Dentin sialophosphoprotein (DSPP), an important odontoblast differentiation marker, is necessary for tooth development and mineralization. Bone morphogenetic protein 2 (BMP2) plays a vital role in odontoblast function via diverse signal transduction systems. We hypothesize that BMP2 regulates DSPP gene transcription and thus odontoblast differentiation. Here we report that expression of BMP2 and DSPP is detected during mouse odontogenesis by in situ hybridization assay, and BMP2 up-regulates DSPP mRNA and protein expression as well as DSPP-luciferase promoter activity in mouse preodontoblasts. By sequentially deleting fragments of the mouse DSPP promoter, we show that a BMP2-response element is located between nucleotides −97 and −72. By using antibody and oligonucleotide competition assays in electrophoretic mobility shift analysis and chromatin immunoprecipitation experiments, we show that the heterotrimeric transcription factor Y (NF-Y) complex physically interacts with the inverted CCAAT box within the BMP2-response element. BMP2 induces NF-Y accumulation into the nucleus increasing its recruitment to the mouse DSPP promoter in vivo. Furthermore, forced overexpression of NF-Y enhances promoter activity and increases endogenous DSPP protein levels. In contrast, mutations in the NF-Y-binding motif reduce BMP2-induced DSPP transcription. Moreover, inhibiting BMP2 signaling by Noggin, a BMP2 antagonist, results in significant inhibition of DSPP gene expression in preodontoblasts. Taken together, these results indicate that BMP2 mediates DSPP gene expression and odontoblast differentiation via NF-Y signaling during tooth development.

Tooth development involves sequential and reciprocal interaction between dental epithelial and mesenchymal cells. The formation of dentin or dentinogenesis originates from the neural crest-derived mesenchymal cells and proceeds in a series of cytodifferentiation stages to form odontoblasts in a specific spatial and temporal pattern originating at the principal cusp tip and advancing toward the base of the tooth (1–3). A consequence of odontoblast cytodifferentiation is the expression of specific gene products that form the dentin extracellular matrix. Dentin extracellular matrix is composed of the inorganic components with mostly hydroxyapatite (about 70%) and the organic matrix that consists of collagenous and noncollagenous proteins (NCPs). Among the NCPs, dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) are expressed predominantly in odontoblasts and transiently in preameloblasts. DSP accounts for 5–8% of the NCPs with high carbohydrate and sialic acid levels, whereas DPP is the principal dentin matrix protein with about 50% of the NCPs. DSP, with high levels of aspartic acid and phosphoserine, is believed to be a nucleator or modulator of function related to dentin mineralization and hydroxyapatite crystal formation (6–8).

DSP and DPP have been shown to be cleavage products of a primary transcript precursor encoded by a single gene termed dentin sialophosphoprotein (DSPP) (9–13). The DSP domain is found at the NH2 terminus (exons 1–4 and the 5′ region of exon 5), and the DPP sequence is located at the COOH portion (remainder of exon 5). Recently, it has been reported that DSPP extracted from porcine teeth consists of three dentin matrix proteins as follows: DSP, DPP, and dentin glycoprotein, which is located between DSP and DPP domains (14). DSPP is expressed predominantly in odontoblasts and transiently in preameloblasts (4, 5). Heterogeneous mutations of human DSPP are associated with dentinogenesis imperfecta type II (DGI-II), type III (DGI-III), and dentin dysplasia type II (DD-II) (15–19). Biological function of DSPP during tooth development has been confirmed by DSPP knock-out mice (20). DSPP

2 The abbreviations used are: NCP, noncollagenous protein; BMP2, bone morphogenetic protein 2; qRT-PCR, quantitative real time PCR; DSPP, dentin sialophosphoprotein; DSP, dentin sialoprotein; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CHX, cycloheximide; ALP, alkaline phosphatase; MAPK, mitogen-activated protein kinase; DPP, dentin phosphoprotein; TGF-β, transforming growth factor β; BSP, bone sialoprotein; nt, nucleotide; E, embryonic day; PN, postnatal day.
homologous deficient mice show teeth similar to human DGI-III. These data indicate that DSPP plays a crucial role in tooth development and mineralization, in particular dentinogenesis, and is one of the key markers during odontoblast differentiation.

Although an atlas of tooth morphogenesis has been elucidated in great detail (1–3), the molecular mechanisms regulating dental cell differentiation, in particular odontoblast differentiation, have not been completely understood. Differentiation of odontoblasts starts at the bell stage of tooth formation and is regulated by several transcription and growth factors that are expressed by odontoblasts during tooth development (1–3, 6, 7). The bone morphogenetic proteins (BMPs) are structurally related to the transforming growth factor β (TGF-β) superfamily and were originally identified by their capacity to induce ectopic bone formation in rodents (21, 22). Members of the BMP family have diverse biological functions during embryonic development (23, 24), including a vital role in osteogenesis (25, 26). Among the BMP family members, BMP2 has been extensively studied for its various biological functions, particularly during chondrogenic and osteogenic differentiation (27, 28). BMP2 is expressed in mesenchymal cells (29, 30) and promotes pluripotent mesenchymal cell commitment to the osteoblast lineage by regulating transcription factors such as Runx2/Smad cascade (31–35) and others (36, 37).

During tooth development, BMP2 is initially expressed in the oral epithelial cells at embryonic day 13 (E13), and at later stages of tooth development its expression shifts to the mesenchymal dental papilla and becomes more intense with dentinogenesis (38–40). Thus, BMP2 is involved in specifying the fate of the dental mesenchymal cells. DSPP expression was observed in odontoblasts at the bell stage and continuously through later stages in mouse teeth by in situ hybridization assay (4, 5, 7). Compared with other tissues such as bone, DSPP is highly expressed in odontoblasts, suggesting its important biological functions during odontogenesis. Although bone and dentin have common characteristics and originate from mesenchymal cells, the molecular mechanisms regulating odontoblast differentiation, particularly in the later stages of tooth development, are different from osteoblasts (4–7).

