Identification of CCAAT/Enhancer-binding Protein α as a Transactivator of the Mouse Amelogenin Gene*

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Yan Larry Zhou and Malcolm L. Snead‡

From the Center for Craniofacial Molecular Biology, University of Southern California, Los Angeles, California 90033

Amelogenin expression is ameloblast-specific and developmentally regulated at the temporal and spatial levels. In a previous transgenic mouse analysis, the expression pattern of the endogenous amelogenin gene was recapitulated by a reporter gene driven by a 2.2-kilobase mouse amelogenin proximal promoter. To understand the molecular mechanisms underlying the spatiotemporal expression of the amelogenin gene during odontogenesis, the mouse amelogenin promoter was systematically analyzed in mouse ameloblast-like LS8 cells. Deletion analysis identified a minimal promoter (−70/−52) containing a CCAAT/enhancer-binding protein (C/EBP)-binding site upstream of the TATA box. In transient transfection assays, C/EBPα up-regulated the promoter activity in a dose-dependent manner. The C/EBP-binding site was necessary for both C/EBPα-mediated transactivation and basal promoter activity. Electrophoresis mobility shift assays demonstrated that C/EBPα bound to its cognate site in the amelogenin promoter and that the binding was specific. Endogenous C/EBPα was detected in LS8 cells, and overexpression of exogenous C/EBPα in LS8 cells was able to increase the expression level of the endogenous amelogenin protein. The activity of the amelogenin promoter in rat parotid Pa-4 cells and Madin-Darby canine kidney cells was minimal, ranging from 20 to 30% of the activity in ameloblast-like cells. Transient transfection experiments showed that C/EBPα transactivated the mouse amelogenin reporter gene in Pa-4 cells, but not in Madin-Darby canine kidney cells. Taken together, these data indicate that C/EBPα is a bona fide transcriptional activator of the mouse amelogenin gene in a cell type-specific manner.

One unique characteristic of tooth development is the formation of mineralizing extracellular matrices. Enamel, the only epithelially derived calcified tissue in vertebrates, is synthesized by ameloblasts. Amelogenins are essential to the proper differentiation of ameloblasts. Amelogenins are tissue-specific and developmentally regulated at the temporal and spatial levels (1–8). A 2263-nucleotide proximal promoter element from the mouse X-chromosomal amelogenin gene has been demonstrated by transgenic mouse analysis to direct the expression of a reporter gene in a temporal and spatial pattern that is essentially identical to that of the endogenous amelogenin gene (5).

During organogenesis, a programmed differentiation of embryonic epithelial cells is often characterized by the highly regulated expression of tissue-specific genes activated in response to inductive interactions with embryonic mesenchyme. The biochemical identities of the inductive signals that direct mammalian epithelial determination and differentiation have been elusive; however, studies of the regulated expression of tissue-specific gene products in developing epithelia may facilitate the molecular dissection of these interactions. The regulated expression of the ameloblast-specific amelogenin gene in the developing mouse tooth organ is an excellent model for studying developmentally regulated gene expression.

We hypothesize that the regulated transcription of amelogenin by specific activator(s) and repressor(s) in ameloblast cell lineage results in the spatiotemporal expression of amelogenin required for proper enamel formation. Gene expression is regulated at several levels, including activation of gene structure, transcription initiation, termination of transcription, nuclear RNA processing, mRNA translation, and mRNA stability. Unlike several other developmentally regulated genes (9), the methylation pattern of CpG islands in the promoter region is not associated with the regulated transcription of the amelogenin gene (10). Extensive homologies exist in the promoter regions of the bovine, human, and murine X-chromosomal amelogenins. There is a 70% identity within the 300-base pair region upstream of the transcription initiation site, suggesting that transcriptional regulation is likely to play an important role in the spatiotemporal expression of amelogenin. For a TATA box-containing promoter, the preinitiation complex is assembled in a highly regulated and defined order (11). The assembly of the preinitiation complex on a core promoter is sufficient to initiate transcription at a minimal level. However, the rate of transcription can be increased by an activator or turned off by a repressor.

The CCAAT/enhancer-binding proteins (C/EBPs)† are a family of related basic region leucine zipper transcription factors involved in the regulation of various aspects of cellular differentiation and function in multiple tissues. Six different members of the family (C/EBPα, -β, -γ, -δ, -ε, and -ζ) have been isolated and characterized. The expression of C/EBPs is tissue- and stage-specific during development. C/EBPs have been shown to play a key role in regulating cellular differentiation, terminal function, and response to inflammatory insults (12–16).

