Ameloblast-specific amelogenin gene expression is spatiotemporally regulated during tooth development. In a previous study, the CCAAT/enhancer-binding protein α (C/EBPα) was identified as a transcriptional activator of the mouse amelogenin gene in a cell type-specific manner. Here, Msx2 is shown to repress the promoter activity of amelogenin-promoter reporter constructs independent of its intrinsic DNA binding activity. In transient cotransfection assays, Msx2 and C/EBPα antagonize each other in regulating the expression of the mouse amelogenin gene. Electrophoresis mobility shift assays demonstrate that Msx2 interferes with the binding of C/EBPα to its cognate site in the mouse amelogenin minimal promoter, although Msx2 itself does not bind to the same promoter fragment. Protein-protein interaction between Msx2 and C/EBPα is identified with co-immunoprecipitation analyses. Functional antagonism between Msx2 and C/EBPα is also observed on the stably transfected 2.2-kilobase mouse amelogenin promoter in ameloblast-like LS8 cells. Furthermore, the carboxy-terminal residues 183–267 of Msx2 are required for protein-protein interaction, whereas the amino-terminal residues 2–97 of Msx2 play a less critical role. Among three family members tested (C/EBPα, β, and γ), Msx2 preferentially interacts with C/EBPα. Taken together, these data indicate that protein-protein interaction rather than competition for overlapping binding sites results in the functional antagonism between Msx2 and C/EBPα in regulating the mouse amelogenin gene expression.

Enamel is the only epithelially derived calcified tissue in vertebrates. Amelogenin, the major organic component of enamel matrix, is essential to the proper regulation of enamel mineralization. Amelogenin proteins comprise ~90% of the enamel matrix proteins. Several mutations in the human X-chromosomal amelogenin gene have been identified from patients with the inherited enamel defect X-linked Amelogenesis imperfecta (1–3). Disruption of amelogenin synthesis during tooth development with either antisense oligonucleotides or ribozymes results in disorganized enamel (4, 5). Amelogenin expression is cell-type specific and developmentally regulated at the temporal and spatial level (6–13). A 2263-nucleotide proximal promoter element from the mouse X-chromosomal amelogenin gene has been demonstrated by transgenic mouse analysis to recapitulate the spatiotemporal expression pattern of the endogenous amelogenin gene (10). Extensive homologies (70% identity) in the 300-nucleotide region upstream of the transcription initiation site exist between the murine, bovine, and human X-chromosomal amelogenin gene, suggesting that this region is likely involved in the transcriptional regulation of tissue-specific amelogenin gene expression. In a previous study, the minimal promoter of the mouse amelogenin gene (−70/+52) was identified, which contains a CCAAT/enhancer-binding protein (C/EBP)α consensus binding site, and C/EBPα activated amelogenin transcription in a cell-type specific manner through binding to its cognate site (14). The C/EBPs consist of a family of related basic region leucine zipper transcription factors that are critical regulators of cellular differentiation and function in multiple tissues. Six different members of the family (C/EBPα, β, γ, δ, ε, and θ) 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 (15–19).

The Msx2 gene family (20) is the mammalian counterpart of the Drosophila msh (muscle segment homeobox) gene. Three unlinked members, Msx1 (21, 22), Msx2 (23), and Msx3 (24), have homeobox sequences very similar to each other and to the Drosophila msh gene. The murine Msx3 is expressed only in the dorsal neural tube (25–27), which appears to exclude the possibility of functional redundancy of Msx3 on the role of Msx1 and Msx2 in tooth development. During odontogenesis, Msx1 is expressed at all stages in dental mesenchymal cells but not in epithelial cells (28, 29). The expression pattern of Msx2 changes with the differentiation of different germ layers. Msx2 is strongly expressed in undifferentiated inner enamel epithelia in which amelogenin expression is barely detectable; but Msx2 is absent in differentiated ameloblasts in which robust expression of amelogenin is detected. On the other hand, Msx2 is weakly expressed/absent in undifferentiated dental papilla mesenchyme, whereas it is strongly expressed in odontoblasts and differentiated dental papilla cells (30). Msx2 has been shown to function as a transcriptional repressor independent of its intrinsic DNA binding activity through the homeodomain. Instead, the repression is mediated by protein-protein interaction.