NF-Y (also known as CBF and CP1) is a heterotrimeric transcription factor that binds to the CCAAT motif found in the promoter of many eukaryotic genes (41, 42). NF-Y is composed of three subunits, NF-YA, NF-YB, and NF-YC. NF-YB and NF-YC core regions are homologous in sequence to the histone-fold motif of histones H2B and H2A, respectively. They are required for heterodimerization, a prerequisite for NF-YA association and CCAAT binding (43, 44). NF-Y interacts with other transcription activators or co-activators (45–50). NF-Y is involved in expression of many genes (51–54) such as collagen (51, 52). Although NF-Y was previously considered to be a constitutive transcription factor, recent studies have found that NF-Y is a serum regulatory protein (55) and responsive to growth factor stimulation such as TGF-β via mitogen-activated protein kinase cascades (56) and interleukin-6 signal pathway (57). The knock-out of NF-YB gene in mice leads to early embryonic lethality and blocks cell proliferation and differentiation (58).

Previously, we and others showed that a functional inverted CCAAT motif in the proximal promoter of mouse and rat DSPP genes and mutations of the CCAAT box resulted in a dramatic decline of DSPP transcriptional activity in mouse odontoblast cell lines (54, 59). In this study, we report that NF-Y is a BMP2 signaling target. BMP2 treatment increased NF-Y expression and led to localization of NF-Y into the nucleus of mouse preodontoblasts. Nuclear accumulation of NF-Y correlated with enhanced endogenous NF-Y recruitment to DSPP promoter in vivo and increases its transcriptional activity. Forced overexpression of NF-Y up-regulated DSPP promoter activity and induced endogenous DSPP expression levels. This study demonstrates for the first time that BMP2 stimulated DSPP expression in mouse preodontoblasts and committed odontoblast differentiation through NF-Y signaling.

**EXPERIMENTAL PROCEDURES**

*Animals and Tissue Preparation—*All experimental procedures involving the use of animals were reviewed and approved by the Institutional Animal Care at the University of Texas Health Science Center at San Antonio. ICR mice were purchased from Harlan Laboratory Animals Inc. (Indianapolis, IN). Mouse tissues from different ages were harvested and prepared for in situ hybridization assay and RNA isolation.

*Cell Culture and Treatment—*MD10-F2 is a mouse immortalized preodontoblast cell line (60). The cells were grown to confluence in 60-mm tissue culture dishes at 33 °C under 5% CO₂ in α-minimum essential medium with 10% fetal calf serum, 100 units/ml penicillin/streptomycin, 50 μg/ml ascorbic acid, and 10 mM sodium β-glycerophosphate (Sigma) and then cultured in α-minimum essential medium without serum and stimulated with 100 ng/ml recombinant human BMP2 in the presence or absence of cycloheximide (CHX) (10 μg/ml) (61, 62) for 0–72 h, and the expression levels of NF-Y subunit, DSP, and β-actin proteins were analyzed by Western blot analysis. BMP2 and Noggin, a BMP2 inhibitor, were purchased from R & D Systems, Minneapolis, MN. CHX was obtained from Sigma.

*Plasmid DNAs—*For all plasmid constructions, standard recombinant DNA technologies were performed as described previously (54). Briefly, the reporter construct p1243, the 5’ to 3’ BglII and HindIII fragment between nt −1,243 and +54 of mouse DSPP gene promoter, was subcloned into the 5’ to 3’ BglII and HindIII sites of pGL-3 basic luciferase vector (Promega, Madison, WI). The p791 was similarly generated from a fragment between nt −791 and +54 containing XhoI and HindIII sites, the p241 from a 295-bp KpnI and HindIII fragment between nt −241 and +54, p97 from a 151-bp BamHI and HindIII fragment between nt −97 and +54, and p72 from a 126-bp XbaI and HindIII fragment between −72 and +54. All the various plasmids contained part of the exon 1 noncoding region. Mutant plasmid, p97mut, was generated by site-specific mutagenesis of p97 DNA construct as a template using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutant oligonucleotides designated for the individual sites (mutated bases in lowercase) were as follows: 97mut, 5’-CAGGGATCCATAGCGAaaATcGgTGAGAA-AATTAT-3’ and 5’-ATAATTTCCTACgctATttCTGCTT-
AGGATCCCTG-3’. All corrective constructs were verified by DNA sequencing. The cDNAs of NF-YA, NF-YB, and NF-YC subunits were generously provided by Dr. Sankar N. Maity (Department of Molecular Genetics, University of Texas M.D. Anderson Cancer Center, Houston). For the generation of the expression constructs, the full length of NF-YA cDNA was digested with EcoRI and XhoI and then subcloned into the EcoRI and XhoI sites of the pcDNA 3.1 vector (Invitrogen). Full-length cDNAs of NF-YB and NF-YC were released with NdeI and XhoI, respectively, and ligated into the NdeI and XhoI sites of the pcDNA 3.1 (43). All constructs were confirmed by DNA sequencing.

