes 6 and 7). In contrast, introducing the mb mutation in this sequence (Table I) did not impair the formation of any complex, even at high concentrations (Fig. 3C, lanes 8 and 9), indicating that the mb mutation completely abolishes the ability of AP1 factors to bind to CE1. Together, the results of our direct and competition EMSA experiments indicate that complex *2 and *3 are formed by an AP1 binding activity while complex *4 binds a NF1-related nuclear protein. Complex *1 appears to contain both types of factors, suggesting that none of them inhibits the ability of the other to bind to its recognition site within CE1.

To determine whether any of the complexes binding to CE1 was osteoblast-specific, we compared EMSA patterns obtained using nuclear extracts from UMR106, NIH3T3, and 3T3L1 cells. Using wild type CE1 the major difference we could observe was the absence of AP1 binding activities *2 and *3 in non-osteoblastic cell lines (Fig. 3D, lanes 1–3). Complexes of intermediate mobility were instead present (Fig. 3D, lanes 2 and 3, black dots). Their formation was abolished by the mb mutation indicating that, like osteoblastic complexes *2 and *3, they contained AP1-binding activities (Fig. 3D, lanes 8 and 9). We thus named them complexes *2/3 to reflect their intermediate mobility compared with complexes *2 and *3. There was a difference of migration between NIH3T3 and 3T3L1 *2/3 complexes suggesting that different AP1 activities bound to CE1 in these cells. We also observed that formation of complex *4, that binds a NF1 nuclear activity, migrated slightly faster with osteoblastic nuclear extracts than with non-osteoblastic extracts (Fig. 3D, lanes 7–9), suggesting that CE1 may bind different NF1 isoforms in these two types of cells.

**CE1 Functions as an Osteoblast-specific Enhancer**—To investigate the function of CE1 and of the two putative binding sites it contains we performed a series of DNA transfection experiments. Multimers of either the wild type, Insa mutant, mb mutant, or Insa/mb double mutant CE1 oligonucleotides were cloned upstream of an inactive heterologous promoter fused to the luciferase reporter gene (Fig. 4A). These different constructs were transfected in UMR106 cells, NIH3T3 cells, and 3T3L1 cells. Five copies of wild type CE1 (p5CE1-luc) increased the activity of an inactive heterologous promoter 44-fold in UMR106 cells compared with less than 4-fold in

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**Table I**

| Name       | Sequence | Reference |
|------------|----------|-----------|
| CE1 (wt)   | GATCCAGCATTTGTGTCTCTAGGCAACTTCATAGTCACAAAAA
            | GTCGTTAACCAAGATCGGTTAGAGTACTCACTGTTTTCTAG | This study |
| Insa       | GATCCAGCATTTGTGTCTCTAGGCAACTTCATAGTCACAAAAA
            | GTCGTTAACCAAGATCGGTTAGAGTACTCACTGTTTTCTAG | This study |
| mb         | GATCCAGCATTTGTGTCTCTAGGCAACTTCATAGTCACAAAAA
            | GTCGTTAACCAAGATCGGTTAGAGTACTCACTGTTTTCTAG | This study |
| Insa/mb    | GATCCAGCATTTGTGTCTCTAGGCAACTTCATAGTCACAAAAA
            | GTCGTTAACCAAGATCGGTTAGAGTACTCACTGTTTTCTAG | This study |
| NF1        | GATCTGGCACCTGTTCAATTTGCGAGCGCAAGACAG
            | CCGTGAGCAGTAAACCCCGGTGCTGCTTTACTAGA | (40) |
| mut NF1    | GATCTGGCACCTGTTCAATTTGCGAGCGCAAGACAG
            | CCGTGAGCAGTAAACCCCGGTGCTGCTTTACTAGA | This study |
| AP1        | GATCCCTCAGTCAAAAA
            | GAGTTTTTCAGTTTTTTCTAG | This study |
| mut AP1    | GATCCCTCAGTCAAAAA
            | GAGTTTTTCAGTTTTTTCTAG | This study |
| hCE1       | GATCCAGCATTTGTGTCTCTAGGCAACTTCATAGTCACAAAAA
            | GTCGTTAACCAAGATCGGTTAGAGTACTCACTGTTTTCTAG | This study |