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1 The abbreviations used are: C/EBP, CCAAT/enhancer-binding protein; EMSA, electrophoresis mobility shift assay; FACS, fluorescence-activated cell sorting; CMV, cytomegavalovirus; TNF, transcription and translation.
tion with either components of basal transcription machinery or other transcription factors (31–35).

To investigate the role of Mxs2 in the regulation of amelogenin gene expression, various amelogenin-promoter reporter constructs were transiently transfected into ameloblast-like LS8 cells with a Mxs2 expression plasmid. The functional relationship between Mxs2 and C/EBPα, a transcriptional activator of amelogenin, was examined with cotransfection assays. The potential of Mxs2 to interfere with the binding of C/EBPα to its cognate site on the amelogenin minimal promoter as well as the ability of Mxs2 itself to bind to the promoter was assessed with electrophoresis mobility shift assays (EMSA). Whether Mxs2 is able to interact with C/EBPα in LS8 cells was further determined with co-immunoprecipitation analyses. The functional antagonism between Mxs2 and C/EBPα was tested in a stably transfected amelogenin-promoter reporter construct. Finally, the ability of Mxs2 to interact with two other C/EBP family members, C/EBPβ and C/EBPγ, was examined with cotransfection assays.

MATERIALS AND METHODS

Cell Culture and Plasmids—A mouse ameloblast cell line (LS8) established by immortalizing primary cultures of enamel organ epithelium with SV40 large T antigen was maintained in Dulbecco’s Modified Eagle’s Medium (Sigma) supplemented with fetal bovine serum (10%) and antibiotics (penicillin (100 units/ml), and streptomycin (100 μg/ml)). The mouse amelogenin-promoter reporter constructs were described in a previous study (14). The expression plasmids of Mxs2FL, Mxs1ΔN, and Mxs2ΔC (31) were generously provided by Dr. Dwight Towner (Washington University).

Transfection and Luciferase Assay—Transient transfection and luciferase assays were performed as described previously (14). Stable cell line LS8/p2207 was established by transfecting LS8 cells with amelogenin-promoter reporter construct p2207. After selection with hygromycin (750 ng/ml), the resistant colonies were isolated, expanded, and screened with luciferase assays for reporter gene activity. Seven clones with luciferase activity 1,000–10,000-fold of that in parental LS8 cells were selected for further studies. One of the selected clones with the highest basal luciferase activity was designated LS8/p2207 and maintained in the presence of hygromycin (750 ng/ml).

Fluorescence-activated Cell Sorting (FACS)—The plasmid pCMV- lacZ (0.2 μg) was cotransfected into LS8/p2207 cells with an empty vector pcDNA3 (4 μg), a Mxs2 expression plasmid pcMxsFL (2 μg) plus pcDNA3 (2 μg), a C/EBPα expression plasmid pcC/EBPα (2 μg) plus pcDNA3 (2 μg), and pcMxs2FL (2 μg) plus pcC/EBPs (2 μg) in 60-mm plates. Twenty-four hours after transfection, cells were harvested, washed in phosphate-buffered saline (pH 7.4), resuspended in 50 μl of staining medium (phosphate-buffered saline, 4% fetal bovine serum, and 0.05% sodium azide) and stained at 37°C for 5 min. Nuclear proteins (50 μl) of prewarmed (37°C) 2 mM di-thiothreitol (DTT) reaction buffer, 10 μM of nuclease-free H2O were added into a 0.6-ml centrifuge tube. The mixture was then placed on ice, and 1 ml of ice-cold isotonized Dulbecco’s modified Eagle’s medium was added. The β-galactosidase positive cells were sorted by flow cytometry (Molto, Cytomation, Inc.).

Protein Purification—An expression plasmid pcQE-Mxs2 was used to produce recombinant 6xHis-Mxs2 protein. Bacterial culture, isopropyl-β-D-galactopyranoside induction, cell lysis, and nickel-nitrilotriacetic acid resin (Qiagen) loading were performed according to the manufacturer’s protocol (17). The recombinant 6xHis-Mxs2 protein was eluted with 20 ml of buffer D (8 M urea, 0.1 M sodium phosphate, 0.01 M Tris-HCl, pH 5.9) followed by 20 ml of buffer E (8 M urea, 0.1 M sodium phosphate, 0.01 M Tris-HCl, pH 4.5). Fractions (3 ml) from each elution were collected and analyzed by SDS-polyacrylamide gel electrophoresis. Fractions containing 6xHis-Mxs2 protein were pooled and dialyzed sequentially against 8, 6, 4, 2, and 1 M urea and distilled H2O at 4°C for 3 h each. After lyophilization, the sample was stored at 4°C.