Transient Transfection Assay—For transient transfection studies, wild-type or mutant reporter plasmid or empty pGL-3 basic plasmid was co-transfected with pRL-TR (Promega) into MD10-F2 cells using the Lipofectamine Plus reagent (Invitrogen). Two days post-transfection, the cells were deprived of serum for 12 h, and 100 ng/ml of recombinant BMP2 was added for 12 h prior to harvesting. To inhibit the BMP2 effect, different doses of Noggin (5–20 μg/ml) were added to the serum-free medium prior to addition of BMP2. Luciferase activity was measured as described previously (54). The data are expressed as the means ± S.E. from at least three separate experiments performed in triplicate.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts from MD10-F2 cells were prepared using the method of Dignam et al. (63). Protein concentration was determined by using the Bradford assay (64). For the EMSA, the double-stranded oligonucleotides were labeled with [γ-32P]ATP and T4 polynucleotide kinase and purified on a 15% polyacrylamide gel. EMSA was performed as described previously (54). For the competition binding reactions, the unlabeled competitor in different fold molar excesses of the labeled probe was included in the reaction. Free and protein-bound DNA complexes were loaded onto a 5% native polyacrylamide gel in 1× Tris/boric acid/EDTA (TBE) buffer, electrophoresed, dried, and exposed to x-ray film. Antibody supershift experiments were performed with specific antibodies to NF-YA, NF-YB, and NF-YC subunits (Santa Cruz Biotechnology Inc., Santa Cruz, CA). This antibody was added to the nuclear extracts 10 min prior to the addition of the radiolabeled probe. All oligonucleotides used in EMSA were obtained from Santa Cruz Biotechnology Inc. (Table 1) and corresponded to consensus DNA-binding sites for each transcription factor.

In Situ Hybridization—Preparation of BMP2 and DSPP probes and details of the in situ hybridization procedures were performed as described earlier (65).

RT-PCR and Quantitative Real Time PCR—Total RNA from MD10-F2 cells and mouse molars at 2, 10, and 13 weeks postnatal was isolated using RNA STAT-60 kit (Tel-Test, Inc., Friendswood, TX), treated with DNase I (Promega, Madison, WI), and purified with the RNaseasy mini kit (Qiagen Inc., Valencia, CA). RNA concentration was determined at A260. The cDNA synthesis and PCR amplification were performed using standard protocols (66). For detection of each NF-Y subunit and β-actin in MD10-F2 cells and tooth tissues, specific sets of primers were designed for each NF-Y subunit and β-actin as follows: NF-YA, forward 5’-AGTCAGTGAGGCGCCAGCTTA-3’ and reverse 5’-ATGAT-CTGCTGGTTTGACC-3’; NF-YB, forward 5’-ATCAGGA-GAAGCGGAAGAC-3’ and reverse 5’-CCGTGACGTCTTCT-TGTTAG-3’; NF-YC, forward 5’-CAAAGCCTGCTGCTCCT-GTCTCCATTAATCCATG-3’; and β-actin, forward 5’-CATCACTATGGCAGACGAC-3’ and reverse 5’-ACTCATCGTACTCTGGTGT-3’. The PCRs were performed as follows: 2 min at 94 °C for 1 cycle, 1 min at 94 °C, 30 s at 58–60 °C, and 1 min at 72 °C for 30 cycles followed by 7 min at 72 °C. After PCR amplification, 10 μl of PCR products were loaded on a 1.5% agarose gel for electrophoresis, stained with ethidium bromide, viewed under ultraviolet light, and photographed by a graphic image (Alpha Annotech Corp., San Leandro, CA). For quantitative real time PCR (qRT-PCR), amplification reactions were analyzed in real time on an ABI 7500 (Applied Biosystems, Foster City, CA) using SYBR Green chemistry, and the threshold values were calculated using SDS2 software (Applied Biosystems) as described earlier (5). The primer sequences used for qRT-PCR were as follows: cyclophilin, forward 5’-GGTGACTCTACACGCCATAA-3’ and reverse 5’-CATGGCCTCCACAATATTCA-3’; DSPP, forward 5’-AATCTGTGGTGCCGTCTCT-3’ and reverse 5’-TATTGACTCGGAGCCATTC-3’. Primers of each NF-Y subunit were described above.

Immunohistochemistry—Mouse MD10-F2 cells were cultured on glass slides and treated with or without 100 ng/ml of recombinant BMP2 (R & D Systems Inc.) at different time points. The cells were rinsed twice with ice-cold PBS and fixed for 10 min at room temperature with cold acetone and methanol (1:1). For quantitative detection of expression of DSPP and NF-Y subunit proteins in MD10-F2 cells, fluorescent immunohistochemistry was performed using antibodies directed against mouse DSP (Alpha Diagnostic International, San Antonio, TX). The anti-DSP antiserum was raised in rabbit (Alpha Diagnostic International) against a recombinant fragment representing the NH2-terminal portion (residues Ile18–Lys371) of mouse DSP (9). High titer polyclonal antiseras as measured by enzyme-linked immunosorbent assays (ELISA) were obtained and then further purified by affinity column. Antibodies against NF-YA, NF-YB, and NF-YC were purchased from Santa Cruz Biotechnology, Inc. Mouse IgG1 (X0931) served as a negative control (Dako, Carpinteria, CA). The samples were incubated overnight at 4 °C with a 1:100 dilution of primary antibodies and then washed with 1× PBS, followed by the secondary antibodies with Alexa Fluo® 488 (Molecular Probes, Eugene, OR). Images of Alexa Fluo® 488 staining of the various proteins in cultures were obtained at the Core Optical Imaging Facility at The University of Texas Health Science Center, San Antonio, under the same parameters in an Olympus wide field microscope and quantitated by means of MetaMorph software (Universal Imaging Corporation, West Chester, PA). For each experiment, all slides were simultaneously processed for a specific antibody, so that homogeneity in the staining procedure was ensured between samples. After the images were captured at the same magnification, the threshold was set and maintained for each slide. The optical density was calculated by use of the morphometric analysis within the software package.