* Substitution mutations are represented in boldface, insertional mutants are boxed.
Fig. 4. Functional analysis of CE1 by DNA transfection. A, schematic representation of multimerized wild-type (wt) CE1 oligonucleotides fused to the AMLP-luc reporter cassette. B, comparison of CE1 multimers activity in osteoblastic cells (UMR106) and in non-osteoblastic mesenchymal cells (NIH3T3 and 3T3L1). Values are given above the basal activity of pAML-luc empty vector. C–E, effect of mutations in the NF1 site (Insa), in the AP1 site (mb), or in both sites (Insa/mb) on CE1 activity in osteoblastic cells (C) and in non-osteoblastic cells (D and E). Values are given in fold activation relative to pAML-luc basal activity. All transfection data represent ratios of luciferase versus β-galactosidase activities, and values are means of at least five independent transfection experiments.

|   | p5CE1-luc (natural orientation) |   | p5CE1-luc (opposite orientation) |
|---|---------------------------------|---|-----------------------------------|
| A |                                 |   |                                   |
| B | p5CE1-luc                       |   |                                   |
|   | RLU (x10^3)                     |   |                                   |
| natural |                              |   |                                   |
| opposite |                              |   |                                   |
| C   |                                 |   |                                   |
|     | CE1                             |   |                                   |
|     | Insa                           |   |                                   |
|     | mb                             |   |                                   |
|     | mb/Insa                         |   |                                   |

Non-osteoblastic cells (Fig. 4B). This stimulatory effect was independent of the orientation of the oligonucleotides, demonstrating that CE1 functions as a true enhancer. When we introduced an inactivating mutation in the AP1 site (mb) or when we mutated simultaneously the NF1 and AP1 sites (Insa/mb), CE1 activity was totally abolished in all 3 cell types (Fig. 4, C–E). In contrast, mutation of the NF1 site alone (Insa) did not significantly decrease CE1 activity in osteoblasts (Fig. 4C), indicating that the NF1 nuclear protein(s) binding to CE1 in these cells do not have a major impact on its activity. This mutation however increased more than 5-folds CE1 activity in NIH3T3 cells and 3T3L1 cells (Fig. 4, D and E), suggesting that the NF1 nuclear factor(s) binding to CE1 in non-osteoblastic cells repress its activity. Considering that Insa did not modify CE1 activity in osteoblastic cells, this result also strongly suggests that these particular NF1 factors are not present in osteoblastic cells.

Together, these experiments demonstrate that CE1 acts as an activator of transcription specifically in osteoblastic cells. They also suggest that this cell-specific activity results from the binding to CE1 of NF1 inhibitory factors present in non-osteoblastic cells but not in osteoblastic cells, and from the binding of AP1 enhancing activities specific to osteoblastic cells.

CE1 Is Evolutionary Conserved—We next searched the Cbfa1 promoter of the rat (12) and human (23) Cbfa1 genes for CE1-related elements. At –399/-359 in the rat promoter and at –257/-217 in the human promoter we located identical regions that share a 93% sequence conservation with mouse CE1 (Fig. 5A). More specifically, these regions harbored a 100% conserved AP1 site and an 87% conserved NF1 site compared with mouse CE1. To determine if they had similar binding abilities we performed EMSA using nuclear extracts from UMR106 and mouse CE1 (mCE1) or rat/human putative CE1 (hCE1) as probes. As shown in Fig. 5B, identical binding patterns were obtained with these 2 probes, indicating that the same nuclear activities can bind to these elements despite their 2-bp difference in the NF1 site. Consistent with this result DNA transfection experiments in UMR106 cells showed that 5 copies of hCE1 were as active as 5 copies of mCE1 (Fig. 5C).

That the CE1 enhancer had been conserved structurally and functionally in the Cbfa1 gene during mammalian evolution underlines its importance.

