Dentin sialophosphoprotein (DSPP) is an extracellular matrix protein that is cleaved into dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) with a highly restricted expression pattern in tooth and bone. Mutations of the DSPP gene are associated with dentin genetic diseases. Regulation of tissue-specific DSPP expression has not been described. To define the molecular basis of this cell-specific expression, we characterized the promoter responsible for the cell-specific expression of the DSPP gene in odontoblasts. Within this region, DNase I footprinting and electrophoretic mobility shift assays delineated one element that contains an inverted CCAAT-binding factor site and a protein-DNA binding site using nuclear extracts from odontoblasts. A series of competitive electrophoretic mobility shift assays analyses showed that the protein-DNA binding core sequence, ACCCCCA, is a novel site sufficient for protein binding. These two protein-DNA binding sequences are conserved at the same proximal position in the mouse, rat, and human DSPP gene promoters and are ubiquitously present in the promoters of other tooth/bone genes. Mutations of the CCAAT-binding factor binding site resulted in a 5-fold decrease in promoter activity, whereas abolishment of the novel protein-DNA binding site increased promoter activity by about 4.6-fold. In contrast to DSPP, expression levels of the novel protein were significantly reduced during odontoblastic differentiation and dentin mineralization. The novel protein was shown to have a molecular mass of 72 kDa. This study shows that expression of the cell type-specific DSPP gene is mediated by the combination of inhibitory and activating mechanisms.

Tooth development is a highly organized process involving complex interactions among many genes. The expression of many of these genes has been characterized during tooth development and matrix mineralization. Dentin is the principal mineralized tissue of teeth and originates from odontoblasts. A series of complex interactions among many genes. The expression of many of these genes has been characterized during tooth development and matrix mineralization. Dentin is the principal mineralized tissue of teeth and originates from odontoblasts. Among noncollagenous proteins, dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) are expressed at high levels in tooth (2, 3). DSP accounts for 5–8% of the dental ECM proteins with high levels of carbohydrate and sialic acid and has a resemblance to other sialoproteins such as bone sialoprotein, osteopontin, dentin matrix protein 1, and matrix extracellular phosphoglycoprotein. DPP is the major noncollagenous dentin ECM protein, representing about 50% of this fraction with high aspartic acid (30–40%) and phosphoserine (38–50%) content suggesting a function related to dentin mineralization. In addition, DPP has an arginine-glycine-aspartic acid (RGD) sequence functional for cell attachment (4). It is thought that the DPP is the archetype of macromolecules that might regulate biomineralization processes by binding to matrix structural proteins, nucleating mineralization, and controlling crystal growth (5–8). DSP and DPP have been shown to be cleavage products of a primary transcript precursor encoded by a single gene termed dentin sialophosphoprotein (DSP) (9). The DSP gene of mouse, rat, human, and pig has been cloned (9–12). This gene consists of five exons and four introns. The DSP domain is found at the NH₂ terminus (exons 1–4 and the 5' region of exon 5), whereas the DPP sequence is located at the COOH region (remainder of exon 5) (9, 12–14). DSP is expressed predominantly in odontoblasts and transiently in preameloblasts (2, 3) as well as at low levels in bone (15). The DSP expression level in rat long bone is about one-fourth the rate of rat dentin, suggesting that the functional role of DSP is mainly involved in tooth formation and mineralization (15). Heterogeneous mutations of human DSP are associated with dentinogenesis imperfecta type II and type III and dentin dysplasia type II (16–19). Patients with these diseases present with discolored teeth, enlarged pulp chambers that fill in with mineralized matrix, a wider predentin zone, decreased dentin width, hypomineralization, and the prevalence of pulp exposures. DSP homologous deficient mice (-/-) show teeth similar to human dentinogenesis imperfecta type III (20). These data indicate that DSP plays an important role in tooth formation and mineralization and that different regulatory mechanisms governing DSP expression are involved in tooth, bone, and other tissues.