Western Blot Analysis—Western blot analysis was performed on whole-cell lysates from MD10-F2 cells with either tran-
siently transfected 5 μg of NF-Y subunit expression plasmids or a mock pcDNA 3.1 vector. After 72 h, cells were washed with 1× cold PBS and lysed with RIPA buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml phenylmethylsulfonyl fluoride, 30 μM aprotinin, 100 mM sodium orthovanadate). Proteins (40 μg/well) were resolved by 10% SDS-PAGE and transferred to a Trans-Blot membrane (Bio-Rad). For the detection of mouse DSP protein, Western blotting assay was performed as described earlier (5). As a loading control, goat anti-mouse β-actin antibody was used (Santa Cruz Biotechnology, Inc.).

Chromatin Immunoprecipitation (ChIP)—ChIP was performed according to the instructions provided by ChIP-IT™ kit (Active Motif, Carlsbad, CA). Briefly, MD10-F2 cells treated or untreated with BMP2, 100 ng/ml, were washed in PBS and incubated for 10 min with 1% formaldehyde. After quenching the reaction with 0.1 M glycine, the cross-linked material was sonicated into chromatin fragments of an average length of 200–800 bp. After pre-cleaning, 1% of each sample was saved as input fraction. Immunoprecipitations were performed with protein G beads and 5 μg of the indicated antibodies as follows: anti-NF-YB goat polyclonal and anti-TFIIB as a positive control (Santa Cruz Biotechnology Inc.). A negative control was provided by this kit. The chromatin solution was precleared by adding protein G beads with salmon sperm DNA for 2 h at 4 °C. Immunoprecipitated material was washed, and cross-links were reversed by incubating samples for 5 h at 65 °C in 200 mM NaCl and 10 μg of RNase A to eliminate RNA. Recovered material was treated with proteinase K, and the DNA fragment was purified by QiaGen columns (Qiagen Inc.). The purified DNA was amplified by PCR using the following primer pairs: mouse DSPP, forward 5′-CACACTGACTCTCAATCAAGG-3′ and reserve 5′-TCGAGGGACCTTGGAAAT-3′; mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5′-CCATGGAGAACGTGGG-3′ and reverse 5′-CAGAGTTCATGGAAGGACC-3′. PCR condition was 95 °C for 4 min, 95 °C for 30 s, 58 °C for 40 s, 72 °C for 2 min for 32 or 36 cycles.

Alkaline Phosphatase Analyses—Quantitative alkaline phosphatase (ALP) activity of the cell lysates was measured using the BCA protein assay reagent (Pierce). The enzyme activity was expressed as nanomoles of p-nitrophenyl phosphate as a substrate (67). Protein concentration was determined using the BCA protein assay reagent (Pierce) and aprotinin, 100 mM sodium orthovanadate.

Data Analysis—All values are expressed as means ± S.E. Analysis of variance with Student’s t test was used to determine significant differences in the control and treated groups.

RESULTS

BMP2 Stimulates DSPP Expression in MD10-F2 Cells—To study DSPP regulation by BMP2, we used a mouse odontoblast cell line called MD10-F2, as these cells have been shown to have odontoblast characteristics and express DSPP (5). Previous studies indicate that BMP2 (100 ng/ml) was sufficient to induce bone sialoprotein (BSP) gene expression in mouse dental periodontal ligament and follicle cells (36). Like BSP, DSPP belongs to the small integrin-binding ligand N-linked glycoprotein (SIBLING) gene family (68). Therefore, BMP2 (100 ng/ml) was added to stimulate MD10-F2 cells. DSPP gene expression in MD10-F2 was measured by qRT-PCR and fluorescent immunohistochemistry (Fig. 1). When MD10-F2 cells were exposed to BMP2, DSPP mRNA expression was increased 3.7-, 2.7-, 2.4-, and 1.7-fold at 12, 24, 48, and 72 h, respectively (Fig. 1, A and B).

Quantitative immunofluorescent assay showed that BMP2 treatment induced not only DSPP protein expression within the cytoplasm but also led to accumulation of DSPP protein in the nucleus (Fig. 1C). The maximal DSPP protein expression level in MD10-F2 cells was at 24 h after BMP2 stimulation.

Identification of a BMP2-response Element in the Mouse DSPP Gene Promoter—To assess whether the BMP2 effect of DSPP expression is mediated by the cis-regulatory region of the mouse DSPP gene, various promoter construct fragments were generated and ligated to a luciferase reporter gene (Fig. 2A). These DNA constructs were transiently transfected into MD10-F2 cells, and their transcriptional activities were measured in the presence or absence of BMP2 (Fig. 2B). Cells transfected with the reporter construct, p1243, encompassing nucleotides −1,243 and +54, gave a 1.6-fold transcriptional activity after 12 h of treatment with BMP2 compared with transfecants without BMP2 (Fig. 2B). BMP2 also gave 1.9- and 1.8-fold increases of promoter activity in cells transfected with p791 (−791 to +54) and p241 (−241 to +54), respectively. The strongest BMP2-response element was located in p97 between nt −97 and +54, resulting in a 3.2-fold increase in promoter activity. However, cells transfected with p72 (nt −72 and +54) was unresponsive to BMP2 stimulation. Therefore, these results indicate that BMP2-response element resided within a 25-bp region between nt −97 and −72 in the mouse DSPP promoter.