To investigate the role of C/EBPα in the regulation of amelogenin gene expression, the 2.2-kilobase mouse amelogenin promoter has been systematically analyzed in mouse ameloblast-like LS8 cells. Our experimental strategy includes

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† To whom correspondence should be addressed: CSAl42, CCMB, University of Southern California, 2250 Alcazar St., Los Angeles, CA 90033. Tel.: 323-442-3178; Fax: 323-442-2981; E-mail: mlsnead@hsc.usc.edu.

† The abbreviations used are: C/EBP(s), CCAAT/enhancer-binding protein(s); MDCK, Madin-Darby canine kidney; EMSA, electrophoresis mobility shift assay; NFY, nuclear factor Y; CBF, CAAT-binding factor.
Materials and Methods

Plasmid Construction—To generate reporter construct p2207, the 5' to 3' Smal-XhoI fragment from pS8 containing 2263 nucleotides of the mouse amelogenin promoter (5) was subcloned into the 5' to 3' Small-XhoI site of pGL3-Basic (Promega). To generate p454, p2207 was digested with KpnI and Smal, treated with exonuclease III followed by blunt end filling, digested and sequenced. For reporters p349, p194, p70, p51, and p70mut, the promoter regions were generated by polymerase chain reaction with p2207 as the template using a common 3'–primer (SN244, 5'-TATTCTGAG TGTATGCT CAGTGAG-3'; the XhoI site is underlined) and respective 5'–primers (SN181, 5'-CGTCTGACT TGAGACG CTGACCG ATTAC-3'; SN180, 5'-CGTCTGACT AACTATTA TTGGCTG TTCAAAGTG-3'). The 5'–primer was synthesized at the Microchemical Core Facility (University of Southern California/Norris Comprehensive Cancer Center). To ascertain the mutations, the plasmids were resolved by restriction mapping and DNA sequencing.

EMSMA—Double-stranded oligonucleotide probes were generated by annealing an antisense strand to a 10-fold excess of sense strand and filling in with [α-32P]dATP (NEF Life Sciences Product) and Klenow (exo-). The GelShift buffer kit (Stratagene) was used for the binding reaction, which was performed as recommended by the manufacturer. The reaction mixtures were resolved on a 6% non-denaturing polyacrylamide gel provided in the kit. The gel was dried, and the bands were visualized by autoradiography. The sequences of the oligonucleotides are as follows: wild-type antisense strand, 5'-GAACACG CAATTG CTTGTGAATGAA-3'; wild-type sense strand, 5'-TTTTCTTCATGCAACACCTTGTGAATGAA-3'; mutant antisense strand, 5'-GAACACG CAACTCT TAGAATGAA-3'; mutant sense strand, 5'-TTTTCTTCATGCAACAACTCTTAGAATGAA-3'.

Western Blot Analysis—Whole cell lysates were prepared from untreated LS8 cells, LS8 cells transfected with pCDNA3 (Invitrogen), and LS8 cells transfected with pcCR/C/EBPa (a C/EBPa expression vector in pcDNA3), respectively. Protein concentrations were determined by the Bio-Rad protein assay kit with bovine serum albumin standards. Equal amounts of protein (10–20 μg) were subjected to SDS-polyacrylamide gel electrophoresis. The resolved proteins on the gel were electrophoretically transferred to Immobilon-P membrane (Millipore Corp.), and the membrane was incubated with a primary antibody (anti-C/EBPa, anti-C/EBPβ, or anti-amelogenin). Horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech) were used to detect the bound antibodies.

Results

The −70–−52 Region Is Essential to Amelogenin Promoter Activity—To identify the cis-element(s) required for the promoter activity of the mouse amelogenin gene, a series of 5'-deletion reporter constructs were tested in LS8 cells with transient transfection assays. Consistent with the transgenic analysis (5), the 2263-base pair region from the mouse amelogenin promoter in the reporter construct p2207 was sufficient to direct the expression of the reporter gene in LS8 cells, a mouse ameloblast-like cell line. Deletional deletion of the region between −2207 and −71 in the mouse amelogenin promoter gave rise to a modest increase (−2-fold) in promoter activity. However, further deletion of a 19-nucleotide stretch (−70 to −52) resulted in a nearly complete ablation of reporter gene activity in LS8 cells, with p51 exhibiting a background level of activity similar to the promoterless construct pGL3-Basic (Fig. 1). Therefore, the −70/+51 region of the mouse amelogenin promoter included in the p70 construct functioned as a minimal promoter in LS8 cells, whereas the −70/−52 region was requisite to promoter activity.

