

Role of Cbfa1 in Ameloblastin Gene Transcription*

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Sangeeta Dhamija and Paul H. Krebsbach‡

From the University of Michigan School of Dentistry, Department of Oral Medicine, Pathology, and Oncology and the Center for Bio restoration of Oral Health, Ann Arbor, Michigan 48109-1078

Ameloblastin is a tooth-specific extracellular matrix protein that is thought to play a role in enamel crystal formation in the developing dentition. The murine ameloblastin promoter functions in a cell type-specific manner and contains *cis*-acting elements that function both to enhance and to suppress transcription. The objective of this study was to determine whether the transcription factor Cbfa1, known to be essential for transcription of other mineralized tissue genes, is also required for ameloblastin transcription. Site-directed mutagenesis of the Cbfa1-binding site (–248 base pairs) termed osteoblast-specific element 2 (OSE2) decreased ameloblastin promoter activity by greater than 50% in ameloblast-like cells. No differences in promoter activity were observed in two other oral tissue-derived cell lines transfected with similar constructs. Nuclear factor binding to the ameloblastin promoter was also shown to be cell type-specific and was altered by site-specific mutations in the OSE2 site. Cbfa1 was specifically shown to participate in the DNA-protein complexes between nuclear factors and the ameloblastin OSE2 site by supershift electrophoretic mobility shift assays. The findings that Cbfa1 interacts with functionally important regions of the ameloblastin promoter while promoter activity is diminished in constructs containing site-directed mutations in the Cbfa1 site indicate that Cbfa1 possesses an important function in transcription of the ameloblastin gene.

The mineralized tissues that comprise the mammalian dentition are formed by the synthesis of a tissue-specific extracellular matrix that facilitates the initiation and orientation of inorganic hydroxyapatite crystallites. Enamel is an oral epithelial-derived mineralized tissue, whereas dentin and cementum are formed by cranial neural crest-derived ectomesenchyme (1, 2). Because enamel is an epithelial-derived tissue that does not contain collagenous proteins, it is unique among the mineralized tissues in vertebrates. Another distinguishing feature of enamel is that, unlike bone and dentin, the organic extracellular matrix that influences the initial crystallite formation is essentially lost during tissue maturation, resulting in a tissue that is greater than 98% mineral (3–5).

Tooth development is dependent on the coordinated expres-

sion of several genes, some of which are unique to the developing tooth (6, 7). Because extracellular matrix molecules are thought to guide the unique size, shape, and orientation of the crystallites forming enamel, it is essential that the mechanisms that regulate their expression be accurately controlled. Precision in the timing of enamel matrix gene expression therefore dictates the supramolecular assembly of the framework that directs crystallite growth and ultimately influences its biomechanical properties (3, 8, 9). The principal secretory products of the enamel matrix-secreting ameloblast have been classified into two major categories: the amelogenins, which make up about 90% of the enamel extracellular matrix, and the nonamelogenins (10, 11). Ameloblastin (12), also known as amelin (13) and sheathlin (14), is a tooth-specific gene that represents the most abundant nonamelogenin enamel matrix protein. Ameloblastin is predominantly expressed in secretory ameloblasts, and is present in the developing enamel matrix (15, 16). Ameloblastin is also transiently expressed in dentin matrix (17) and Hertwig's root sheath epithelial cells (18), but its role in dentin and cementum formation has not been established. Localization of ameloblastin near crystal growth sites suggests a role in enamel crystal formation (19, 20). Therefore, resolving the molecular events directing the control of ameloblastin gene expression during tooth formation is an essential element in understanding how the enamel matrix is secreted and assembled and how it functions to guide biomineralization.

The cloning and initial characterization of murine ameloblastin promoter suggested that regulatory regions of the murine ameloblastin promoter function in a cell-specific manner (21). Sequential 5'-deletion mutants encompassing DNA sequences from –1616 to –781 bp¹ exhibited high promoter activity in ameloblast-like LS8 cells, whereas the promoter activity decreased to 50% of the full-length construct in the –781- and –477-bp regions. The –217-bp promoter region regained promoter activity that approached the activity of the full-length promoter construct, suggesting that both positive and negative *cis*-acting regions may be involved in ameloblastin transcriptional regulation. The ameloblastin promoter contains several potential *cis*-regulatory sites within functionally important promoter regions including AP1, CF2-II, Kruppel, and osteoblast-specific element 2 (OSE2) sites (21).