EMSA—Double-stranded oligonucleotide probes were generated by annealing a sense-strand to a 40-nucleotide 5′-end and filling in with [α-32P]dATP (New England Science Products) and Klenow (exo−). The GelShift buffer kit (Stratagene) was used for the binding reaction. The reaction mixtures were resolved in a 6% nondenaturing polyacrylamide gel provided in the kit. The gel was dried, and the bands were visualized by autoradiography. The sequences of the oligonucleotides were: amel antisense strand 5′-GGACGACCATACGTTTCTGTAATG-
promoter in a dose-dependent manner in ameloblast-like LS8 cells.

C/EBPα and Msx2 Antagonized Each Other in Regulating Mouse Amelogenin Promoter Activity—C/EBPα has been demonstrated to function as a transcriptional activator of the mouse amelogenin gene through its cognate binding site in the −70−52 region of the mouse amelogenin promoter (14). To investigate the functional relationship between C/EBPα and Msx2 in regulating the mouse amelogenin promoter activity, C/EBPα and Msx2 expression plasmids were cotransfected into LS8 cells with either amelogenin-promoter reporter construct p2207 or p70. Three different Msx2 expression plasmids were used in the study to generate amino-terminal FLAG epitope-tagged Msx2 proteins. Msx2FL was a full-length protein, including murine Msx2 residues 2–267. Msx2ΔN was an aminoterminal deletion containing residues 98–267, whereas Msx2ΔC was a carboxyl-terminal deletion containing residues 2–183.

Increasing amounts of Msx2FL were able to attenuate the C/EBPα-mediated transactivation of amelogenin-promoter reporter construct p2207 (Fig. 2A, lanes 1–5) or p70 (Fig. 2B, lanes 1–5), whereas Msx2FL by itself was a potent transcriptional repressor. On the other hand, C/EBPα was capable of overcoming the repressive effect of Msx2FL on the reporter construct p2207 (Fig. 3A, lanes 1–5) or p70 (Fig. 3B, lanes 1–5) in a dose-dependent manner. As a first step to identify the
Msx2 and C/EBPα Antagonism on the Amelogenin Promoter

In the presence of Msx2FL, there was a functional antagonism between Msx2 and C/EBPα. These transfection analyses indicated that there was a difference in the activities of the two Msx2 deletion mutants. Msx2 interfered with the binding of C/EBPα to its cognate site on the mouse amelogenin promoter. As a first step to understand the mechanism underlying the functional antagonism between Msx2 and C/EBPα, the effect of Msx2 on the binding of C/EBPα to the mouse amelogenin promoter was assessed with an EMSA. Various forms of Msx2 protein were generated using an in vitro transcription-coupled translation system (TNT-coupled wheat germ extract system, Promega). A functional C/EBP cognate site has been identified in the −70/−52 region of the mouse amelogenin promoter (14). A 32P-labeled double-stranded oligonucleotide containing the C/EBP cognate site (Fig. 4C, amel probe) was able to form a C/EBPα-containing protein-DNA complex using nuclear extracts prepared from LS8 cells overexpressing C/EBPα protein (Fig. 4A, lane 12). Only modest changes in the intensity of the C/EBPα-probe complex were observed when different amounts of either sham-treated TNT extracts (Fig. 4A, lanes 3 and 9) or TNT-expressed luciferase protein (Fig. 4A, lanes 10 and 11) were included in the EMSA reactions. On the contrary, TNT-expressed Msx2FL diminished the intensity of the EMSA complex to 20% (Fig. 4A, lanes 3 and 9).

Functional antagonism of C/EBPα-mediated transactivation of the mouse amelogenin promoter by Msx2. In the presence of 250 ng of p2207 (A) or p70 (B) reporter construct, 500 ng of empty vector (pcDNA3), 250 ng of C/EBPα expression plasmid (pcC/EBPα) with 250 ng of pcDNA3, 250 ng of pcC/EBPα with 125 ng of pcDNA3 plus 125 ng of Msx2FL expression plasmid (pcMsx2FL), 250 ng of pcC/EBPα with 250 ng of pcMsx2FL, and 250 ng of pcMsx2FL with 250 ng of pcDNA3 were cotransfected into LS8 cells, respectively. Similar parameters for transfection experiments were used for Msx2N and Msx2C, respectively. pCMV-lacZ was used as an internal control for transfection efficiency. The relative luciferase activity is the normalization of luciferase activity with 250 ng of pcDNA3 were cotransfected into LS8 cells, respectively. Similar parameters for transfection experiments were used for Msx2N and Msx2C, respectively. pCMV-lacZ 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, and the basal level of p2207 (A) and p70 (B) was set as 1, respectively.