In ameloblast-like LS8 cells, the −2207/−71 region appeared to have a limited effect on the promoter activity of the mouse amelogenin gene. To determine whether a cis-element(s) responsible for tissue specificity of the amelogenin promoter is located in the −2207/−52 region, the same set of reporter constructs was transiently transfected into non-ameloblast cells. Rat parotid Pa-4 and MDCK cells were used since they represent terminal differentiated oral and non-oral epithelial
cells, respectively. The reporter gene activity of p2207 in Pa-4 and MDCK cells was 3–5-fold above that of the promoterless construct pGL3-Basic, in comparison with 15-fold observed in LS8 cells. Progressive deletion to −70 led only to a modest increase in reporter gene activity: <2-fold in Pa-4 cells and 2–3-fold in MDCK cells. However, the reporter gene activities of p51 were comparable in Pa-4, MDCK, and LS8 cells (Fig. 1). The transfection studies confirmed that the mouse amelogenin promoter is ameloblast-specific as demonstrated in a previous transgenic animal analysis (5). Given the fact that the corresponding amelogenin reporter constructs (p2207 versus p70) exhibited comparable promoter activities in Pa-4 and MDCK cells that were consistently between 20 and 30% of the activity in LS8 cells, the −2207/−71 region appeared to contribute little to the tissue specificity of the mouse amelogenin promoter. However, similar to that in LS8 cells, the −70/−52 region was necessary for promoter activity in both Pa-4 and MDCK cells.

C/EBPα Is a Transcriptional Activator of the Mouse Amelogenin Promoter—A putative C/EBP transcription factor-binding site was found in the −70/−52 region of the mouse amelogenin promoter. To test whether C/EBPα could function as a transcriptional activator of the mouse amelogenin promoter, a C/EBPα expression vector was cotransfected into LS8 cells with either the amelogenin promoter-reporter construct p2207 or p70. In response to increasing amounts of C/EBPα, the reporter gene activity was increased up to 11-fold for p2207 (Fig. 2A) and up to 27-fold for p70 (Fig. 2B). These data indicate that C/EBPα transactivates the mouse amelogenin promoter in a dose-dependent manner in LS8 cells.

To determine whether C/EBPα alone is sufficient to activate the mouse amelogenin promoter in non-ameloblast cells, similar cotransfection experiments were performed in Pa-4 and MDCK cells. Cotransfection of C/EBPα had little effect on the promoter activity of both p2207 and p70 in MDCK cells. However, in Pa-4 cells, the reporter gene activity was increased up to 4-fold for p2207 (Fig. 2A) and up to 11-fold for p70 (Fig. 2B) in response to increasing amounts of C/EBPα. In the absence of exogenous C/EBPα, the reporter gene activity of p2207 in Pa-4 cells was 24% of the basal level in LS8 cells; cotransfection of C/EBPα was able to increase the level to 140%. A similar result was obtained for p70. In the absence of exogenous C/EBPα, the reporter gene activity of p70 in Pa-4 cells was 24% of the basal level in LS8 cells; cotransfection of C/EBPα was able to increase the level to 140%. Taken together, these data indicate that C/EBPα functions as a transcriptional activator of the mouse amelogenin gene in a cell type-specific manner.

The Putative C/EBP-binding Site Is Necessary for Amelogenin Promoter Activity—To understand the function of the putative C/EBP-binding site, a mutation was introduced into the core sequence of the C/EBP site in the context of p2207 (p2207mut) and p70 (p70mut) (Fig. 3A); the responsiveness to C/EBPα was then determined in LS8 cells with transient cotransfection assays. Mutation or deletion of the C/EBP site consistently abolished the basal promoter activity of these reporter constructs (p2207mut, p70mut, and p51). Furthermore, C/EBPα-mediated transactivation of these constructs was reduced to the background level, similar to that of promoterless pGL3-Basic, whereas the reporter gene activity of the wild-type constructs was increased an order of magnitude by cotransfection with C/EBPα (Fig. 3B). Taken together, these data indicate that the putative C/EBP-binding site in the −70/−52 region of the mouse amelogenin promoter is required not only for C/EBPα-mediated transactivation, but also for basal promoter activity in LS8 cells. Interestingly, the putative C/EBP-binding site identified in the −70/−52 region of the mouse amelogenin promoter is conserved between species, as shown in the alignment of murine, bovine, and human X-chromosomal amelogenin promoter nucleotide sequences (Fig. 3C).