Binding of NF-Y to BMP2-response Element in the Mouse DSPP Promoter—To identify transcription factors that bind to the BMP2-response element between nt −97 and −72, a double-stranded oligonucleotide (Table 1) was used as an EMSA probe. Nuclear extracts from MD10-F2 cells formed a protein-DNA complex with the wild-type probe (Fig. 3A, lane 1). Computer analysis of this DNA sequence shows that this element contains a number of binding sites for known transcription factors. These include AP-1 (fos/jun), NF-kB, GATA, GT-1, AP-3, NF-Y, CP2, and CCAAT/enhancer-binding protein (C/EBP) (Fig. 3B). Based on this observation, we performed a series of competitive EMSAs (Fig. 3A). The result shows that the protein-DNA complex could be competed away by 50- and 100-fold molar excesses of the unlabeled homologous wild-type and NF-Y consensus oligonucleotides (Fig. 3A, lanes 2 and 3 and 10 and 11) but not with other oligonucleotides (lanes 4–9 and 12–19). To further confirm that the DNA sequence within the BMP2-response element contains an NF-Y-binding site, antibody supershift experiments were performed using three anti-NF-Y antibodies. As shown in Fig. 3D, the NF-Y binds to the inverted CCAAT box within the BMP2-response element (−97/−72). Notably, the inverted NF-Y-binding site in the mouse DSPP gene promoter is also present at the same position in rat and human DSPP gene promoters (Fig. 3C).

To test whether NF-Y interacts directly with the CCAAT box in vivo, we performed ChIP assays. Anti-NF-YB antibody-immunoprecipitated protein-DNA complexes were de-cross-
linked, and the purified DNA was used as a template for PCR, using primers corresponding to the mouse DSPP-5′-flanking region (Fig. 3E). As expected, a 181-bp PCR product was amplified from the DNA fragment immunoprecipitated by anti-NF-YB antibody (Fig. 3F, lane 5). Positive and negative controls were set up using anti-TFIIB antibody and no IgG antibody, respectively, as well as using total DNA before immunoprecipitation as the input for PCR (Fig. 3F, lanes 2–4). These results indicate that the NF-Y physically binds to the inverted CCAAT box in the mouse DSPP promoter in vivo.

Expression of DSPP, BMP2, and NF-Y during Tooth Development—In situ hybridization studies showed BMP2 mRNA expression in dental papilla at embryonic day 15 (E15) (Fig. 4B). BMP2 mRNA expression was also evident in the surrounding tissues. By comparison, DSPP mRNA was no signal at E15 (Fig. 4H) but expressed in odontoblasts and preameloblasts at the bell stage (E16) (Fig. 4J). At postnatal day (PN) 1, both BMP2 and DSPP expressions were apparent in odontoblast cells (Fig. 4, F and L). In contrast to DSPP, expression of BMP2 mRNA was more widely distributed, being detected in ameloblast and dental pulp cells. At PN3 and 2 weeks (2 weeks) after birth, the signal for BMP2 and DSPP expressions was still seen in odontoblast cells (supplemental Fig. 1). Thus, the data indicate that BMP2 expression was relevant to DSPP expression during the later stages of odontoblast differentiation and maturation. Because ALP activity is a marker of odontoblast differentiation and cell mineralization (54), we also measured ALP activity in confluent MD10-F2 cells treated or untreated with BMP2 for 3 or 6 days. The results show that BMP2 treatment led to higher ALP activity as compared with cells without BMP2 (Fig. 4M). These studies indicate that BMP2 promotes MD10-F2 cell differentiation. For NF-Y gene expression, total RNA was extracted from MD10-F2 cells and mouse tooth molars at 2, 10, and 13 weeks after birth, and mRNA expression of each NF-Y subunit was measured by RT-PCR using NF-Y subunit-specific primers. Fig. 4N shows that all three NF-Y subunits were expressed in the MD10-F2 cells and mouse tooth molars at the three ages studied.
BMP2 Regulates DSPP Expression in Odontoblasts

**BMP2 Regulates NF-Y Expression in MD10-F2 Cells**—To determine whether expression of NF-Y is regulated by BMP2, MD10-F2 cells were treated with BMP2 at various time points and then processed for qRT-PCR and fluorescent immunohistochemistry for NF-Y subunit expressions and their subcellular distributions. As shown in Fig. 5, A and B, the three NF-Y subunit mRNAs were up-regulated by BMP2. NF-YA, NF-YB, and NF-YC mRNAs peaked at 24, 12, and 12 h, respectively, after BMP2 treatment. Immunofluorescent assay was performed to visualize cellular localization of each NF-Y subunit. The results show that BMP2 increased protein expression of each NF-Y subunit within the cytoplasm and nucleus (supplemental Fig. 2, A–C). However, dynamic changes of subcellular distribution of individual NF-Y subunits differed from each other. NF-YA accumulated in the nucleus at 12 h and continued for 72 h. NF-YB nuclear localization peaked at 24 h after BMP2 treatment and clearly differed from NF-YA expression. NF-YC nuclear accumulation occurred at 24 and 48 h in MD10-F2 cells and then progressively decreased its nuclear accumulation by 72 h.

To assess whether NF-Y accumulation in the nucleus also increases NF-Y binding to its target site in the mouse DSPP promoter in vivo, we performed ChIP assay. Chromatin was prepared from MD10-F2 cells treated with or without BMP2 at given time points and immunoprecipitated with antibody specific for NF-YB. Following immunoprecipitation with NF-YB antibody and de-cross-linking, the purified DNA fragments were amplified by semi-quantitative PCR to the inverted CCAAT box of the mouse DSPP regulatory region (supplemental Fig. 3). Based on the above results, both p97wt and p97mut DSPP-luciferase reporter constructs were transiently transfected into MD10-F2 cells over 12–72-h periods by immunoblotting analysis. These results suggest that BMP2 is required for NF-Y protein synthesis in MD10-F2 cells.