**FIG. 2.** Functional antagonism of C/EBPα-mediated transactivation of the mouse amelogenin promoter by Msx2. In the presence of 250 ng of p2207 (A) or p70 (B) reporter construct, 500 ng of empty vector (pcDNA3), 250 ng of C/EBPα expression plasmid (pcC/EBPα) with 250 ng of pcDNA3, 250 ng of pcC/EBPα with 125 ng of pcDNA3 plus 125 ng of Msx2FL expression plasmid (pcMsx2FL), 250 ng of pcC/EBPα with 250 ng of pcMsx2FL, and 250 ng of pcMsx2FL with 250 ng of pcDNA3 were cotransfected into LS8 cells, respectively. Similar parameters for transfection experiments were used for Msx2N and Msx2C, respectively. pCMV-lacZ 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, and the basal level of p2207 (A) and p70 (B) was set as 1, respectively.

**FIG. 3.** Functional antagonism of Msx2-mediated repression of the mouse amelogenin promoter by C/EBPα. In the presence of 250 ng of p2207 (A) or p70 (B) reporter construct, 500 ng of empty vector (pcDNA3), 250 ng of Msx2FL expression plasmid (pcMsx2FL) with 250 ng of pcDNA3, 250 ng of pcMsx2FL with 125 ng of pcDNA3 plus 125 ng of C/EBPα expression plasmid (pcC/EBPα), 250 ng of pcMsx2FL with 250 ng of pcC/EBPα, and 250 ng of pcC/EBPα with 250 ng of pcDNA3 were cotransfected into LS8 cells, respectively. Similar parameters for transfection experiments were used for Msx2N and Msx2C, respectively. pCMV-lacZ 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, and the basal level of p2207 (A) and p70 (B) was set as 1, respectively.
intensity of the C/EBPα-probe complex to 27% (Fig. 4B, lanes 4–8), whereas the highest concentration of bovine serum albumin had little effect (Fig. 4B, lane 9). Neither purified Msx2 protein nor bovine serum albumin alone bound to the probe (Fig. 4B, lanes 2 and 3).

To ascertain that both TntT-expressed and -purified Msx2 proteins were capable of binding to DNA, a 3H-labeled double-stranded oligonucleotide containing a Msx2 cognate site (Fig. 4C, msx2 probe) was used in gel shift analyses. The TntT-expressed full-length (Msx2FL) and amino-terminal deleted (Msx2ΔN) Msx2 protein efficiently bound to the msx2 probe (Fig. 4C, lanes 7, 8, and 12), whereas the TntT-expressed carboxyl-terminal deleted Msx2 protein (Msx2ΔC) and luciferase protein failed to form an EMSA complex, respectively (Fig. 4C, lanes 9 and 10). An EMSA complex with the msx2 probe was also observed for the purified Msx2 protein (Fig. 4C, lane 12).

Neither of the various Msx2 proteins nor the luciferase control protein could bind to the amel probe that contained a C/EBPα binding site instead of a Msx2 cognate site (Fig. 4C, lanes 2–5 and 11). The EMSA analyses indicated that Msx2 protein was able to interact with the binding of C/EBPα to the mouse amelogenin promoter in a dose-dependent manner. Given that the fact Msx2 protein itself did not bind to the mouse amelogenin promoter, the observed interference appeared to result from protein-protein interaction instead of competition for binding to overlapping cognate sites on the promoter. Deletion of either amino- or carboxyl-terminal domain of Msx2 only had a modest effect on the ability of Msx2 to interfere with the binding of C/EBPα to the amelogenin promoter in the EMSA analyses (Fig. 4A). However, the same deletions resulted in a dramatic decrease in the antagonistic potency of Msx2 on C/EBPα in the transfection studies (Fig. 2).