C/EBPα Binds to the Mouse Amelogenin Promoter—The results described above provide functional evidence of a role for C/EBPα as a positive regulator of mouse amelogenin gene expression. To determine whether C/EBPα can bind to the mouse amelogenin promoter, a gel mobility shift assay (EMSA) was performed using the double-stranded mouse amelogenin C/EBP oligonucleotide, 5′-TTTTTC ATTCAGAAACCCTGATTGGCTGTC-3′ (C/EBP-binding site is in boldface). A protein-DNA complex was formed using nuclear extract prepared from LS8 cells as evidenced by the shifted band (Fig. 4A, lane 2). The intensity of the band was increased when nuclear extract prepared from C/EBPα-transfected LS8 cells was used (Fig. 4A, lane 3). In addition, an antibody specific to C/EBPα was able to supershift the protein-DNA complex (Fig. 4A, lane 4), whereas the antibody alone did not bind to the probe (lane 5). The addition of a 10-, 50-, or 100-fold molar excess of unlabeled C/EBP oligonucleotide inhibited the binding of C/EBPα to the labeled probe (Fig. 4B, lanes 3–5). However, no inhibition was observed (Fig. 4B, lanes 6–8) with a molar excess of an oligonucleotide encoding a mutated C/EBP-binding site, 5′-TTTTTC ATTCAGctagc GATTGGCTGTC-3′ (mutated site is in lowercase letters). These data demonstrate that C/EBPα is able to
bind to the mouse amelogenin promoter and that the binding is specific.

As shown in Fig. 4D, the C/EBP consensus sequence within the mouse amelogenin promoter does not include a CCAAT box. Furthermore, a \( ^{32} \)P-labeled double-stranded oligonucleotide containing a mutated C/EBP-binding site (Fig. 4D, MUT) was unable to form a detectable protein-DNA complex, even though the CCAAT box remained intact (Fig. 4C, lanes 7–10). In contrast, the wild-type probe (Fig. 4D, WT) formed a protein-DNA complex in a dose-dependent manner (Fig. 4C, lanes 2–5). These data indicate that the C/EBP-binding site, independent of the CCAAT box, is required for the binding of C/EBP\( \alpha \) to the mouse amelogenin promoter.

**C/EBP\( \alpha \) Up-regulates the Expression of the Amelogenin Protein in LS8 Cells**—To ascertain that C/EBP\( \alpha \) is a bona fide positive regulator of mouse amelogenin gene expression, two additional questions were asked. First, what is the expression status of C/EBP\( \alpha \) in ameloblasts? Second, is C/EBP\( \alpha \) able to regulate the expression of the endogenous amelogenin gene in the context of chromatin? The expression status of C/EBP\( \alpha \) was analyzed in an ameloblast-like cell line (LS8) by Western blotting. An antibody specific to C/EBP\( \alpha \) recognized a 42-kDa protein, which could be specifically competed off with a blocking peptide to the antibody (Fig. 5A), indicating that authentic C/EBP\( \alpha \) is expressed in LS8 cells. Furthermore, the overexpression of exogenous C/EBP\( \alpha \) in LS8 cells was achieved by transiently transfecting 2 \( \mu \)g of C/EBP\( \alpha \) expression vector (Fig. 5B, C/EBP alpha panel, compare lane 3 with lanes 1 and 2, respectively). The expression level of the endogenous amelogenin protein in LS8 cells was then determined. A \( \approx \)2-fold increase in the amelogenin protein level was observed in LS8 cells overexpressing C/EBP\( \alpha \) (Fig. 5B, histogram, third bar versus first bar), whereas the level of amelogenin expression in empty vector-transfected LS8 cells remained essentially the same as that in untransfected control LS8 cells (Fig. 5B, histogram, second bar versus first bar). With a typical 20–30\% transfection efficiency in our transient transfection experiments, the observed 2.5-fold overall increase (Fig. 5B, histogram, third bar) in the amelogenin protein level upon C/EBP\( \alpha \) activation actually represented a 6–9-fold increase in the cells that overexpressed exogenous C/EBP\( \alpha \). Therefore, the existence of C/EBP\( \alpha \) and its capability of activating the endogenous amelogenin gene in the ameloblast-like LS8 cells clearly indicate that C/EBP\( \alpha \) is a genuine regulator of amelogenin gene expression.