NF1-A Acts as an Inhibitor of CE1 Activity in Non-osteoblastic Cells—To establish that a bona fide NF1 nuclear activity bound to CE1, we performed antibody supershift experiments in EMSA. Addition of an antibody recognizing specifically all NF1 proteins to nuclear extracts from UMR106, NIH3T3 and 3T3L1 cells induced the formation of an additional band of slow mobility (Fig. 6A), indicating that NF1 proteins bind to CE1 both in osteoblastic and non-osteoblastic cells. Four NF1 genes (NF1-A, -B, -C, and -X) have been identified, encoding nuclear factors binding the same DNA core sequence (24). The transfection experiments presented Fig. 4 indicated that the NF1 isoforms binding to CE1 in osteoblastic cells had no significant impact on its activity while those present specifically in non-osteoblastic cells were playing a major inhibitory role. We therefore compared NF1 gene expression in osteoblastic and non-osteoblastic cells, searching for an NF1 isoform only expressed in the latter. All NF1 isoforms display a well-conserved N-terminal region and within this region a common 486-bp product can be generated using degenerated oligonucleotides (24). When subjected to particular restriction digests, this product however leads to fragments specific of each NF1 subtype (17). Using total RNA from UMR106, NIH3T3, and 3T3L1 cells co-amplification of the 486-bp common product with Hprt in a semiquantitative RT-PCR assay showed that non-osteoblastic cells have a higher level of total NF1 gene expression compared with UMR106 cells (Fig. 6B, upper panel). More importantly, when this 486-bp common product was digested to reveal each NF1 isoform we could never detect the presence of NF1-A transcripts in osteoblastic cells while they were abundantly expressed in non-osteoblastic cells (Fig. 6B, lower panel, star). This result suggested that NF1-A could be the NF1 isoform specifically present and inhibiting CE1 activity in non-osteoblastic cells.

To test this hypothesis we analyzed which impact each NF1 isoform had on the Cbfa1 promoter activity by co-transfecting p976-luc with expression vectors for NF1-A, -B, -C, and -X. Both in osteoblastic and non-osteoblastic cells, expression of NF1-A decreased p976-luc activity whereas expression of the 3 other NF1 isoforms had no significant effect (Fig. 6C). This effect was specifically mediated by CE1 because: 1) expression of NF1-A in osteoblasts decreased the activity of p5CE1-luc but not of its Insa mutant counterpart, and 2) increased expression of NF1-A in non-osteoblastic cells decreased p5CE1-luc activity and completely abolished the de-repressing effect induced by its Insa mutation (Fig. 6D). Taken together, these experiments indicated that CE1 was negatively regulated by NFA-1 in non-osteoblastic cells. This isoform being expressed in non-osteoblastic cells (Fig. 4B). This stimulatory effect was independent of the orientation of the oligonucleotides, demonstrating that CE1 functions as a true enhancer. When we introduced an inactivating mutation in the AP1 site (mb) or when we mutated simultaneously the NF1 and AP1 sites (Insa/mb), CE1 activity was totally abolished in all 3 cell types (Fig. 4, C–E). In contrast, mutation of the NF1 site alone (Insa) did not significantly decrease CE1 activity in osteoblasts (Fig. 4C), indicating that the NF1 nuclear protein(s) binding to CE1 in these cells do not have a major impact on its activity. This mutation however increased more than 5-folds CE1 activity in NIH3T3 cells and 3T3L1 cells (Fig. 4, D and E), suggesting that the NF1 nuclear factor(s) binding to CE1 in non-osteoblastic cells repress its activity. Considering that Insa did not modify CE1 activity in osteoblastic cells, this result also strongly suggests that these particular NF1 factors are not present in osteoblastic cells.

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NF1-A Acts as an Inhibitor of CE1 Activity in Non-osteoblastic Cells—To establish that a bona fide NF1 nuclear activity...
blastic cells but not in osteoblastic cells explained the NF1-mediated repression of CE1 occurring specifically in the former cells.