Msx2 interacted with C/EBPα in LS8 Cells—To investigate whether Msx2 could interact with C/EBPα, a co-immunoprecipitation analysis was performed in LS8 cells. A C/EBPα expression plasmid was cotransfected into LS8 cells with an empty vector, Msx2FL, Msx2ΔN, and Msx2ΔC expression plasmid, respectively. Comparable amounts of C/EBPα protein were expressed in all four transfected cell populations (Fig. 5, lanes 1–4, panels III and IV). The C/EBPα protein was co-immunoprecipitated efficiently with Msx2FL (Fig. 5, lane 2, panel I) but to a lesser extent with Msx2ΔN (Fig. 5, lane 3, panel I). However, C/EBPα protein was barely detected in the Msx2ΔC-containing immunocomplex (Fig. 5, lane 4, panel I). A similar amount of FLAG-tagged Msx2 protein was present in each immunoprecipitated complex (Fig. 5, lanes 2–4, panel II), whereas no protein was detected in empty vector-transfected cells (Fig. 5, lane 1, panel II) using the same monoclonal anti-FLAG antibody (M2Ab) as that in the immunoprecipitation process. The reciprocal experiment was also performed, in which immunoprecipitation with a C/EBPα-specific antibody was followed by Western blot analysis using the anti-FLAG M2Ab (data not shown). The Msx2FL protein was readily detected in the C/EBPα-containing immunocomplex and so was Msx2ΔN to a less extent. However, the migration rate of the C/EBPα-specific antibody light chain was very close to that of Msx2ΔC in SDS-polyacrylamide gel electrophoresis, thereby compromising the detection of Msx2ΔC band in Western blot analyses. These data indicated that Msx2 interacted with C/EBPα at the protein level in LS8 cells. Moreover, the carboxyl-terminal domain (residues 183–267) of Msx2 was required, whereas the amino-terminal domain (residues 2–97) of Msx2 contributed somehow to the interaction between Msx2 and C/EBPα protein. Taken together with the transfection data in Figs. 2 and 3, the carboxyl-terminal domain of Msx2 was indispensable for the repressive effect as well as the antagonism between Msx2 and C/EBPα on the promoter activity of the mouse amelogenin gene, whereas the amino-terminal domain of Msx2 played a less critical role.

Functional Antagonism between Msx2 and C/EBPα Was Observed on the 2.2-Kilobase Mouse Amelogenin Promoter in the Context of the Chromosome—Several stable cell lines were...
established by stably transfecting the amelogenin-promoter reporter construct p2207 into LS8 cells. The basal level of luciferase activity in these stably transfected cells was 1,000- to 10,000-fold of that in the parental LS8 cells (data not shown), indicating that the 2.2-kilobase mouse amelogenin promoter in the context of chromosome was very efficient in directing the expression of the reporter gene luciferase. LS8/p2207, one of the cell lines with the highest basal luciferase activity, was selected for further studies on the Mx2- and C/EBPα-mediated regulation of the mouse amelogenin promoter. Transient transfection of a C/EBPα expression plasmid into LS8/p2207 cells resulted in a 3–4-fold increase in the reporter gene activity (Fig. 6A, lane 2), whereas little effect on the basal promoter activity was observed for each of the Mx2 constructs alone (Fig. 6A, lanes 3–5). Furthermore, cotransfection of Mx2FL with C/EBPα was able to decrease the C/EBPα-mediated transcriptional depression from 3.3-fold to 2-fold of the basal activity, whereas either Mx2ΔN or Mx2ΔC interfered little with C/EBPα function (Fig. 6A, lanes 6–8).

In our transient transfection experiments, 20–30% transfection efficiency was consistently achieved. In other words, 70–80% of the LS8/p2207 cells assayed for luciferase activity were not transfected with an expression plasmid, but these nontransfected cells still contributed to the background signal. To circumvent this problem, FACS analyses were performed. A β-galactosidase expression plasmid pCMV-lacZ was included in each transfection. After incubation with a fluorescent β-galactosidase substrate, di-β-d-galactopyranoside, cells containing the plasmid pCMV-lacZ were sorted with flow cytometry and assayed for luciferase activity. Given the fact that pCMV-lacZ only comprised 5% of the total amount of the plasmid DNA in each transfection, the β-galactosidase positive cells should also contain the plasmid of interest (Mx2 or C/EBPα). A 6–7-fold increase and 75% decrease in reporter gene activity were observed for C/EBPα and Mx2FL, respectively. Furthermore, cotransfection of equal amounts of C/EBPα and Mx2FL expression plasmids gave rise to a 3-fold increase in reporter gene activity (Fig. 6B). Therefore, the 2.2-kilobase mouse amelogenin promoter in the chromosome context was responsive not only to the C/EBPα-mediated activation and Mx2-mediated repression but also to the functional antagonism between C/EBPα and Mx2.