Mouse and rat DSP genes have been characterized.
ized (13, 21). The mouse DSPP promoter activity of various deleted constructs has been assayed using a mouse odontoblastic cell line (MO6-G3) shown to express DSPP (22). Promoter deletion analysis revealed that several enhancer and silencer elements within 1500 bp upstream of the transcription initiation site control tissue-specific expression of the DSPP gene in MO6-G3 cells (13). Furthermore, an immunohistochemical study showed that the first 500 bp upstream of the DSPP transcription start site is sufficient to activate LacZ reporter gene expression restricted to odontoblasts of transgenic mice (23). However, the precise trans-acting transcription factors associated with cis-acting elements underlying tissue-specific DSPP gene expression are unknown. Gene expression is controlled by transcriptional regulators, activators and repressors, which interact either directly or indirectly with cis-regulatory elements and modulate the expression levels of their target genes (24, 25).

CCAAT-binding factor (CBF; also known as NF-Y and CP1) is a heterotrimeric transcription factor that binds to the CCAAT box found in the promoter of many eukaryotic genes (26, 27). CBF consists of three subunits, CBF-A, CBF-B, and CBF-C, all of which are required for DNA binding and transcriptional activation (28). The CCAAT box is one of the most common cis-acting elements present in 30% of eukaryotic gene promoters located between −60 and −100 bp upstream of the transcription initiation site (29). CBF physically and functionally interacts with other transcription activators or co-activators (30–34). The role of this gene is involved in many gene promoter activities such as cell cycle-regulatory classes of genes (35, 36). Studies have identified CBF activation of collagen type I and bone sialoprotein genes expressed in mouse fibroblasts (37–39). Expression of osteocalcin is also regulated in osteoblasts by this protein (40). However, it remains unknown how CBF and other transcription factors regulate DSPP gene expression in odontoblasts. In the present study, we investigated the role of CBF and a novel silencer transcription factor in the regulation of DSPP expression in odontoblasts.

EXPERIMENTAL PROCEDURES

Cell Culture—Mouse immortalized odontoblast cells (MO6-G3) were grown at 33 °C in α-minimal essential medium containing 50 μg/ml ascorbic acid, 10 mM sodium pyruvate, human urine (HeLa) cells were cultured in minimal essential medium at 37 °C under 5% CO2. All of the cells were grown in media supplemented with 10% fetal calf serum and 100 units of penicillin/streptomycin.

DNA Transfection and Enzyme Assay—For generation of p241mut-3 and p241mut-4 constructs, sequences in the promoter was subcloned into the 5′/H11032 reporter construct p1243, the 5′/H11032-3′/H11022 segment of the mouse DSPP gene promoter was cloned into the pcDNA-hygro vector (Promega). As an internal control, a PCR fragment containing the first 500 bp upstream of the DSPP promoter was cloned into pcDNA-hygro vector containing a hygromycin-resistant gene (30). Promoters with inserted PC1 were transfectected into the host cells (MO6-G3 or HeLa) and positive colonies were selected by hygromycin. Genomic DNA from individual positive colonies of the host cells was used to determine the copy number of the various DSPP promoter-luciferase reporter gene constructs. DNA constructs of p1243, p791, p241, and p97 were co-transfected with the pcDNA-hygro vector (Invitrogen) containing a hygromycin-resistant gene into MO6-G3 cells using the LipofectAMINE Plus reagent (Invitrogen) as specified by the manufacturer. After 24 h, cells were trypsinized and seeded at 86-well plates, and 0.2 ng of hygromycin was added to each well. After 48-h incubation in 5% CO2, the medium was changed and incubation continued for another 20 h. For competition binding reactions, the unlabeled competitor in different -fold molar excesses of the labeled probe was included in the reaction. Free DNA and protein-bound DNA complexes were loaded onto a 5% native polyacrylamide gel in 1× Tris-boric acid-EDTA buffer, electrophoresed, dried, and exposed to x-ray film. Antibody supershift experiments were performed with specific antibodies to CBF-A, CBF-B, and CBF-C 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 of consensus DNA binding sites of transcription factors used in EMSA were obtained from Santa Cruz Biotechnology (Table 1).