**C/EBP\( \beta \) Has a Marginal Effect on Amelogenin Gene Expression**—As a first step to determine whether other C/EBP family members play a role in mouse amelogenin gene expression, C/EBP\( \beta \) was studied. The expression status of C/EBP\( \beta \) was analyzed in LS8 cells by Western blotting. An antibody specific to C/EBP\( \beta \) recognized a 32-kDa protein, which could be specifically competed off with a blocking peptide to the antibody (Fig. 6A), indicating that C/EBP\( \beta \) is expressed in LS8 cells. To test whether C/EBP\( \beta \) can function as a transcriptional activator of the mouse amelogenin promoter, a C/EBP\( \beta \) expression vector was cotransfected into LS8 cells with either amelogenin promoter-reporter construct p2207 or p70. C/EBP\( \beta \) was much less potent than C/EBP\( \alpha \) in activating amelogenin reporter gene activity: 2–3-fold for C/EBP\( \beta \) versus 8–15-fold for C/EBP\( \alpha \) (Fig. 6B). The transfection data indicate that the capability of C/EBP\( \beta \) in transactivating the mouse amelogenin gene is very limited. To further determine whether endogenous C/EBP\( \beta \) in LS8 cells can bind to the amelogenin promoter, a gel mobility shift analysis was performed using the wild-type oligonucleotides (Fig. 4D) as a labeled probe. A protein-DNA complex was formed using nuclear extract prepared from LS8 cells as evidenced by the shifted band (Fig. 6C, lane 2). However, an antibody specific to C/EBP\( \beta \) was not able either to supershift or to disrupt the protein-DNA complex (Fig. 6C, lane 3), and the antibody alone was not able to bind to the probe (lane 4). This EMSA data argued that there was no detectable level of C/EBP\( \beta \) present in the protein-DNA complex. Taken together, the results indicate that C/EBP\( \beta \) has only a marginal effect, if any, on the transcriptional regulation of the mouse amelogenin gene.

**DISCUSSION**

The Mouse Amelogenin Promoter Proximal Regulatory Region—The proximal regulatory region of the mouse amelogenin promoter is the region immediately upstream of the TATA box. This region includes nucleotides –70 to –33 together with the TATA box and drives \( \sim \)190\% of the promoter activity in ameloblast-like LS8 cells relative to the 2.2-kilobase promoter tested in transgenic animals (5). The slight increase in promoter activity suggests that a silencer element(s) may be located in the region between –2207 and –70, which is consistent with a previous report in which an upstream fragment from the bovine amelogenin promoter decreased the activity of a hetero-
C/EBPα Activates Amelogenin

Gene Expression—We tested the hypothesis that C/EBPα is involved in the regulation of mouse amelogenin gene expression. Amelogenin expression is ameloblast-specific and developmentally regulated at the temporal and spatial levels. Nucleic acid hybridization experiments (1–3) demonstrated that the transcription of amelogenin is restricted to inner enamel epithelial cells that undergo terminal differentiation to the ameloblast phenotype, which is characterized by withdrawal from the cell cycle, polarization, and columnar morphology. Furthermore, the same promoter region as that in the p2207 construct is able to recapitulate the spatiotemporal expression pattern of the endogenous amelogenin gene in transgenic mouse analyses (5). In the present study, the promoter of the mouse amelogenin gene was systematically analyzed in ameloblast-like LS8 cells, rat parotid Pa-4 cells, and MDCK cells. The minimal promoter was identified, in which a 19-nucleotide stretch containing the C/EBP transcription factor-binding site was required for basal promoter activity.