OSE2, first described in the osteocalcin gene 2 promoter and later in several other genes involved in biomineralization, has the consensus core sequence AACCAC that binds the transcription factor Cbfa1 (core-binding factor A1) (22). Cbfa1, also known as PEBP2 (polyoma enhancer-binding protein 2) or AML3 (acute myloid leukemia 3) is an essential transcription factor for osteoblast differentiation and function (23). Alternate

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‡ Recipient of Independent Scientist Award DE00426 from the NIDCR, National Institutes of Health. To whom correspondence should be addressed: University of Michigan School of Dentistry, Dept. of Oral Medicine, Pathology, and Oncology, 1011 N. University Ave., Ann Arbor, MI 48109-1078. E-mail: paulk@umich.edu.

¹ The abbreviations used are: bp, base pair(s); OSE, osteoblast-specific element; wt, wild type; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay; HGF, human gingival fibroblast(s); HPF, human pulp fibroblast(s).

splicing of the first three exons of Cbfa1 mRNA results in at least three Cbfa1 isoforms, Cbfa1/Osf2, Cbfa1/Iso, and Cbfa1 original, that contain exons 1–3, lack exon 1, or lack exons 1 and 2, respectively (24). The largest isoform, Cbfa1/Osf2, has been described as the predominant isoform functioning in osteoblast lineage progression and is referred to as Cbfa1 in this report (23).

Recently, several genes related to osteoblast differentiation and bone matrix formation have been reported to contain OSE2 sequences in their promoter regions, suggesting that Cbfa1 may be a principal gene controlling osteoblast function (23, 25–28). Furthermore, targeted disruption of Cbfa1 in mice results in cessation of skeleton formation and haploinsufficiency in humans causes cleidocranial dysplasia, a genetic disorder with malformations in a number of tissues including bone (29–31). In addition to a critical role in bone formation, Cbfa1 also has been suggested to support a more general role in the differentiation of mineralized connective tissues. For example, dental abnormalities such as supernumerary teeth and delayed tooth eruption associated with cleidocranial dysplasia suggest that Cbfa1 may also participate in tooth formation and eruption.

To understand the involvement of Cbfa1 in ameloblastin gene transcription, we have analyzed ameloblastin promoter function in cells derived from three different oral tissues. The finding that Cbfa1 interacts with functionally important regions of the ameloblastin promoter, whereas promoter activity is diminished in constructs containing site-directed mutations in the Cbfa1 site suggests that Cbfa1 is involved in the transcription of the ameloblastin gene.

EXPERIMENTAL PROCEDURES

Plasmids, Cell Lines, and Transient Transfections—pAmbn-1616-Luc and pAmbn-271-Luc are luciferase reporter plasmids containing 1616 and 271 bp of 5' sequences of the ameloblastin promoter, respectively (21). Mutant plasmids pAmbn-1616 m-Luc and pAmbn-271 m-Luc each contain a 2-bp mutation at the OSE2 site at position –241 and –246 bp relative to the transcription start site, respectively (see Table I). Mutant constructs were generated by site-directed mutagenesis using the GeneEditor™ mutagenesis kit (Promega). Briefly, mutated oligonucleotide probes (see Table I) and an antibiotic selection oligonucleotide were annealed to the double-stranded wild type plasmid. Following synthesis and ligation of the mutant strand, the mutant plasmids were selected with the GeneEditor™ antibiotic selection mix. The DNA sequence of each mutant construct was confirmed by sequencing with the ThermoSequenase kit (Amersham Pharmacia Biotech) prior to transfection.

The mouse enamel organ epithelial cell line, LS8, is an immortalized ameloblast-like cell line that expresses enamel-specific genes such as amelogenin and ameloblastin (32). Human gingival fibroblasts and pulp fibroblasts were generated from primary explants. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 units penicillin/streptomycin. The cells were co-transfected with the reporter construct that uses *Photinus pyralis* (firefly) luciferase as a reporter for transcriptional activity and the pRL-TK vector (Promega) as an internal control for transfection efficiency. pRL-TK uses *Renilla reniformis* (sea pansy) luciferase as a reporter allowing discrimination between the bioluminescence of the two constructs in a single-tube assay (dual luciferase assay system; Promega). For each transfection, cells (3×10^5 cells/35-mm plate) were incubated with 1 μ g of each promoter-reporter plasmid, 0.1 μ g of pRLTK, and 5 μ l of LipofectAMINE (Life Technologies, Inc.) in serum-free medium (OptiMem; Life Technologies Inc.) for 6 h. The transfection medium was removed and replaced with growth medium, and the cells were incubated for 48 h. The cells were harvested in 1 \times passive lysis buffer (Promega), and the firefly and *Renilla* luciferase activities were measured with a luminometer (Monolight 2010, Analytical Luminescence Laboratory), and *Renilla* activity was used for normalizing the transfection data. Each plasmid construct was transfected in triplicate, and the mean and standard deviation were determined from at least three independent experiments.