**Binding of JunD/FosB AP1 Complex to CE1 Is Restricted to Osteoblastic Cells**—We next investigated which Fos/Jun AP1 family members were binding to CE1 by using antibodies specific for each of the 4 Fos-related factors and the 3 Jun-related factors in EMSA. These supershift experiments were performed using wild type CE1 oligonucleotide and nuclear extracts from UMR106, NIH3T3, and 3T3L1 cells. As shown in Fig. 7A, cFos and cJun antibodies did not have any effect, indicating that these AP1 family members do not bind CE1. Conversely, addition of a Fra2-specific antibody produced a supershifted band with all 3 nuclear extracts (Fig. 7A, lanes 5, 13, and 21), indicating that Fra-2 binds equally to CE1 in osteoblastic and non-osteoblastic cells and is therefore unlikely to mediate a cell-specific role. More importantly, three major differences were observed when comparing osteoblastic and non-osteoblastic supershift patterns. First, a FosB-specific antibody prevented the formation of osteoblastic complex *2* (Fig. 7A, lane 3, diamond) while it did not affect formation of non-osteoblastic complexes *2/3* (Fig. 7A, compare lane 3 to lanes 11 and 19). Second, a JunD-specific antibody reduced formation of osteoblastic complexes *1, 2* and *3* (Fig. 7A, lane 8) while non-osteoblastic complexes *2/3* were not affected (Fig. 7A, compare lane 8 to lanes 16 and 24). Third, addition of a Fra-1-specific antibody did not have any effect on osteoblastic nuclear extracts whereas it reduced formation of complexes *1 and 2/3* in non-osteoblastic cells (Fig. 7A, compare lanes 12 and 20 to lane 4). These 3 differences between osteoblastic and nonosteoblastic binding factors were further analyzed by Western blot analysis and DNA co-transfection experiments.

According to the above supershift analysis JunD bound CE1 only when UMR106 extracts were used (Fig. 7A, empty circles). To determine whether this specificity resulted from JunD osteoblast-specific expression, osteoblastic and non-osteoblastic extracts were analyzed by Western blot. As shown in Fig. 7B, JunD was detected at similar levels in both types of cells, indicating that the osteoblast-specific binding of JunD to CE1 is not associated with its exclusive presence in these cells. We therefore hypothesized that binding of JunD to CE1 could be inhibited in non-osteoblastic cells and that overcoming this inhibition by increasing JunD level in these cells would reveal its potential. To test this hypothesis we co-transfected p5CE1-luc or p976-luc with a JunD expression vector or its empty counterpart in NIH3T3 and 3T3L1 cells. JunD forced expression induced a 2-fold increase of p976-luc activity (Fig. 7C). A weaker but significant increase was also observed using the p5CE1-luc construct (Fig. 7C, 5CE1). This effect was specific since it could be abolished by the mb mutation (Fig. 7C, mb). Together, these results suggest that JunD is an activator of CE1 whose absence of binding to this element in non-osteoblastic cells could explain CE1 inactivity in these cells. However, the mild activation observed upon JunD co-expression with both the *Cbfa1* promoter fragment and CE1 multimeric constructs suggests that this is not a major mechanism regulating CE1 cell-specific activity.

The antibody supershift analysis also detected that Fra1 bound CE1 only when non-osteoblastic nuclear extracts were used (Fig. 7A, black dots). Furthermore, Western blot analyses indicated that Fra1 was much less abundant in UMR106 nuclear extracts than in non-osteoblastic extracts (Fig. 7B). These two results suggested that CE1 could be repressed by Fra1 in non-osteoblastic cells only because they highly express this factor. To test whether Fra1 could indeed act as an inhibitor of CE1 activity we co-transfected Fra family members with p5CE1-luc or p976-luc in osteoblastic cells. Neither Fra1 nor Fra2 expression decreased CE1 (or CE1mb) activity (Fig. 7D). This result indicates that Fra1 is not a repressor of CE1 activity whose higher expression in non-osteoblastic cells compared with osteoblastic cells would explain CE1 inactivity in the former.

Lastly, the EMSA supershift experiments showed that FosB bound to CE1 only when osteoblastic nuclear extracts were used (Fig. 7A, diamond). Because a Western blot analysis demonstrated that FosB is expressed as well in non-osteoblastic cells as in osteoblastic cells (Fig. 7B), we concluded that FosB ability to bind CE1 could be specifically inhibited in non-osteoblastic cells. As for JunD, this observation raised the hypothesis that FosB could be an activator of CE1 whose binding to