C/EBPs are composed of a family of basic region leucine zipper domain-containing transcription factors that are critical regulators of cell differentiation (12–16). C/EBPα has been demonstrated to mediate cell cycle arrest; cellular differentiation; and transcriptional regulation of tissue-specific genes in adipocytes, hepatocytes, keratinocytes, pneumocytes, and ovarian follicles (20–34). In liver and adipose tissue, peak levels of C/EBPα mRNA are detected only in differentiated tissues (35, 36). C/EBPα functions as a transcriptional activator in adipocytes, and the accumulation of C/EBPα late in preadipocyte differentiation is correlated with the expression of differentiation markers (37–39). Here, we demonstrate that C/EBPα functions as a transcriptional activator of the mouse amelogenin gene by the following evidence. 1) The overexpression of C/EBPα in mouse ameloblast-like LS8 cells can up-regulate the promoter activity of the mouse amelogenin gene in a dose-dependent manner. 2) The ability of C/EBPα to transactivate the mouse amelogenin gene is cell type-specific. 3) The C/EBP-binding site located in the −70/−52 region in the mouse amelogenin promoter is necessary for both C/EBPα-mediated transactivation and basal promoter activity in LS8 cells. 4) C/EBPα is capable of specifically binding to the C/EBP site in the promoter. 5) C/EBPα is expressed in ameloblast-like LS8 cells, and overexpression of C/EBPα can increase the expression level of the endogenous amelogenin protein.

Consistent with the notion that a repressive element(s) is located in the −2207/−70 region of the mouse amelogenin promoter, the transactivation of the p2207 reporter construct by C/EBPα is less pronounced than that of p70. This finding suggests that the putative repressor(s) can interfere with the transactivating function of C/EBPα, probably through protein–protein interactions. The interference may be due to direct interactions with C/EBPα and/or interruption of the interactions between C/EBPα and basal transcriptional machinery. The mouse amelogenin promoter is cell type-specific. In non-ameloblast cell lines, all reporter constructs tested so far have very low promoter activity, and deletion of the −2207/−71 region leads only to a modest increase in reporter gene activity. There are several possible explanations for this observation. 1) The cis-element(s) conferring tissue specificity is located in the −70/−52 region. 2) The −2207/−71 region lacks the cis-element(s) that binds to tissue-specific repressor(s). 3) The −2207/−71 region contains a tissue-specific silencer(s); however, deletion of this region is not sufficient to increase promoter activity due to the lack of certain transcriptional activator(s) in non-ameloblast cells. Interestingly, the ability of C/EBPs to transactivate the mouse amelogenin gene is cell type-dependent. The presence of repressor(s) and/or the absence of coacti-

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**Figure 3.** Requirement of the C/EBP-binding site in the amelogenin promoter for C/EBPα-mediated transactivation and basal promoter activity. A. Schematic representation of the proximal regulatory region of the amelogenin promoter in the reporter construct. The transcription initiation site is indicated by the arrow and designated as position +1. The sequence of the −70/−33 region is shown, in which the 5′-boundary of the promoter in the p51 construct is indicated by −51. A C/EBP-binding site is located in the region between −70 and −51. The mutated nucleotides in the core sequence of the C/EBP-binding site are in lowercase. B. Results of transfection experiments. The reporters p2207mut and p70mut are constructs with a mutated C/EBP-binding site in the context of p2207 and p70, respectively. Equal amounts of various reporter constructs were transiently transfected into LS8 cells with 250 ng of C/EBPα expression plasmid or empty vector pcDNA3. pCMV-βgal was used as an internal control for transfection efficiency. The relative luciferase activity is the normalization of luciferase activity with β-galactosidase activity. The mean ± S.D. from at least three independent experiments is represented. C. Sequence alignment of the amelogenin promoter. The −70/−51 region and flanking sequence of the murine X-chromosomal amelogenin promoter is aligned with the corresponding regions of the bovine and human X-chromosomal amelogenin promoters. Dashes indicate identical nucleotides in all three genes. The C/EBP consensus sequence is identified in the alignment in **boldface**. WT, wild-type; MUT, mutated.
vator(s) may account for the inability of C/EBPα to transactivate the mouse amelogenin promoter in MDCK cells.