**NF-Y-binding Site Is Required for BMP2-mediated DSPP Promoter Activity**—To access the CCAAT box essential for BMP2-mediated DSPP promoter activity, we generated two DSPP-luciferase promoter constructs with wild-type and mutant NF-Y-binding site (Fig. 7A). To further determine whether abrogation of BMP2 signaling pathway results in a decrease of mouse DSPP expression, we transiently transfected p97wt and p97mut luciferase reporter constructs into MD10-F2 cells, and then their transcriptional activity was determined in the presence or absence of CHX, a protein synthesis inhibitor. Regulation of NF-Y subunits and DSPP expression by BMP2 in the presence or absence of CHX was observed for the indicated time periods in MD10-F2 cells (Fig. 6). The results showed that CHX treatment led to decreases of both basal expression and BMP2-stimulated increases of NF-Y subunit and DSPP protein levels in MD10-F2 cells over 12–72-h periods by immunoblotting analysis. These results suggest that BMP2 is required for NF-Y protein synthesis in MD10-F2 cells.

**Inhibition of BMP2 Effect on NF-Y Expression by Cycloheximide**—To better clarify the mechanism of BMP2 action, a protein synthesis inhibitor, CHX, was applied. Regulation of NF-Y subunits and DSPP expression by BMP2 in the presence or absence of CHX was observed for the indicated time periods in MD10-F2 cells (Fig. 6). The results showed that CHX treatment led to decreases of both basal expression and BMP2-stimulated increases of NF-Y subunit and DSPP protein levels in MD10-F2 cells over 12–72-h periods by immunoblotting analysis. These results suggest that BMP2 is required for NF-Y protein synthesis in MD10-F2 cells.

**TABLE 1**

EMSAs and competitions were performed with the oligonucleotide duplexes listed below.

Only the 5′ to 3′ strand of the duplexes is shown. The inverted CCAAT box is underlined. WT, wild type; mut, mutant.

### Name | Sequencea (5′ to 3′)
---|---
Wild type (WT) | 5′-GATCCCTAACGCAATGATGTTGAGAA-3′
Mutant (mut) | 5′-GATCCCTAACGACTAATTGAGAA-3′
NF-Y (WT) | 5′-GAGCCGATACCGTTTAACTTTCTCTCT-3′
NF-Y (mut) | 5′-GAGCCGATACCGATTAATTCACTCTCTC-3′
C/EBP | 5′-TCAATTGGCACCATCTCCA-3′
Ap-1 | 5′-CTGGAGGAATGACGACGCAAG-3′
NF-kB | 5′-AGTTTGGGACCTTCCCCACGCC-3′
Ap-3 | 5′-GATCTTGGGAAATCCTGACATC-3′
GATA | 5′-CACTTGGTACACAGAGAAGGTATACGCTT-3′
CP2 | 5′-GACGAGACACAAAACACCCAAA-3′
GT-1 | 5′-CTTTGTGTTGATATGCTGTC-3′

**a** Substitution mutations are represented in lowercase letters.

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**FIGURE 2. The DSPP promoter contains a BMP2-response element.** A, schematic representation of the constructs used in the luciferase (Luc) assay. B, BMP2 stimulates DSPP promoter activity. Transient MD10-F2 transfectants in the presence or absence of BMP2 (100 ng/ml) for 12 h were used to determine transcriptional activity of those chimeric constructs. The value obtained from the control group (pGL3 basic only) was taken as 1-fold, and fold increases were calculated by dividing the individual value by the control group value and plotted as a graph showing the mean ± S.E. from three independent experiments in triplicate. Significant differences comparing BMP2-treated groups with BMP2-untreated groups are shown with the following probability levels: *, p < 0.05; **, p < 0.001.

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The DSPP promoter contains a BMP2-response element. To better clarify the mechanism of BMP2 action, a protein synthesis inhibitor, CHX, was applied. Regulation of NF-Y subunits and DSPP expression by BMP2 in the presence or absence of CHX was observed for the indicated time periods in MD10-F2 cells (Fig. 6). The results showed that CHX treatment led to decreases of both basal expression and BMP2-stimulated increases of NF-Y subunit and DSPP protein levels in MD10-F2 cells over 12–72-h periods by immunoblotting analysis. These results suggest that BMP2 is required for NF-Y protein synthesis in MD10-F2 cells.
promoter activity, MD10-F2 cells were treated with BMP2 in the presence of the BMP inhibitor, Noggin (24). As shown in Fig. 7C, the effect of BMP2 on DSPP promoter activation was inhibited by Noggin in a dose-responsive fashion. These data demonstrate that BMP2-mediated DSPP gene expression through NF-Y signaling and the CCAAT box is important, not only for the NF-Y binding but also for BMP2-mediated DSPP expression in mouse preodontoblast cells.

Effect of NF-Y Overexpression on Mouse DSPP Gene Expression—To determine the effect of NF-Y on the mouse DSPP promoter activity, we examined forced NF-Y expression in MD10-F2 cells using the p97wt and p97mut reporter gene constructs. As shown in Fig. 8A, overexpression of NF-Y caused a 3.2-fold increase of p97wt promoter activity as compared with that of the mock-transfected group. However, NF-Y overexpression had no effect on the promoter activity in the p97mut construct. We then ascertained whether overexpression of NF-Y could induce endogenous DSP. MD10-F2 cells were transiently transfected with either empty or NF-Y expression vector, and Western blot assay shows that expression of DSP protein levels in NF-Y transfectants were about 3-fold higher than that of the control transfectants (Fig. 8B).
DISCUSSION

Odontoblast differentiation is regulated by many transcription and growth factors. BMPs have a prominent role in tooth development and formation. DSPP has been characterized as a unique marker of odontoblast differentiation (4, 5). To investigate the molecular mechanism of BMP2 regulation during odontoblast differentiation, we analyzed the effects of BMP2 on DSPP expression in mouse preodontoblast cells. In this study, we have identified an NF-Y transcription factor-binding site in the proximal mouse DSPP promoter that mediates BMP2-stimulated DSPP expression.