Little Functional Interaction with Mx2 Was Observed for Two Other C/EBP Family Members—To determine whether Mx2 is also able to interact with other C/EBP family members in regulating the mouse amelogenin promoter activity, C/EBPβ and C/EBPγ expression plasmid was transfected into LS8 cells together with a Mx2 expression plasmid, respectively. The amelogenin-promoter construct p2207 or p70 was used as a reporter in this study. C/EBPα not only potently activated the basal promoter activity but also efficiently overcame the Mx2-mediated transcriptional repression of p2207 (Fig. 7A, lanes 1–6) or p70 (Fig. 7B, lanes 1–6). C/EBPβ by itself weakly activated the basal promoter activity of the reporter construct, and an increasing amount of C/EBPβ modestly antagonized Mx2 repression of p2207 (Fig. 7A, lanes 7–12) or p70 (Fig. 7B, lanes 7–12). C/EBPγ had little effect on either the basal promoter activity or the Mx2-mediated transcriptional repression of p2207 (Fig. 7A, lanes 13–18) or p70 (Fig. 7B, lanes 13–18). These cotransfection data indicated that C/EBP family members functioned differentially in the regulation of the mouse amelogenin promoter. Furthermore, only C/EBPα, but not C/EBPβ or C/EBPγ, was able to efficiently antagonize the repressive effect of Mx2 on the amelogenin gene in the functional analyses.

**FIG. 6.** Functional antagonism between Mx2 and C/EBPα on the mouse amelogenin promoter in a stable cell line LS8/p2207. The reporter construct p2207 was transfected into LS8 cells to establish a stably transfected cell line LS8/p2207, in which the reporter gene luciferase is constitutively expressed at high level under the control of the 2.2-kilobase mouse amelogenin promoter. A, LS8/p2207 cells in 12-well plates were transfected, respectively, with equal amounts (500 ng) of empty vector (vector), C/EBPα expression plasmid (C/EBPα) and Mx2 expression plasmid (Mx2FL, Mx2ΔN, and Mx2ΔC), or cotransfected with C/EBPα and Mx2 expression plasmid together (C/EBPα+Mx2FL, C/EBPα+Mx2ΔN, and C/EBPα+Mx2ΔC). pCMV-lacZ was used as an internal control for transfection efficiency. The relative luciferase activity is the normalization of luciferase activity with β-galactosidase activity. B, LS8/p2207 cells in 60-mm dishes were cotransfected with pCMV-lacZ (0.2 μg) and expression plasmids for C/EBPα, Mx2FL, or C/EBPα plus Mx2FL as described under “Materials and Methods.” The β-galactosidase positive cells were sorted by FACS analysis and subjected to luciferase assay. The relative luciferase activity is the normalization of luciferase activity with protein concentration of the cell lysate. The mean ± S.D. from three independent experiments is represented, and the level of luciferase activity in the presence of empty vector was set as 1.

**DISCUSSION**

The Mx2-mediated Transcriptional Repression of the Mouse Amelogenin Gene Is DNA Binding Independent—We tested the hypothesis that Mx2 is involved in the regulation of mouse amelogenin gene expression. Amelogenin expression is ameloblast specific and spatiotemporally regulated during tooth development. The transcription of amelogenin is restricted to inner enamel epithelial cells that undergo terminal differentiation to the ameloblast phenotype (6–8). In transgenic mouse analyses, the same 2.2-kilobase mouse amelogenin promoter as that used in the p2207 construct is able to recapitulate the spatiotemporal expression pattern of the endogenous amelogenin gene (10). Furthermore, the minimal promoter of the mouse...
amelogenin gene has been identified, in which a 19-nucleotide stretch (~71~52 region) is required for the basal promoter activity (14).