Electrophoretic Mobility Shift Assay—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 by Chen et al. (44). Briefly, nuclear extracts (1–5 μg) from MO6-G3 and HeLa cells were preincubated in 20-μl reactions containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, and 2 μg of poly(dI-dC). After 5 min at room temperature, 20 fmol of 32P-labeled oligonucleotide duplex probes were added, and incubation continued for another 20 min. For competition binding reactions, the unlabeled competitor in different -fold molar excesses of the labeled probe was included in the reaction. Free DNA and protein-bound DNA complexes were loaded onto a 5% native polyacrylamide gel in 1× Tris-boric acid-EDTA buffer, electrophoresed, dried, and exposed to x-ray film. Antibody supershift experiments were performed with specific antibodies to CBF-A, CBF-B, and CBF-C 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 of consensus DNA binding sites of transcription factors used in EMSA were obtained from Santa Cruz Biotechnology (Table 1).

Preparation of Nuclear Extracts—Nuclear extracts from MO6-G3 cells were prepared using the methods of Dignam et al. (42). Cell cultures contained 2 μg/ml aprotinin, leupeptin, bestatin, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol added just prior to use. All manipulations were performed at 4 °C. Protein concentrations were determined using the Bradford assay (43).

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picric acid, and 0.1N HCl solution added for 5 min. The wells were stained. Each well had 1% acid fuchsin, 0.13% water again. The modified van Gieson stain was used as a counter-

Two percent silver nitrate solution was added to each well under direct exposure to UV light for 25 min. The plates were washed with water and air-dried.

The cell lysates were harvested with passive lysis buffer (Promega) 48 h after the start of transfection. Luciferase activity was measured with the dual luciferase assay, and protein concentrations of the cell extracts were determined using the Bradford assay. The fold increase was plotted as a graph. The data are the mean ± S.E. from five independent experiments in triplicate.

Western Blot Analysis—The cells were washed with phosphate-buffered saline and lysed with 300 μl of radioimmunoprecipitation buffer (1× phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml phenylmethylsulfonyl fluoride, 30 μg/ml aprotinin, 100 mM sodium orthovanadate). Protein (30 μg/well) was resolved by 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (Bio-Rad). The secondary antibody conjugate to alkaline phosphatase was used at a dilution of 1:500. The secondary antibody conjugate to horseradish peroxidase-conjugated anti-rabbit IgG was used at a dilution of 1:20,000. Immunoreactivity was determined using the ECL chemiluminescence detection system (Amersham).

EMSAs and competitors were performed with the oligonucleotide duplexes listed below. Only the 5′ to 3′ strand of the duplexes is shown.

**Table 1. Oligonucleotide sequences**

| Name | Sequence (5′ to 3′) |
|------|---------------------|
| DE1 (wt) | 5′-GATCTTAACGCAAAAGGCTGAG-3′ |
| DE1 (mut) | 5′-GATCTTAACGCAAAAGGCTGAG-3′ |
| CBF (wt) | 5′-AGACCTGACGAAATCAGG-3′ |
| CBF (mut) | 5′-AGACCTGACGAAATCAGG-3′ |
| Ap-1 | 5′-CCCTTCTAGTTCTGCGGAA-3′ |
| Ap-3 | 5′-CCCTTCTAGTTCTGCGGAA-3′ |
| C/EBP | 5′-TTCAAGTCGACAGCCTCC-3′ |
| GRE | 5′-TCCCTCAGCTTTACCGGT-3′ |
| NF-κB | 5′-TTCCCTCAGCTTTACCGGT-3′ |
| Sp-1 | 5′-ATTCTAGTCGACAGCCTCC-3′ |
| Tef-1 | 5′-ATGAGGATCGGTCCGAG-3′ |

Substitution mutations are represented in lowercase.

Stained as described by the instructions using the alkaline phosphatase substrate kit (Bio-Rad). ALP activity was measured using 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt and p-nitro blue tetrazolium chloride as substrates.