RNA Isolation and Analysis—Total RNA from MC3T3, LS8, human gingival fibroblast and human pulp fibroblasts was extracted by a

guanidinium isothiocyanate method (Trizol; Life Technologies, Inc.). RNA was quantified by optical density (A_{260}) and stored at -80°C until analyzed. One μ g of total RNA was used for cDNA synthesis (cDNA synthesis system; Life Technologies, Inc.). cDNAs were amplified by PCR using mouse and human specific primers complementary to the 5' region of Cbfa1 (accession numbers AF010284 and AF053949). Mouse primers Cbfa-12, 5'-GCCTCACAACAACCACAGAA-3', and Cbfa-1–360, 5'-TTGCTGCTGCTGCGCAGCCACCAC-3', generated a PCR product of 348 bp and human primers hOsf2I, 5'-CAGAACCACAAGT-GCGGTGC-3', and hOsf2II, 5'-TGCTGAAGAGGCTGTTTGATGC-3', generated a 402-bp product. The PCR reaction conditions were as follows: 200 μ M dNTPs, 0.8 μ M for each primer, and 30 cycles of 30 s of denaturation at 94°C , 30 s of annealing at 58°C , and a 3-min extension at 72°C . PCR products were separated in 1% agarose gels and were visualized by ethidium bromide. The identity of the PCR products was confirmed by Southern blot analysis and DNA sequencing using standard molecular biology techniques (33).

Southern Blot Analysis—One μ l of the PCR reaction was fractionated in 1% agarose gel. DNA was transferred to the nylon membrane by a capillary transfer method, UV cross-linked, and probed for Cbfa1 expression. The probe was prepared by gel purifying a 1.5-kilobase EcoRI fragment from a full-length Cbfa1-containing cDNA plasmid (gift from P. Ducy) (23). The probe was random prime-labeled using the Prime it II kit (Stratagene, La Jolla CA). The membrane was prehybridized in a high temperature prehybridization solution containing 6 \times SSPE, 5 \times Denhardt's solution, 0.5% SDS, 50 μ g/ml denatured salmon sperm DNA, and 10% Dextran sulfate for at least 3 h at 65°C . The labeled probe (1×10^6 cpm/ml) was added to hybridization solution (6 \times SSPE, 5 \times Denhardt's solution, 0.5% SDS, 100 μ g/ml denatured DNA, and 10% Dextran sulfate) and was hybridized overnight at 65°C . The blot was washed in 1 \times SSC, 0.1% SDS at room temperature for 30 min (three washes of 10 min each) and 0.1 \times SSC, 0.1% SDS at room temperature for 20 min (two washes of 10 min each), followed by a final wash in the latter solution at 65°C . The membrane was exposed to the x-ray film overnight and autoradiographed.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assays (EMSA)—Nuclear extracts were prepared from cultured cells by the method of Dignam *et al.* (34). The buffers were supplemented with protease inhibitors (1 μ M phenylmethylsulfonyl fluoride and 1 mM each of leupeptin and pepstatin). Protein concentration of the nuclear extracts was determined by the bicinchoninic protein assay (Pierce) using bovine serum albumin as a standard. The wild type and mutated oligonucleotide probes corresponding to OSE2 sites in ameloblastin and the osteocalcin promoters (see Table I) were generated by oligonucleotide synthesis in the University of Michigan Biomedical Research Core Facility. Oligonucleotides were annealed, end-labeled with T4 polynucleotide kinase and [γ - ^{32}P]ATP, and gel-purified prior to EMSA. For EMSA, up to 10 μ g of nuclear extract was incubated for 30 min at room temperature in mobility shift buffer (12 mM Hepes, pH 7.9, 50 mM KCl, 4 mM MgCl_2 , 1 mM EDTA, 0.1 mM ZnSO_4 , 1 mM dithiothreitol, 5% glycerol, and 2 μ g poly(dI-dC) with 30,000 cpm of ^{32}P end-labeled double-stranded DNA probe in a 30- μ l volume. For competition experiments, the nuclear extract was incubated with the indicated concentrations of double-stranded oligonucleotide competitors at room temperature for 5 min prior to incubation with the probe. DNA-protein complexes were resolved on a 5% nondenaturing polyacrylamide gel containing 4.5 mM Tris-HCl (pH 7.5), 4.5 mM boric acid, and 1 mM EDTA. The gels were dried and autoradiographed. For the supershift assays, 1 μ l of anti Cbfa1 (gift from Dr. Patricia Ducy) (23) was incubated with nuclear extracts for 10 min prior to the addition of probe.