At the onset of odontoblast differentiation at E16 during mouse tooth development, DSPP gene expression gradually increases during dentinogenesis and the spatial-temporal

FIGURE 4. DSPP, BMP2, and NF-Y gene expression in tooth organs during development. A–L, in situ hybridization of mouse tooth developmental stages from E15 to PN1 with BMP2 (B, D, and F) and DSPP (H, J, and L) antisense probes. Hematoxylin was shown in A, C, E, G, I, and K. od, odontoblasts; am, ameloblasts; p, dental pulp cells; c, cartilage; eo, enamel organ; dp, dental papilla; e, enamel; d, dentin. Bar scale is 200 μm. M, effect of BMP2 on ALP activity in MD10-F2 cells. After the cells were treated with BMP2 (100 ng/ml) or without BMP2 (con) for 3 and 6 days, ALP activity was determined as described under “Experimental Procedures.” Data were means ± S.E. of three wells, significantly different from the control without BMP2. *, p < 0.001. N, RT-PCR amplification of the three NF-Y subunits were carried out. Ten μl of PCR products were analyzed on 1.5% agarose gels. Amplification products of mouse β-actin served as an internal control. M, 100-bp DNA ladder. Arrows indicate expected PCR products of the three NF-Y subunits, NF-YA, NF-YB, and NF-YC, and β-actin genes. Con, negative control; 10-F2, MD10-F2; 2 wk, 10 wk, and 13 wk indicate mouse molars at aged 2, 10, and 13 weeks after birth, respectively.
expression pattern of DSPP gene is largely restricted to dental tissues (4, 5). Mutations in the DSPP gene are associated with DGI-II and DGI-III as well as DD-II, indicating that DSPP is associated with tooth development, in particular dentinogenesis (15–20). BMP2 plays an important role in dental differentiation as well as tooth development and growth (23, 36). BMP2 signaling induces mesenchymal cell differentiation into odontoblasts (69). In this study, we chose DSPP gene expression as a paradigm to dissect the molecular mechanisms of BMP2-mediated odontoblastic differentiation. We initially examined expression of BMP2 and DSPP genes during tooth development. In situ hybridization assay revealed that during tooth development both BMP2 and DSPP gene expressions existed throughout odontoblast differentiation from E16 to 2 wk tested (Fig. 4 and supplemental Fig. 1). These observations suggest that BMP2 is relevant to DSPP gene expression and odontoblast differentiation. Moreover, in vitro study shows that BMP2 up-regulated expression of DSPP and ALP genes in MD10-F2 cells (Figs. 1 and 4M), indicating that BMP2 promoted preodontoblast differentiation. Interestingly, we observed that DSPP accumulates in the nucleus in response to BMP2 (Fig. 1C). Evidence for dentin matrix protein nuclear localization was also found for dentin matrix protein 1, a SIBLING family member (70, 71). Recently, Jadlowiec et al. (72, 73) found the DPP domain of DSPP up-regulated bone- and tooth-related genes via integrin/Smad and MAPK signaling pathways. This implies that DSPP is not only a structural protein but may play a more complicated biological role during odontogenesis. The molecular mechanisms for the nuclear accumulation of DSPP in response to BMP2 remain unknown. It has been reported that BMP2 stimulated expression of BSP and osteocalcin genes and promoted mesenchymal cell differentiation into osteoblast cells mediated through the Runx2/Smad signal transduction pathway (31–34). MAPK is also involved in response to BMP2 and regulates BSP and osteocalcin gene expressions in dental follicle and periodontal ligament cells (36). In addition, other transcription factors are known to be targeted by TGF-β/BMPs signaling (56). Therefore, we focused on testing whether BMP2 regulates DSPP expression at transcriptional levels. Various deleted fragments of the 5′-flanking mouse DSPP regulatory region were ligated upstream of the luciferase reporter gene and then transiently transfected into mouse MD10-F2 preodontoblast cells. We identified an element between nt −97 and −72 in the proximal promoter of the mouse DSPP gene responsible for BMP2 stimulation. Notably, sequence of this element is
NF-Y is a heterotrimeric protein complex that binds to the CCAAT motif found in the promoter of many eukaryotic genes (40, 41). NF-Y is composed of three subunits, NF-YA, NF-YB, and NF-YC. The A and C subunits associate through a subdomain that binds DNA and resembles the α-helical structure found in core histone proteins (43). The B subunit associates with the A/C heterodimer to form a functional CCAAT-binding complex. The transactivating activity of NF-Y is mediated by the association of the A/C heterodimer with the histone acetyltransferase P/CAF (47) and the association of the A subunit with the p300 that has an intrinsic histone acetyltransferase activity (48). It indicates that NF-Y is able to modify chromatin structure, activating its target gene expression.