Southwestern Blot Analysis—Thirty micrograms of MO6-G3 cell nuclear extracts or BSA were separated on a 4−20% gradient SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Nuclear
proteins on the membrane were prehybridized with a binding buffer (50 mM NaCl, 0.1 mM EDTA, pH 7.5, 0.1% SDS, 1 μg of poly(dI-dC)/ml, 50 ng of sonicated salmon sperm DNA/ml) containing 5% nonfat milk overnight at 4 °C. The membrane was then washed three times with the binding buffer plus 0.25% nonfat milk and hybridized in 4 ml of the same buffer containing 30 pmol (3 × 10^7 cpm) of a ^32P-labeled 33-bp DNA probe positioned between nt −127 and −95 of mouse DSPP proximal promoter. Hybridization was carried out overnight at room temperature. The membrane was washed three times (10 min each) with binding buffer, dried, and subjected to autoradiography.

Partial Purification of Nuclear Binding Protein by UV Cross-linking—Nuclear extracts from MO6-G3 cells were allowed to bind to the 33-bp DNA probe in the presence of nonspecific competitor, poly(dI-dC), and then subjected to gel electrophoresis as described above for the EMSA. Afterward, the wet gel was exposed to UV light (254 nm) for 1 h to cross-link the DNA to the nuclear protein(s). The bands were then revealed by autoradiography. The gel pieces containing the protein-DNA complex were cut out, diced, and placed into Eppendorf tubes. Elution buffer was added (20 mM Tris-HCl, pH 8.0, 1% SDS), and the sample was homogenized with a microhomogenator and transferred to a new tube. The tube was left on a rotator for incubation overnight at 37 °C and centrifuged. The supernatant was concentrated with a microcon YM-10 (Millipore Corp., Bedford, MA) until the volume was 250 μl. The sample was precipitated with acetone and centrifuged, the supernatant was removed, and the pellet was air-dried. The precipitated macromolecules were solubilized in loading buffer and resolved on
RESULTS

Stable Transfection Analysis of Mouse DSPP Promoter Constructs—To determine the cis-acting element(s) necessary for the promoter activity of the mouse DSPP gene, a series of DSPP promoter-luciferase reporter constructs bearing progressive 5'-deletions were tested in MO6-G3 cells with stable transfection assays. Fig. 1 shows that the p1243 construct was sufficient to drive the expression of the reporter gene in MO6-G3 cells. Analysis of the promoter activity suggested that potential transcriptional enhancers existed in regions between nt 1243 and 792 and between nt 791 and 242, whereas a suppressor element was located in a region between 241 and 98 base pairs, since the promoter activity increased a 5-fold in p97 after deletion of this region. In addition, the strongest promoter activity of the mouse DSPP gene was located within a 151-bp fragment between nt 97 and 54, indicating that this fragment contained the essential control elements for expression of the DSPP gene. These results were similar to those reported using a transient transfection assay (13).
Identification of Protein-DNA Binding Sites within a Near Upstream Region of the Mouse DSPP Gene—The nucleotide sequence of up to 1.5 kb of the mouse and rat DSPP gene promoter has been characterized (13, 21). Also, the DNA sequence of the human DSPP gene promoter was obtained from the human genome data base (GenBank™ number AC093063). Computer analysis of the nucleotide sequence with the existing data bases suggests consensus binding sites for a number of known transcription factors within the proximal promoter (first 500 bp upstream of the transcription initiation site). These include several potential binding sites for Sp1, Ap-1 (fos/jun), Ap-3, CBF, CCAAT/enhancer-binding protein, Tcf-1, Msv-1, NF-κB, and a glucocorticoid receptor (half-site). Several unidentified binding sites have also been found in this region (13, 21). However, binding of these transcription factor(s) to their cis-acting element(s) has not been reported to date. In order to identify any known or novel nuclear factors that may bind within a 300-bp upstream region of the DSPP gene promoter, since both transient and stable transfection analyses demonstrated that positive and negative regulatory elements were located within this region, we initially conducted DNase I protection analysis with nuclear extracts from the mouse odontoblastic cells.