RESULTS

Cbfa1-binding Sites in the Ameloblastin Promoter—Sequence analysis of the mouse ameloblastin promoter for putative *cis*-acting regions revealed two potential Cbfa1-binding elements termed OSE2 at positions –248 and –1391 bp relative to the transcription start site (Fig. 1). These elements have a consensus core sequence (AACCAC) as described in several other genes involved in bone mineralization (23). The proximal OSE2 site located at position –248 bp has a reversed orientation. This Cbfa1 site is located within a promoter region that expresses a high level of ameloblastin promoter activity in ameloblast-like cells but is inactive in other oral tissues-derived cells such as human gingival fibroblasts (HGF) and human pulp fibroblasts (HPF) (21). The distal OSE2 site (–1391

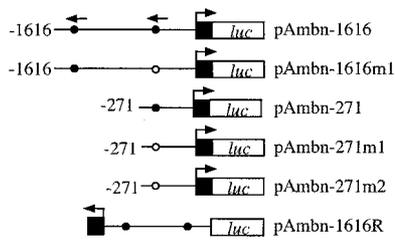


FIG. 1. **Ameloblastin reporter genes.** Schematic representations of the promoter constructs used in this study. The constructs contain 5'-flanking regions of the ameloblastin promoter including the transcription start site (*bent arrow*), 57 bp of untranslated sequence (*filled bar*), and the luciferase reporter gene (*open bar*). The *filled circles* designate the location of wild type Cbfa1 sites, and the *open circles* designate the mutated Cbfa1 sites. The *arrows* indicate the orientation of the OSE2 sites.

bp) is located outside the minimal promoter region and we therefore studied the role of the proximal OSE2 site in ameloblastin promoter function.

The Proximal OSE2 Site Is Required for Full Ameloblastin Promoter Activity—Site-directed mutagenesis of the proximal OSE2 site (−248 bp) was performed to determine the importance of this OSE2 site in ameloblastin promoter activity in ameloblast-like cells. Two different oligonucleotides possessing 2-bp mutations within the OSE2 core sequence (Ambn m1 and Ambn m2) were used to generate mutant constructs pAmbn-271 m1 and pAmbn-271 m2, respectively (Table I and Fig. 1). Both substitution mutations were generated in the context of pAmbn-271-luc, a construct that contains 271 bp of 5'-flanking sequence of the ameloblastin promoter and exhibits high level promoter activity in LS8 cells and minimal activity in human gingival fibroblasts and human pulp fibroblasts (Fig. 2A). When promoter activity of the wild type promoter and the mutant promoter constructs were compared in transient transfection assays, promoter activity in constructs containing the mutated OSE2 sites was significantly reduced and consistently ranged between 40 and 60% of the wild type promoter (Fig. 2A). Promoter activity was also diminished when the proximal OSE2 site was mutated in the context of the much larger full-length ameloblastin promoter, pAmbn-1616 (Fig. 2B). The wild type construct pAmbn-1616 also had high expression levels in ameloblast-like cells and minimal activity in either gingival or pulp fibroblasts. Although the decrease in pAmbn-1616 m1 promoter activity was not statistically significant, the consistent reduction in promoter activity with a 2-bp mutation in the proximal OSE2 site in the context of a 1.6-kilobase promoter confirms the findings with pAmbn-271 and suggests that this site is required for full transcriptional activity.

Cbfa1 Is Expressed in MC3T3, LS8, HGF, and HPF Cells—Cbfa1 is expressed in the mammalian dentition and is temporally and spatially regulated during tooth development (35). Reverse transcriptase-PCR analysis showed that Cbfa1 transcripts were expressed in ameloblast-like LS8 and preosteoblastic MC3T3-E1 murine cell lines. Cbfa1 transcripts were also expressed in human gingival and pulp fibroblasts. Both human- and mouse-specific primers were designed to amplify the 5' region of either human or mouse Cbfa1. The predicted band of 348 bp was observed in both LS8 and MCT3-E1 cells, and the identities were confirmed by Southern blot analysis (Fig. 3A). PCR products of 402 bp were observed in HGF and HPF (Fig. 3B). All PCR products were confirmed by DNA sequencing.

Binding of Cbfa1 to Ameloblastin Promoter Is Altered by Mutations in the OSE2 Site—The significant decrease in promoter activity produced by site-directed mutagenesis of the OSE2 site in the context of either pAmbn-271 or pAmbn-1616 suggested that specific binding of transcription factors to this

TABLE I
Oligonucleotide probes

The double underlines designate the mutated nucleotides. The osteocalcin OSE2 sequence is underlined. The dotted underline represents the ameloblastin OSE2 site in the reverse orientation. Ambn designates the ameloblastin promoter, and OG2 designates the osteocalcin gene 2 promoter.