The formation of a protein-DNA complex detected in LS8 nuclear extract (Fig. 4A, lane 2) most likely resulted from endogenous C/EBPα in LS8 cells. This notion is supported by the observation that exogenous C/EBPα was able to increase the intensity of the complex (Fig. 4A, lane 3). The protein-DNA complex in EMSA could not be completely supershifted by a C/EBPα-specific antibody (Fig. 4A), which might be due to the following reasons. First, the epitope recognized by the antibody

Fig. 4. EMSA of the C/EBP-binding site. Nuclear extracts were prepared from nearly confluent LS8 cells (NE) and from LS8 cells transfected with a C/EBPα expression plasmid (NE/α). The extracts were incubated with 32P-labeled double-stranded oligonucleotides (5'-TTTTTCTATTGCTGTTTC-3') containing the murine amelogenin C/EBP-binding site. A, for supershift analysis, the extracts were preincubated with a C/EBPα-specific antibody (Ab; sc-61, Santa Cruz Biotechnology). B, for competition (comp) assay, 10-, 50-, and 100-fold molar excesses of unlabeled wild-type (WT) or mutated (MUT) double-stranded oligonucleotides were included during the binding reaction. C, increasing amounts of nuclear extract (NE/α) were incubated with wild-type or mutated 32P-labeled double-stranded oligonucleotide probes. Complexes were separated by electrophoresis and visualized by autoradiography. D, the sequences of the oligonucleotides used in EMSA studies are aligned. The C/EBP-binding site is in boldface. The mutated nucleotides in the core sequence of the C/EBP-binding site are in lowercase. The CCAAT box in the complementary strand (ATTGG) is underlined.

Fig. 5. Detection of C/EBPα and C/EBPα-induced expression of the endogenous amelogenin protein in LS8 cells. A, equal amounts of LS8 cell lysate were electrophoresed, transferred to nitrocellulose, and immunoblotted with a C/EBPα-specific antibody (sc-61, Santa Cruz Biotechnology) without (-) or with (+) preincubation with blocking peptides (sc-61p, Santa Cruz Biotechnology). The arrow indicates C/EBPα. The numbers indicate the molecular masses of marker proteins (in kilodaltons). B, whole cell lysates were prepared from untransfected LS8 cells (control bar; lane 1) or from LS8 cells transfected with 2 μg of empty vector pcDNA3 (vector bar; lane 2) or 2 μg of C/EBPα expression plasmid (C/EBP alpha bar; lane 3). Similar amounts of protein were loaded into each lane, as shown in the loading control panel; electrophoresed; transferred to nitrocellulose; and immunoblotted with either an amelogenin-specific antibody (Amelogenin panel) or a C/EBPα-specific antibody (C/EBP alpha panel). For better visualization of overexpressed exogenous C/EBPα in LS8 cells, the exposure time with the ECL system was optimized in the following manner. After immunoblotting with a C/EBPα-specific antibody (C/EBP alpha panel), the membrane was cut in half. Exogenous C/EBPα in lane 3 was detected with a 15-s exposure, whereas the endogenous C/EBPα proteins in lanes 1 and 2 were detected with a 600-s exposure, a 40-fold difference. The expression level of the endogenous amelogenin protein was quantitated by densitometry, normalized with the loading control, and plotted.
blocking peptides (sc-150p, Santa Cruz Biotechnology). The arrow indicates C/EBPβ. The numbers indicate the molecular masses of marker proteins (in kilodaltons). B, shown are the results of transient cotransfection experiments. Equal amounts of reporter construct p2207 or p70 were transiently transfected into LS8 cells with 250 ng of empty vector pcDNA3 (vector bars) or C/EBPα (C/EBP alpha bars) or C/EBPβ (C/EBP beta bars) expression plasmid.

Our results demonstrate that the C/EBPα-binding site in the proximal region of the mouse amelogenin promoter is necessary not only for C/EBPα-mediated transactivation, but also for basal promoter activity in ameloblast-like cells, and that over-expression of C/EBPα is sufficient to increase the protein level of the endogenous amelogenin gene. These findings suggest that C/EBPα plays a key role in the developmentally regulated expression of the amelogenin gene. Amelogenin, the major organic component of enamel matrix, has been demonstrated to play an important role in proper enamel mineralization. Amelogenins consist of ~90% of the enamel matrix proteins. Several mutations in the human X-chromosomal amelogenin gene have been identified in patients with the inherited enamel defect X-linked amelogenesis imperfecta (44–46). Disruption of
amelogenin promoter and/or through disrupting the transcriptional regulation of the amelogenin gene. Transcription derepression and activation are probably required for the activation of the amelogenin gene during tooth development. Both transcriptional repressors to regulate the spatiotemporal expression of the amelogenin gene, and amelogenin expression will provide insights into the understanding of the mechanism involved in amelogenin gene expression, but also facilitates the study of potential repressors acting through C/EBPα.

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