Fig. 2 shows the DNase I footprinting pattern of the 308-bp mouse DSPP promoter fragment encompassing nt −254 to +54 in the presence of MO6-G3 cell nuclear extracts. Three regions were protected: the first at nt −69 to −52 (A region), the second at nt −123 to −74 (B region), and the third at nt −209 to −191 (C region). Examination of the B region revealed partial asymmetry in DNase I protected sites between nt −123 and −95 and between nt −95 and −74, suggesting that two proteins might interact with this region. The p97 fragment covers partial sequences from nt −97 to −74 in B region and had the highest promoter activity in MO6-G3 cells, whereas the p241 contained the entire B region DNA sequence and showed the lowest promoter activity (Fig. 1); therefore, the 49-bp sequence of the B region was subdivided into two elements: DSPP element 1 (DE1) located from nt −97 to −74 and DSPP element 2 (DE2) from nt −127 to −95.

CBF Binds to DE1 in MO6-G3 Cell Nuclear Extracts—A double-stranded oligonucleotide covering DE1 (Table I) was used for a probe in EMSA using MO6-G3 cell nuclear extracts as the source of proteins. A protein-DNA complex was seen (Fig. 3A, lane 1). Computer analysis of DE1 sequence showed that this element contains a few potential protein-DNA binding sites including an inverted CCAAT box (Fig. 3B). Based on this observation, we performed a series of competition and mutant EMSA analyses (Fig. 3A). The results showed that the protein-DNA complex could be completely competed away with 75- and 150-fold molar excesses of either the unlabeled homologous DE1 or CBF consensus oligonucleotides (Fig. 3A, lanes 2 and 3 and lanes 6 and 7) but not with even a 150-fold molar excess of a mutant CBF binding site DE1 (lanes 4 and 5) and mutant CBF consensus oligonucleotides (lanes 8 and 9) as well as other oligonucleotides (lanes 10–15). To further confirm that the DE1 is a CBF binding site, supershift experiments were performed using three anti-CBF antibodies specific for CBF-A, CBF-B, and CBF-C subunits or a nonspecific serum. Incubation of the

**Fig. 5. Biological activity of the wild-type and mutant DSPP promoter region in MO6-G3 and HeLa cells.** A and C, illustration of wild-type and mutant DSPP promoter-luciferase reporter gene constructs. The diagram shows the relative positions and sequences of CBP- and DF2-responsive elements with mutated sequences shown in lowercase type. These constructs carrying mutations in either core CBF or DE2 elements alone or combined are indicated. B and D, the MO6-G3 cells were transfected with these wild-type and mutant DSPP promot-
nuclear extracts with the three anti-CBF subunit antibodies prior to the addition of the labeled double-stranded DE1 and CBF consensus oligonucleotides led to the formation of slower migrating protein-DNA complexes (Fig. 3D, lanes 3–9 and 13–19), whereas the nonspecific serum had no effect (lanes 10 and 20). These results demonstrated that the DE1 is an inverted CBF binding site that binds all three CBF subunits. Notably, the inverted CBF binding site in the mouse DSPP gene promoter is also present at the same position in rat and human DSPP gene promoters (Fig. 3C).

**DE2 Interacts with a Novel Nuclear Factor**—Since computer analysis showed that several potential transcription factor-DNA binding sites exist in DE2 (Fig. 3B), we investigated whether transcription factor(s) were able to bind to DE2. Incubation of a labeled 33-mer DE2 oligonucleotide with M06-G3 cell nuclear extracts formed a protein-DNA complex (Fig. 4A, lane 1). To identify which transcription factor(s) are capable of interacting with DE2, we next performed a series of competition EMSA experiments. The results showed that the protein-DNA complex was only abolished with the unlabeled homologous DE2 oligonucleotide (Fig. 4A, lanes 2 and 3) but not with any other oligonucleotides tested (Fig. 4A, lanes 4–15). These results suggest that DE2 bound by this nuclear factor is unique and not homologous to any potential known transcription factor-DNA binding sequences in this element (Fig. 3B). The nuclear factor binding to DE2 was named DSPP factor 2 (DF2). To identify a consensus sequence in the 33-mer DE2, we performed EMSA experiments of partial deletions and mutations within DE2, and the binding site of DF2 was narrowed to a 17-mer DNA sequence between nt −121 and −104 (Fig. 4B and Table II). Furthermore, through a series of competition experiments, the core site of DE2 sequence was defined as ACCCCCA (Fig. 4C and Table II). These experiments also showed that DF2 only binds to the labeled wild-type probe and not the mutant probes (Fig. 4D). Although the labeled mutant 2 probe with the nuclear extracts produced a higher retarded band (Fig. 4E, marked with an asterisk), the binding was substantially abolished with the unlabeled homologous mutant 2 oligonucleotide (lane 2) but not with the unlabeled wild-type DE2 oligonucleotide (lane 3). This indicates that the binding of the nuclear factor to the mutant 2 oligonucleotide is different from the wild-type DE2 oligonucleotide. Furthermore, examination of the existing computer data base programs revealed that the core DE2 DNA sequence is not overtly homologous to known transcription factor-DNA binding elements. Interestingly, the core DE2 element sequence in the mouse DSPP gene promoter is highly conserved between species, as shown in alignment of rat and human DSPP promoter nucleotide sequences (Fig. 3C).