Ambn wt	5'-TCCCGAAGCTC <u>ACCA</u> AAAATGATTGTCT-3'
Ambn m1	TCCCGAAGCTCA <u>AG</u> AAAATGATTGTCT
Ambn m2	TCCCGAAGCTCT <u>CCG</u> AAAATGATTGTCT
OG2 wt	CTGCAATCA <u>CCACC</u> CAGCA

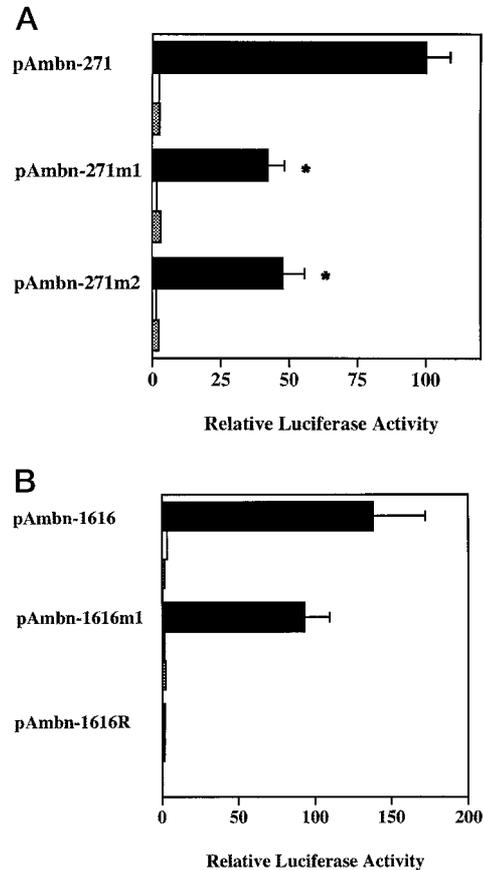


FIG. 2. **The OSE2 site is required for full ameloblastin promoter activity.** Wild type or site-directed ameloblastin mutants were transiently transfected into cells derived from three different oral tissues. The ameloblastin-luciferase constructs (Fig. 1) were co-transfected with a control plasmid (pRLTK) and assayed 48 h after transfection. The percentage of luciferase activity elicited by each construct is expressed as a percentage of the activity obtained by the control plasmid. *A*, comparison of mutant constructs to wild type construct pAmbn-271. *B*, comparison of mutant constructs to wild type construct pAmbn-1616. The *open bars* designate the activity in pulp fibroblasts, the *gray bars* designate activity in gingival fibroblasts, and the *black bars* designate activity in the ameloblast-like LS8 cells. The *error bars* represent the standard deviation for three samples in at least three independent experiments. *, $p < 0.05$ compared with the wild type constructs.

site may serve an essential function in transcription of the ameloblastin gene. Therefore, EMSAs were performed to determine whether Cbfa1 or other nuclear proteins expressed by LS8 cells would interact with the OSE2 site present within the ameloblastin promoter. A radiolabeled, double-stranded oligonucleotide probe was prepared from this region (Ambn wt in Table I) of the ameloblastin promoter and was incubated with nuclear extracts from LS8 cells or human pulp fibroblasts (Fig. 4). Two discrete DNA-protein complexes were formed between the Ambn wild type (wt) probe and nuclear extracts derived from LS8 cells (Fig. 4, *A*, lane 2, and *B*, lane 2). In contrast, a single complex formed with the OSE2-containing Ambn wt

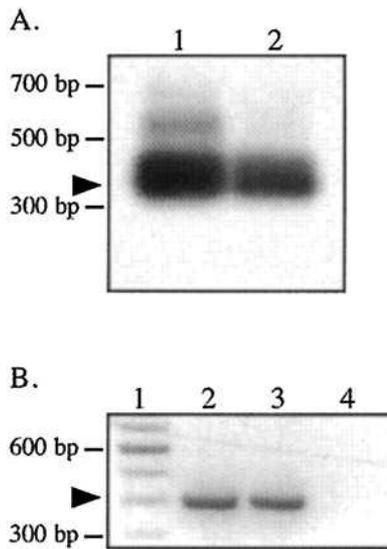


FIG. 3. Cbfa1 expression in ameloblast and nonameloblast cells. cDNA generated from total RNA was subjected to PCR amplification using Cbfa1-specific primers (see "Experimental Procedures"). A, following separation in a 1% agarose gel, DNA was transferred to a nylon membrane and subsequently hybridized with a Cbfa1 cDNA probe labeled with [γ - 32 P]ATP. Lane 1 represents MC3T3-E1 osteoblast-like cells. Lane 2 represents LS8 ameloblast-like cells. Molecular weight markers are indicated on the left, and the arrowhead designates the predicted size of PCR product (348 bp). B, reverse transcriptase-PCR of RNA from human gingival and pulp cells. The PCR products were gel-purified, cloned, and sequenced to confirm the presence of human Cbfa1. Lane 1 contains molecular weight markers, lane 2 represents HGF, lane 3 represents HPF, and lane 4 represents a control with no DNA template. The arrowhead designates the predicted size of the PCR product (402 bp). The primer pairs used in A were mouse-specific (Cbfa1-12 and Cbfa1-360), and those in B were human-specific (hOsf2I and hOsf2II).