The expression patterns of Msx2 during initiation and development of the murine teeth have been identified with in situ hybridization (30). In molar teeth, the expression pattern of Msx2 changes with the differentiation of each germ layer dependent manner (Fig. 1). Msx2 expression is not DNA binding mediated. First, there is no Msx2 consensus binding site in the mouse amelogenin minimal promoter that is effectively repressed by Msx2. Second, Msx2 itself does not bind to the amelogenin minimal promoter as shown in Fig. 4C. The repression is most pronounced on the full-length promoter (p2207) and decreases gradually with the shortening of the promoter. This is consistent with the observation that the longer promoter constructs have lower basal activity than the minimal promoter does, which may be because of the endogenous Msx2 protein expressed in LS8 cells. Msx2 transcripts are detected in LS8 cells with reverse transcriptase-polymerase chain reaction (data not shown), and very low levels of endogenous Msx2 protein are expressed in LS8 cells as detected by an antipeptide antibody specific for Msx2 (Fig. 1B). During odontogenesis, Msx1 is expressed in all stages in dental mesenchymal cells but not in epithelial cells (28, 29). In cotransfection assays, Msx1 can repress amelogenin-promoter reporter constructs only modestly in ameloblast-like LS8 cells (data not shown).

Functional Antagonism between Msx2 and C/EBPα Results from Protein-Protein Interaction—Msx2ΔC, the carboxyl-terminal deletion form of Msx2, interacts very poorly with C/EBPα in vitro, evidenced by the cotransfection and immunoprecipitation analyses in LS8 cells. However, Msx2ΔC is able to interfere with the binding of C/EBPα to its cognate site on the mouse amelogenin minimal promoter in vitro, although less potently than Msx2FL, the full-length protein. Residues 182~193 in murine Msx2 consist of homeodomain helix 3 that is the DNA recognition helix. The nuclear localization signal of Msx2 is overlapped with homeodomain helix 3. Deletion of residues 183~267, as in Msx2ΔC, not only results in the loss of DNA binding activity but also affects nuclear localization (31). Therefore, different subcellular localization most likely accounts for the inability of Msx2ΔC to antagonize C/EBPα in cotransfection assays, with Msx2ΔC in cytoplasm and C/EBPα in nucleus. Little C/EBPα protein is co-immunoprecipitated with Msx2ΔC from a whole cell lysate of LS8 cells in which both Msx2ΔC and C/EBPα are overexpressed. The weak interaction between Msx2ΔC and C/EBPα cannot withstand the stringent washing condition in co-immunoprecipitation assays; however, the high local concentration of Msx2ΔC in gel shift analyses likely enables Msx2ΔC proteins to interfere with the binding of C/EBPα to its cognate site. Therefore, in vivo, the nuclear localization signal in Msx2 is necessary for the functional antagonism between Msx2 and C/EBPα. However, in co-immunoprecipitation assays, being accessible to each other alone is not sufficient for effective interaction between C/EBPα and Msx2 in the absence of Msx2 amino acid residues 183~267. Deletion of the amino-terminal domain (residues 2~97) of Msx2 attenuates, but does not abolish the interaction between Msx2 and C/EBPα, suggesting that Msx2 amino acid residues 2~97 play a less critical role. In the future, it will be of interest to further delineate the domains(s) responsible for the interaction between Msx2 and C/EBPα.

Msx2-mediated transcriptional repression has been extensively studied in osteoblasts. Gene repression by Msx2 is independent of the intrinsic DNA binding activity of the Msx homeodomain. Protein-protein interactions are essential to the repressive function of Msx2 (31~33). Two repressive mechanisms have been proposed, general repression and promoter-specific repression. Msx2 can bind to transcription factor F for RNA polymerase II (TFIIF), a component of the preinitiation complex, thereby repressing basal transcription (31). The DNA binding activity of other interacting partners is required to achieve promoter specificity for Msx2-dependent transcriptional repression (32~35). Dlx5, another homeodomain transcription factor, up-regulates transcription of the osteoblast-
specific osteocalcin gene through binding to its cognate site. Msx2 antagonizes the function of Dlx-5 by forming a Max2-Dlx5 heterodimer that cannot bind DNA (33). Msx2 also abrogates the induction of the osteocalcin promoter by fibroblast growth factor 2 through inhibiting a DNA binding activity to the fibroblast growth factor 2-response element without Msx2 itself binding to this element (32). However, the identity of this DNA binding activity remains unclear.

The mechanism underlying the Msx2-mediated transcriptional repression on the mouse amelogenin promoter appears to fall into the second category, in which Msx2 inhibits the DNA binding activity of C/EBPα, a transcriptional activator of amelogenin promoter. By antagonizing C/EBPα, Msx2 fulfills its role as a promoter-specific transcriptional repressor of the mouse amelogenin gene in ameloblasts.