**CBF Acts as an Activator, whereas DF2 Is a Repressor for Mouse DSPP Promoter Activity**—To determine the biological function of the inverted CBF binding site in the mouse DSPP promoter, a mutation that disrupts CBF binding was introduced into the core sequence of the CBF binding site in the context of p97mut (Fig. 5A). The results showed that the promoter activity of the mutant construct resulted in a 5-fold decline compared with the wild-type construct in M06-G3 cells (Fig. 5B). These data indicated that the CBF binding site enhances the mouse DSPP gene promoter activity in M06-G3 cells. In contrast, point mutations of the core DE2 element sequence in p241mut-1 and p241mut-2 resulted in 4.5–4.7-fold higher promoter activity than that of p241wt (Fig. 5, C and D). These results suggest that the core site of DE2 is essentially responsible for inhibiting DSPP promoter activity in this region of p241 (Fig. 1). To study whether there is any functional effect between CBF and DF2 binding sites, double mutant DNA constructs of both CBF and DE2 binding sites were generated in the context of p241mut-3 and p241mut-4 (Fig. 5C) and transfected into MO6-G3 cells. As shown in Fig. 5D, the promoter activity in the p241mut-3 and p241mut-4 was less than 50% compared with mutations of only the DF2 binding site in the context of p241mut-1 and p241mut-2. These results suggested that inhibition of DSPP gene expression by DF2 may involve interaction with CBF. To further assess effects of the DE2 element on cell-specific expression of the DSPP gene, p241mut-1 and p241wt constructs were transfected into HeLa cells. The results showed that the mutation of the DF2 binding site in p241 resulted in a 14-fold increase of promoter activity in HeLa cells but had about a 4.3-fold increase of its activity in MO6-G3 compared with that of p241wt (Fig. 5E). Moreover, four DSPP promoter-luciferase reporter constructs were transfected into HeLa cells. As shown in Fig. 5F, there were not significant differences in luciferase activities among the various reporter constructs as compared with the control group except for p97. These data indicate that the DSPP gene promoter region, in particular the DE2 site, controls cell type-specific expression of the DSPP gene.

Identification of the Core DE2 Element in Other Bone/Tooth Gene Promoters—To further identify whether the core sequence of DE2 exists in other ECM gene promoter regions, promoter sequences of other abundant ECM genes were scanned using computer data base programs, and the core DE2 sequence in these gene promoter regions was identified (Table III). Next we determined whether the core DE2 sequence in these gene promoters was able to bind DF2. Double-stranded