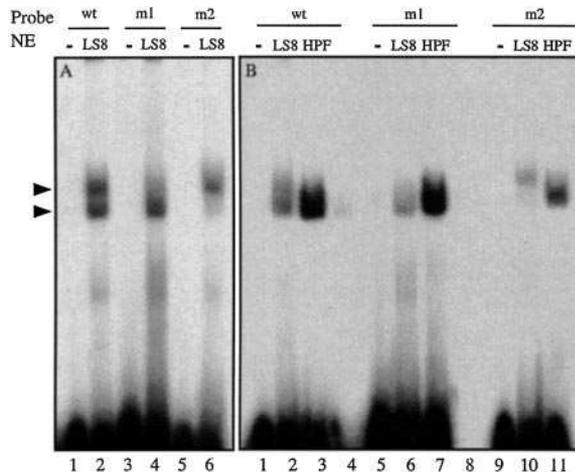


FIG. 4. Effects of OSE2 mutations on DNA-protein interactions. DNA-protein interactions between nuclear extracts and oligonucleotide probes containing wild type or mutant ameloblastin promoter sequence (shown in Table I) were analyzed by EMSA. A, nuclear extracts from LS8 cells were incubated with [γ - 32 P]ATP-labeled wild type (lane 2), Ambn m1 (lane 4), or Ambn m2 (lane 6) oligonucleotide probes. B, nuclear extracts from LS8 or HPF were incubated with [γ - 32 P]ATP-labeled wild type (lanes 2 and 3) Ambn m1 (lanes 6 and 7) or Ambn m2 (lanes 10 and 11) oligonucleotide probes. Lanes 1, 5, and 9 are wild type, m1, and m2 probes without nuclear extracts. Lanes 4 and 8 are blank lanes. The arrowheads designate the two DNA-protein complexes formed with LS8 nuclear extracts and the wild type ameloblastin probe. NE, nuclear extracts.

probe and nuclear extracts from the pulp fibroblasts (Fig. 4B, lane 3), suggesting that distinct differences in *trans*-acting factors between the two cells types contribute to the cell-specific promoter activity demonstrated in ameloblast-like cells.

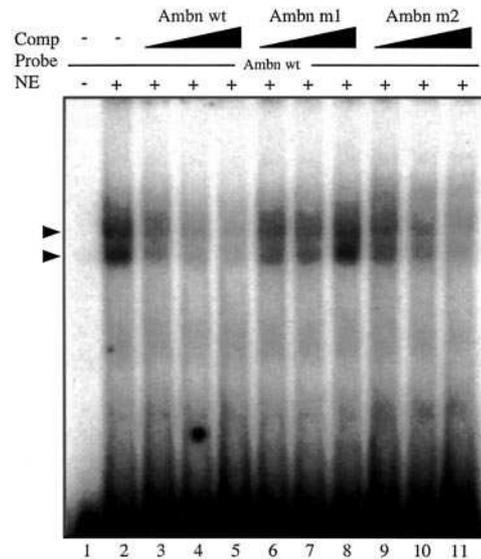


FIG. 5. Competitive inhibition of DNA-protein interactions in LS8 cells. DNA-protein interactions between the wild type ameloblastin probe (Table I) and nuclear extracts from LS8 cells were analyzed by EMSA. Competition analysis was performed with 50-, 100-, and 150-fold molar excess of the indicated unlabeled oligonucleotide probes, Ambn wt (lanes 3-5), Ambn m1 (lanes 6-8), and Ambn m2 (lanes 9-11). The arrowheads designate the two DNA-protein complexes formed with LS8 nuclear extracts and the wild type ameloblastin probe. NE, nuclear extracts.

To determine the specificity of nuclear factor binding to the OSE2 site, EMSAs were performed with the Ambn wt probe and similar oligonucleotides containing substitution mutations within the OSE2 core sequence. The oligonucleotide probes contained the same substitution mutations that were used to illustrate reduced functional activity in transfection analyses (Table I and Fig. 2). The results of EMSA experiments demonstrated that binding of ameloblast-derived nuclear proteins to the mutated oligonucleotide probe m1 led to an abrogation of the upper DNA-protein complex observed in wt binding (Fig. 4, A, lane 4, and B, lane 6), whereas the binding was unchanged in HPF (Fig. 4B, lanes 3 and 7). In contrast, the lower DNA-protein complex was abolished when LS8-derived nuclear proteins were incubated with the m2 probe (Fig. 4, A, lane 6, and B, lane 10). The intensity of binding in DNA-protein complexes with probe m2 and HPF nuclear extracts was diminished but not completely abolished (Fig. 4B, lane 11). Taken together, the results from EMSA and transfection experiments suggest that nuclear factors from ameloblast-like cells show a degree of specificity for the OSE2 core sequence in this functionally important region of the ameloblastin promoter.