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**Fig. 5. Functional conservation of CE1 during mammalian evolution. A**, alignment of CE1 putative sequences present in the mouse, rat, and human *Cbfa1* promoters. The NF1 and AP1 core sites are boxed. Nucleotide differences are indicated, dashes mark a conserved nucleotide. **B**, EMSA comparison of labeled double-stranded wild type mouse CE1 (lane 1, m*CE1*) and human/rat CE1 (lane 2, h*CE1*) oligonucleotides used as probes in presence of UMR106 nuclear extracts. **C**, similar activity of m*CE1* and h*CE1* multimers fused to the AMLP-luc reporter cassette in osteoblastic cells (UMR106). Values are given in activity above pAML-P-luc basal activity. Data represent ratios of luciferase versus β-galactosidase activities and values are means of at least six independent transfection experiments.
NF1 sites have been identified in the promoter of multiple genes. Considering that to fulfill the same function in mouse and human Cbfa1 has to be expressed in a similar fashion such a conservation of its transcriptional regulation of expression during mammalian evolution is not surprising. We predict however that in addition to CE1 other cis-acting elements are likely to be involved in controlling Cbfa1 expression. Indeed, Cbfa1 is highly expressed in undifferentiated osteochondro progenitor cells but it is down regulated in differentiating non-hypertrophic chondrocytes, only to be re-expressed at high levels in pre-hypertrophic chondrocytes (14). We believe that achieving such variation of expression is likely to require more than one cis-acting element and in particular should involve both enhancer and repressor elements.

AP1 family members have already been implicated in the regulation of osteoblast differentiation. Notwithstanding that AP1 family members are differentially expressed during osteoblast differentiation (26), AP1 binding sites have been identified in the promoter of several genes expressed in osteoblasts, where they often mediate the responsiveness of these promoters to growth factors (27–29). More recently, the role of AP1 family members has been emphasized in vivo by two studies showing respectively that Fra1 or FosB overexpression in mice enhanced bone formation by osteoblasts and led to osteosclerosis (25, 30). Sabatakos et al. (25) additionally showed that overexpression of a short form of ∆FosB in osteoblasts induces a significant increase of the expression of Cbfa1 as well as of bone matrix protein encoding genes. By showing that FosB isoforms are major activators of CE1 our results provide a molecular explanation for this observation. Interestingly, although Fra1 transgenic animals also displayed an osteosclerotic phenotype, Fra1 overexpressing osteoblasts did not show a significant increase of Cbfa1 expression (30), nor did we observe an increase of CE1 activity upon Fra1 co-transfection experiments. These observations and our results together suggest that the high bone mass phenotype observed in the Fra1- and ∆FosB-overexpressing mice may have different molecular bases.

To our knowledge this study for the first time implicates NF1 transcription factors in the regulation of osteoblast-specific gene expression. NF1 isoforms are encoded by 4 distinct genes whose patterns of expression are broad although some isoforms are preferentially expressed in specific tissues (24). Functional NF1 sites have been identified in the promoter of multiple genes, some of them cell-specific (31–33). In most cases, NF1 cis-acting elements acted as activators of transcription, al-

In summary, our analysis of the AP1 complexes binding to CE1 suggests that binding of JunD/FosB(∆FosB) is responsible for CE1 activity in osteoblastic cells and that the absence of binding of this particular complex in non-osteoblastic contributes to the absence of CE1 activity in these cells.

DISCUSSION

To study the early transcriptional events controlling osteochondro progenitor differentiation, we have embarked on the systematic analysis of the promoter of mouse Cbfa1. In this study we show that the first 976 bp of this promoter are active only in osteoblastic cells. We then narrowed down this cell specificity to a 40-bp region, CE1, located at –415 compared with the Cbfa1 start site of transcription and show that CE1 acts as an osteoblast-specific enhancer in DNA transfection experiments. CE1 contains a NF1 and an AP1 binding sites, functioning respectively as a repressor specifically in non-osteoblastic cells and as an activator only in osteoblastic cells. Further analyses determined that binding of particular AP1 and NF1 factors triggers these cell specificities. Indeed, NF1-A is the only NF1 isoform able to repress CE1 activity and it is only expressed in non-osteoblastic cells, therefore repressing CE1 activity only in these cells. Likewise, FosB(∆FosB)/JunD is an activator of CE1 that can bind to its AP1 site only in osteoblastic cells.

Our analysis also showed that CE1 is present in mouse, rat, and human Cbfa1 promoters where it binds NF1 and AP1 nuclear activities in a similar fashion and activate transcription to a similar extent. These observations suggest that CE1 plays the same role in regulating Cbfa1 expression in these 3 species. Considering that to fulfill the same function in mouse and human Cbfa1 has to be expressed in a similar fashion such a conservation of its transcriptional regulation of expression during mammalian evolution is not surprising. We predict however that in addition to CE1 other cis-acting elements are likely to be involved in controlling Cbfa1 expression. Indeed, Cbfa1 is highly expressed in undifferentiated osteochondro progenitor cells but it is down regulated in differentiating non-hypertrophic chondrocytes, only to be re-expressed at high levels in pre-hypertrophic chondrocytes (14). We believe that achieving such variation of expression is likely to require more than one cis-acting element and in particular should involve both enhancer and repressor elements.