Various transcription factors in concert with C/EBPα have been shown to synergistically activate the responsive promoters (37–42). Negative regulation of C/EBP-mediated transactivation through protein-protein interaction has also been reported (43–46). To our knowledge, Msx2, a homeodomain protein, is the first nonbasic region leucine zipper protein identified to date that functions as a transcriptional repressor through its interaction with C/EBPα protein. However, the nature of the interaction remains to be delineated. Our data demonstrate that the interaction between C/EBPα and Msx2 requires the carboxyl-terminal domain of Msx2, although the interaction is not mediated by the binding of Msx2 to the mouse amelogenin minimal promoter. Notably, this domain contains the third helix of the homeodomain, which is responsible for the binding of Msx2 to its cognate sites.

Transcription factors of the nuclear factor-κB families have been reported to have interacted directly with C/EBPβ via the Rel homology domain of nuclear factor-κB and the basic leucine-zipper domain of C/EBPβ (47, 48). The glucocorticoid receptor, transcription factor v-myb or Sp1, interacts directly with C/EBPβ, resulting in synergistic activation of the target genes (49–51). Among these factors, Sp1 synergizes with C/EBPβ but not with C/EBPα, suggesting that the C/EBP family members may interact differentially with Msx2. In the present study, Max2 preferentially interacts with C/EBPα but not with either C/EBPβ or C/EBPγ in the regulation of the mouse amelogenin promoter activity.

A previous study has demonstrated an important role for C/EBPα in regulating the mouse amelogenin promoter (14). A C/EBP binding site (−70 to −61) is located at the minimal promoter of the mouse amelogenin gene and cotransfection of a C/EBPα expression plasmid transactivates amelogenin-promoter reporter constructs in a cell type-specific manner. Mutation or deletion of the C/EBP site within the amelogenin promoter not only results in the loss of C/EBPα-mediated transactivation but also abolishes the basal promoter activity (Fig. 1A (14)). Furthermore, both the endogenous amelogenin gene and the stably transfected amelogenin-promoter reporter construct (p2207) in ameloblast-like LSS cells are responsive to the transactivation mediated by C/EBPα (Fig. 6 (14)). These data indicate that C/EBPα is likely to play a critical role in regulating ameloblast-specific expression of the amelogenin gene.

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 (52–66). In liver and adipose, peak levels of C/EBPα mRNA are detected only in differentiated tissues (67, 68). C/EBPα functions as a transcriptional activator in adipocytes, and the accumulation of C/EBPα late in preadipocyte differentiation is correlated to the expression of differentiation markers (69–71).

Msx2 has been demonstrated to regulate cellular proliferation and differentiation during development (72–75). Msx2 prevents differentiation and stimulates proliferation in primary cultured chick calvarial osteoblast (72). In transgenic mouse analyses, enhanced expression of Msx2 transiently inhibits osteoblast differentiation. As a consequence, the increase in osteoblast precursors in growth centers of the developing skull results in augmented bone growth and ultimately craniosynostosis (73). Tissue-specific gene expression during development has been shown to be regulated by Msx2. For example, Msx2 has been suggested to repress the expression of osteocalcin gene in the craniofacial skeleton at stages immediately preceding odontoblast and osteoblast terminal differentiation (76). It is conceivable that Msx2 may function in an analogous way to regulate the amelogenin gene during ameloblast differentiation.

In summary, we demonstrate that the functional antagonism between Msx2 and C/EBPα results from the Msx2-mediated interference with the binding of C/EBPα to its cognate site on the mouse amelogenin minimal promoter. Protein-protein interaction rather than competition for overlapping binding sites are responsible for the observed antagonism. Furthermore, the carboxyl-terminal residues 183–267 of Msx2 are required for the interaction, whereas the amino-terminal residues 2–97 play a less critical role. These data, together with the identification of C/EBPα as a transactivator of amelogenin gene in a previous study (14), support our interpretation that Msx2-mediated repression and C/EBPα-mediated activation operate in concert to regulate the spatiotemporal expression of amelogenin gene during tooth development.

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Functional Antagonism between Msx2 and CCAAT/Enhancer-binding Protein α in Regulating the Mouse Amelogenin Gene Expression Is Mediated by Protein-Protein Interaction

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