Cbfa1 Participates in the DNA-Protein Interactions between Nuclear Factors and the Ameloblastin OSE2 Sequence—The association of Cbfa1 in DNA-protein binding to the Ambn wt promoter was further established by competition studies with wild type and mutant probes known to specifically bind Cbfa1 (Table I). The murine OSE2 is a *cis*-acting element in the promoter of the mouse osteocalcin gene that binds Cbfa1 and together with other *cis*-acting sites participates in the determination of the osteoblast phenotype (22). Mutations in this site abolish Cbfa1 binding and affect osteoblast differentiation.

The specificity of the Cbfa1 binding to the ameloblastin promoter was demonstrated by competition assays (Fig. 5). Increasing amounts of unlabeled wild type and mutant probes were used as competitors. As also demonstrated in Fig. 4, nuclear extracts from LS8 cells form two DNA-protein complexes with the wt probe (Fig. 5, lane 2). Both of these complexes were competed by 50-, 100-, and 150-fold molar excess

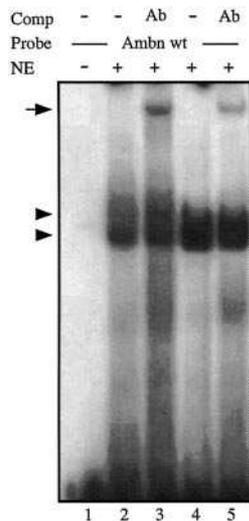


FIG. 6. Identification of Cbfa1 in DNA-protein complexes between the ameloblastin promoter and nuclear factors derived from ameloblast-like cells and pulp cells. DNA-protein interactions between the wild type ameloblastin probe (Table I) and nuclear extracts from LS8 cells (lanes 2 and 3) and pulp cells (lanes 4 and 5) were analyzed by EMSA. Anti-Cbfa1 antisera (lanes 3 and 5, Ab) were incubated with the nuclear proteins prior to the addition of probe. The arrow designates the supershifted DNA-protein complex in the presence of anti-Cbfa1 antisera. The filled arrowheads designate the two DNA-protein complexes formed with LS8 nuclear extracts and the wild type ameloblastin probe. NE, nuclear extracts.

unlabeled wild type probe (Fig. 5, lanes 3–5). The binding pattern observed with the m2 probe and nuclear extracts from LS8 cells was similar to binding with the wt probe but exhibited slightly less competition. (Fig. 5, lanes 9–11). No significant competition was seen with probe m1 (Fig. 5, lanes 6–8), suggesting that the mutated Cbfa1 site could not compete for Cbfa1 binding.

To ascertain the involvement of Cbfa1 in the DNA-protein complex formation, nuclear extracts from LS8 cells were co-incubated with and without anti-Cbfa1 antisera. DNA-protein complexes exhibited a modified electrophoretic mobility when anti Cbfa1 antisera were added to the reaction (Fig. 6, lane 3). A portion of the DNA-protein complex was supershifted by Cbfa1-specific antiserum in the reaction mixture, confirming the interaction of Cbfa1 in the ameloblastin promoter. A supershifted complex by anti Cbfa1 antiserum addition was also observed in HPF extracts (Fig. 6, lane 5).

DISCUSSION

Cbfa1 has been shown by *in situ* hybridization to be expressed in dental tissues such as the dental papilla and ameloblasts and is hypothesized to serve as a mediator of epithelial-mesenchymal interactions controlling the morphogenesis of the enamel organ (35). Our findings suggest a role for Cbfa1 in controlling the transcription of ameloblastin, an enamel matrix gene that may play an important role in the formation of the dentition. The murine ameloblastin promoter contains two Cbfa1 sites within 1616 bp of the transcription start site. The Cbfa1 site at –248 bp is located within a short proximal promoter region that exhibits high levels of promoter activity in ameloblasts. Progressive 5'-deletion from this region of the promoter to –100 bp abolishes detectable promoter activity and suggests that key DNA-protein interactions in this region direct the cell-specific transcriptional activity of the ameloblastin gene (21). Site-directed mutations within the ameloblastin Cbfa1 site significantly reduced the activity in reporter gene constructs and altered DNA-protein interactions with ameloblast nuclear proteins. These data, together with the observa-

tions that Cbfa1 is expressed and participates in the binding of nuclear factors to the ameloblastin Cbfa1 site, suggest that Cbfa1 possesses an important function in transcription of the murine ameloblastin gene.