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Fig. 6. Identification of NF1-A as a repressor of CE1 activity in non-osteoblastic cells. A, supershift experiments in EMSA using a NF1-specific antibody and the wild type CE1-labeled probe in presence of nuclear extracts from osteoblastic cells (UMR106) and non-osteoblastic cells (NIH3T3 and 3T3L1). Stars indicate supershifted complexes. B, RT-PCR analysis of NF1 isoforms expression in osteoblastic cells (UMR106) and non-osteoblastic cells (NIH3T3 and 3T3L1). Note the absence of NF1-A expression in UMR106 cells compared with NIH3T3 and 3T3L1 cells (star). C, repression of p5CE1-luc activity by co-transfection of a NF1-A expression vector in osteoblastic cells (UMR106) and non-osteoblastic cells (3T3L1). Forced expressions of NF1-B, -C, or -X do not induce significant effects. D, repression of p5CE1-luc activity by co-transfection of a NF1-A expression vector in osteoblastic cells (UMR106) and non-osteoblastic cells (3T3L1). The InsA mutation abolishes this effect in osteoblastic cells. In non-osteoblastic cells NF1-A forced expression blocks the effect of the InsA mutation but does not induce a decrease below the level of wild type p5CE1-luc activity. All transfection data represent ratios of luciferase versus β-galactosidase activities, and values are means of at least six independent transfection experiments.
though some repressor activities have been reported (24). Although CE1 binds a NF1 nuclear activity in all cell types we analyzed, our DNA transfection experiments show that within CE1 the NF1 binding site is neutral in osteoblastic cells or repressive in non-osteoblastic cells. Indeed, upon mutation of the NF1 binding site in osteoblastic cells, CE1 activity is not significantly impaired, indicating that the NF1 isoforms expressed in these cells do not contribute significantly to its positive activity. In contrast, this mutation de-repressed CE1 activity in non-osteoblastic cells. The mb mutation abolishes this effect. D, forced expressions of Fra family members in osteoblastic cells (UMR106) do not have any effect on p976-luc or p5CE1-luc activities. E, induction of p976-luc and p5CE1-luc activities by co-transfection with FosB-related expression vectors in non-osteoblastic cells. The mb mutation abolishes this effect. All transfection data represent ratios of luciferase versus β-galactosidase activities, and values are means of at least six independent transfection experiments.

In summary, two peculiarities characterize CE1. First, it is activated in a cell-specific manner via non-cell-specific cis-acting elements. Indeed, both NF1 and AP1 families of transcription factors contain members whose expression pattern is broad (36). Considering also that all NF1 (AP1) isoforms can bind to identical DNA core sequences, it is remarkable that these particular families of transcription factors could mediate a cell-specific activity. Second, CE1 specific activity in osteoblastic cells is the net result of a combination of cell-specific absence of repression and of cell-specific activation. Such combination allows a finer regulation than a simple "On/Off" switching mechanism. Such fine-tuning could find its purpose when Cbfa1 expression needs to be moderately and/or transiently modified. For instance transient expression of NF1-A at specific stage(s) of development could decrease Cbfa1 promoter activity to limit its expression and thereby slow down the rate...
of differentiation of osteochondro progenitors. Three lines of evidence support such hypothesis. First, NF1-A is expressed transiently in 11.5 dpc limb buds (36), i.e. at a stage where proliferation of osteochondro progenitors should be favored compared with their differentiation (37), and therefore Chfa1 expression should be limited. Second, a negative NF1 cis-acting element has been identified in the promoter of cartilage matrix protein (CMP), a gene encoding an extracellular protein synthesized by chondrocytes (31). Like Chfa1, CMP is expressed in prehypertrophic cells during chondrocyte differentiation (14, 38), suggesting that NF1-A could modulate the expression of several molecular determinants of this process. Lastly, the rare NF1-A deficient mice that survive beyond birth are runted and present cranio-facial abnormalities, two phenotypes that dis-

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