Recently, NF-Y has been reported to be a regulatory protein that is responsive to several molecular stimulations (55–57). Caretti et al. (55) showed that serum up-regulated NF-Y recruitment into promoters of several cell cycle genes in NIH3T3 cells and activated their gene expressions. Interleukin-6 induced NF-YA expression and up-regulated expression of myeloid differentiation primary response (MyD) gene, promoting murine M1 myeloid leukemic cell differentiation (57). Our study shows that BMP2 up-regulated NF-Y gene expression and activated nuclear NF-Y subunit accumulation with increased binding to chromatin of CCAAT box in the mouse DSPP promoter in MD10-F2 cells (Fig. 5 and supplemental Fig. 2). NF-Y expression by BMP2 was inhibited by the protein synthesis inhibitor cycloheximide (Fig. 6), suggesting that BMP2 regulates NF-Y synthesis. Furthermore, our data demonstrate that mutating the NF-Y-binding site in the mouse DSPP promoter interrupted promoter activity in response to BMP2 stimulation (Fig. 7B). Noggin, a BMP antagonist, was able to block the BMP2 effect on DSPP-luciferase promoter activity in a dose-responsive fashion (Fig. 7C), verifying the role of BMP2 in DSPP expression through the NF-Y-binding site. NF-Y forced overexpression up-regulated DSPP-luciferase
BMP2 Regulates DSPP Expression in Odontoblasts

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REFERENCES

1. Thesleff, I. (2003) J. Cell Sci. 116, 1647–1648
2. Maas, R., and Bei, M. (1997) Crit. Rev. Oral Biol. Med. 8, 4–39
3. Milewich, I., and Sharpe, P. T. (2003) Hum. Mol. Genet. 1, R69–R73
4. D’Souza, R. N., Cavender, A., Sunavala, G., Alvarez, J., Ohshima, T., Kulikarni, A. B., and MacDougall, M. (1997) J. Bone Miner. Res. 12, 2040–2049
5. Chen, S., Rani, S., Wu, Y., Unterbrink, A., Gu, T. T., Glaubak-Heinrich, I., Chuang, H. H., and MacDougall, M. (2005) J. Biol. Chem. 280, 29717–29727
6. Linde, A., and Goldberg, M. (1993) Crit. Rev. Oral Biol. Med. 4, 679–728
7. Butler, W. T. (1998) Europ. J. Oral Sci. 106, 204–210
8. He, G., Ramachandran, A., Dahl, T., George, S., Schultz, D., Cookson, D., Veis, A., and George, A. (2005) J. Biol. Chem. 280, 33109–33114
9. MacDougall, M., Simmons, D., Luan, X., Nydegger, J., Feng, J., and Gu, T. T. (1997) J. Biol. Chem. 272, 835–842
10. Ritchie, H. H., Hou, H., Veis, A., and Butler, W. T. (1994) J. Biol. Chem. 269, 3698–3702
11. Gu, K., Chang, S., Ritchie, H. H., Clarkson, B. H., and Rutherford, R. B. (2000) Europ. J. Oral Sci. 108, 35–42
12. Yamakoshi, Y., Hu, J. C., Liu, S., Zhang, C., Oida, S., Fukae, M., and Simmer, J. P. (2003) Europ. J. Oral Sci. 111, 60–67
13. Qin, C., Cook, R. G., Orkiszewski, R. S., and Butler, W. T. (2001) J. Biol. Chem. 276, 904–909
14. Yamakoshi, Y., Hu, J. C., Fukae, M., Zhang, H., and Simmer, J. P. (2005) J. Biol. Chem. 280, 17472–17479
15. Xiao, S., Yu, C., Couch, X., Yuan, W., Wang, Y., Bu, L., Fu, G., Qian, M., Yang, J., Shi, Y., Hu, L., Han, B., Wang, Z., Huang, W., Liu, J., Chen, Z., Zhao, G., and Kong, X. (2001) Nat. Genet. 27, 34520
16. Zhang, X., Zhao, J., Li, C., Gao, S., Qiu, C., Liu, P., Wu, G., Qiang, B., Lo, W. H., and Shen, Y. (2001) Nat. Genet. 27, 151–152
17. Dong, J., Gu, T. T., Jeffords, L., and MacDougall, M. (2005) Ann. J. Med. Genet. 132, 305–309
18. Rajpar, M. H., Koch, M. J., Davies, R. M., Mellody, K. T., Kiely, C. M., and Dixon, M. J. (2002) Hum. Mol. Genet. 11, 2559–2565
19. Kim, J. W., Nam, S. H., Jang, K. T., Lee, S. H., Kim, C. C., Hahn, S. H., Hu, J. C., and Simmer, J. P. (2004) Hum. Genet. 115, 248–254
20. Streaneth, T., Thyagarajan, T., Hall, B., Longenecker, G., DaSooua, R., Hong, S., Wright, J. T., MacDougall, M., Sauk, J., and Kulikarni, A. B. (2003) J. Biol. Chem. 278, 24874–24880
21. Urist, M. R. (1965) Science 150, 893–899
22. Wozney, J. M., Ross, V., Celeste, A. J., Mitosch, L. M., Whitters, M. J., Kric, R., Hewick, R., and Wang, E. A. (1988) Science 242, 1528–1534
23. Hogan, B. L. (1996) Genes Dev. 10, 1580–1594
24. Wu, X. B., Schneider, A., Yu, W., Rajendran Iqbal, G. J., Yamamoto, M., Alam, M., Brunet, L. J., Blair, H. C., Zaidi, M., and Abe, E. (2003) J. Clin. Investig. 112, 924–993
25. Wozney, J. M., and Rosen, V. (1998) Clin. Orthop. 346, 26–37
26. Chen, D., Zhao, M., and Mundy, G. R. (2000) Growth Factors 22, 233–241
27. Reddy, A. H. (1997) Cytokine Growth Factor Rev. 8, 11–20
28. Duy, P., and Karsenty, G. (2000) Kidney Int. 57, 2207–2214
29. Vazquez, R., Shenker, B., Datar, S., and Lepor, P. S. (1998) J. Cell Physiol. 174, 331–341
30. De Luca, F. D., Barnes, K. M., Uyeda, J. A., De-Levi, S., Abad, V., Palese, T., Mericq, V., and Baron. J. (2001) Endocrinology 142, 430–436
31. Duy, P., Zhang, R., Geoffroy, V., Ridall, A. L., and Karsenty, G. (1997) Cell 89, 747–754