Cbfa1 is a member of the runt domain gene family because of its homology with the *Drosophila* pair-rule gene runt (36). Runt domain proteins bind their cognate regulatory elements in several genes in cells with functions diverse as neuronal and skeletal development (23, 31, 36, 37). Cbfa1 is known to participate in the regulation of osteocalcin and osteopontin in osteoblasts (25, 28) and may be involved in regulating the expression of osteoblast-related genes when overexpressed in myoblasts and fibroblasts *in vitro* (23). Evidence also exists for the role of Cbfa1 in the activation of osteoblast differentiation *in vivo*. Targeted disruption of the runt domain of Cbfa1 inhibits bone formation (29, 31), and mutations in human Cbfa1 cause autosomal dominantly inherited cleidocranial dysplasia (30). More recently Cbfa1 has been described as a positive regulator of chondrocyte differentiation (38–40) and may be involved in the coupling of bone formation and resorption by regulating the expression of osteoprotegerin an osteoblast-secreted glycoprotein that functions as a potent inhibitor of osteoclast differentiation and bone resorption (41, 42).

Although Cbfa1 is clearly important for the regulation of genes involved in bone formation, it does not function exclusively in bone. Cbfa1 is expressed in ameloblasts and odontoblasts, two cell types that synthesize the extracellular matrix of enamel and dentin, respectively (35, 43). In addition to the well described skeletal defects in mice with targeted disruption of Cbfa1, these mice also exhibit defects in the development of the dentition. Although early events regulating the initiation of odontogenesis appear to be unaffected, the absence of Cbfa1 adversely affects late stage differentiation of ameloblasts and odontoblasts in molar teeth, thus delaying the normal developmental process (35). In humans, the development of the dentition may also be delayed in patients with cleidocranial dysplasia (44). Other dental findings in cleidocranial dysplasia include irregular dentin matrix, hypoplastic enamel, and supernumerary teeth (45–47). Therefore, Cbfa1 may play a broader role in the development of mineralized tissues of vertebrates including both bones and teeth.

We investigated the role of Cbfa1 in the transcriptional regulation of ameloblastin in ameloblast and nonameloblast cell lines. Despite Cbfa1 expression in both ameloblast and nonameloblast cell types, the nonameloblast cells did not support ameloblastin promoter activity in transient transfection experiments. In addition, the DNA-protein interaction patterns in these cell types were clearly different than in the LS8 cell line. Although the current data support a role for Cbfa1 in the transcription of the ameloblastin gene, important questions remain unanswered. Despite the finding that promoter activity was diminished in constructs containing site-directed mutations in the Cbfa1 site, total elimination of promoter activity was not observed in these experiments. The pAmbn-271 m1 construct displayed significantly reduced promoter activity, and the mutant probe (Ambn m1) exhibited altered binding patterns with extracts from LS8 cells (Fig. 4). Two major DNA-protein complexes were observed upon binding of LS8 nuclear extracts to the wild type DNA probe. However, incubation of LS8 nuclear extracts with Ambn m1 did not lead to the formation of the more slowly migrating DNA-protein complex (upper bands in Fig. 4, A, lane 4, and B, lane 6), suggesting that Cbfa1 is present in the upper, more slowly migrating DNA-protein complex. Further evidence for the presence of Cbfa1 in the upper complex is provided by the observation that Ambn m1 was unable to compete for nuclear factor binding to the wild type probe (Fig. 5). In the supershift experiments the upper

complex was not attenuated (Fig. 6) and may reflect an unfavorable stoichiometry of proteins and DNA used in the assay rather than a failure to compete for binding. Like Ambn-271 m1, the pAmbn-271 m2 construct had diminished promoter activity (Fig. 2A). However, the m2 probe was able to weakly compete for nuclear extract binding (Fig. 5, lanes 9–11), again supporting the presence of Cbfa1 in the upper complex.

The observations that HGF and HPF express Cbfa1 transcripts and that a DNA-protein complex was supershifted with nuclear extracts from HPF despite not having the upper complex highlight differences in the transcriptional environment and promoter context between ameloblast and nonameloblast cell types. In addition to Cbfa1, the ameloblastin promoter contains several other putative *cis*-acting regulatory elements including AP-1, TCF-1, CACCC binding sites, and sites for two zinc finger proteins, CF2-II and Krueppel (21). Investigations into potential cooperative or synergistic interactions among these sites should contribute to a more complete understanding of the ameloblastin transcriptional program.

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Sangeeta Dhamija and Paul H. Krebsbach

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