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**Table II**

| Oligonucleotide | Sequence (5′ to 3′) | Binding activity |
|-----------------|--------------------|-----------------|
| DE2 −127 to −95 | CACACTGACTTTAAAACCCCCACATCAGGGAT | + |
| DSPP −127 to −104 | CACACTGACTTTAAAACCCCCACATCAGGGAT | + |
| DSPP −121 to −95 | GACCTTTAAAAACCCCCACATCAGGGAT | + |
| DE2-del-9 −127 to −95 | CACACTGACTCT----CACATCAGGGAT | – |
| DE2-del-3 −127 to −95 | CACACTGACTTTAAAAC-C-CACATCAGGGAT | – |
| DE2-del-2 −127 to −95 | CACACTGACTTTAAACCCCATCAGGGAT | – |
| DE2-mut-4 −127 to −95 | CACACTGACTATGAAAACCCCCACATCAGGGAT | – |
| DE2-mut-2 −127 to −95 | CACACTGACTTTAAGCCCCCATCAGGGAT | – |
| Mutant 1 | t | – |
| Mutant 2 | g | – |
| Mutant 3 | a | – |
| Mutant 4 | g | – |
| Mutant 5 | a | – |
| Mutant 6 | g | – |
| Mutant 7 | t | – |
DNA oligonucleotides were generated, labeled as probes, and incubated with nuclear extracts from MO6-G3 cells. Like the protein-DNA complex from the DE2 probe, the same positioned migrating protein-DNA complexes formed with these probes using the nuclear extracts (Fig. 6). Furthermore, these protein-DNA complexes could be competed away with a 100-fold molar excess of either the unlabeled homologous or DE2 oligonucleotides. Thus, these data indicated that the core DE2 element sequence was sufficient for DE2 binding, and the existence of this core sequence in other ECM gene promoters plays a functional role(s) in regulating these ECM genes.

Expression of DF2 during Odontoblastic Differentiation and Mineralization—In situ hybridization studies have shown that DSPP expression is gradually increased during odontoblastic differentiation and dentin mineralization (2, 53). To study whether DF2 is correlated with DSPP expression, MO6-G3 cells at days 5 and 20 postconfluence were evaluated. Development of mineralized nodules was observed in MO6-G3 cell cultures at days 5 and 20 as visualized by the von Kossa staining technique (Fig. 7A). However, higher density and larger sized mineralized nodules were seen in MO6-G3 cells at day 20 of culture. We next investigated ALP activity in MO6-G3 cells at days 5 and 20, since ALP is a marker of osteoblasts and odontoblasts related to mineralization (54–56). These results showed higher ALP activity at day 20 as compared with day 5 (Fig. 7B). Furthermore, DSPP protein expression at these two time points was detected using a specific anti-DPSS antibody and shown to parallel ALP expression (high level at day 20) (Fig. 7C). Conversely, DF2 expression was significantly lower in EMMA performed with nuclear extracts from day 20 MO6-G3 cell cultures (Fig. 7D, lanes 3 and 5), whereas CBF was evenly expressed at the two time points tested (Fig. 7D, lanes 7–10). In addition, a higher expression level of DF2 was seen in HeLa cell nuclear extracts that do not express DSPP as compared with MO6-G3 cell nuclear extracts (Fig. 7E). Thus, the level of DF2 expression is probably relevant to DSPP expression and other ECM proteins.

Identification of Molecular Mass of DF2—To initially characterize DF2, we determined the molecular mass of this protein by Western blot analysis. A single DNA-protein hybridization band was seen at ~72 kDa using the MO6-G3 cell nuclear extracts (Fig. 8A, lane 1) but not observed with BSA (lane 2). Next, a UV cross-linking assay was carried out, and the DE2 probe was incubated with MO6-G3 cell nuclear extracts in an EMSA. Protein-DNA complexes were exposed to UV light and purified. The isolated product was electrophoresed through a 4–20% gradient SDS-PAGE gel, and the gel was stained with Coomassie Brilliant Blue. As shown in Fig. 8B, a single band was detected, and the protein migrated slightly higher than the protein itself, since this complex contained the 32P-labeled 33-bp DE2 probe. Subtracting the contribution of the DE2 DNA fragment would result in the same calculated molecular weight estimated by the Southwestern blot analysis.

**DISCUSSION**

The spatial and temporal expression of DSPP is largely restricted to mineralized tissues (2–3, 15). The expression level of DSPP in dentin is about 400-fold higher than that found in bone, suggesting that different regulatory mechanisms govern DSPP gene transcription in tooth, bone, and other tissues (15). Differences in the tissue-specific expression profiles of genes are due, in part, to regulation of their transcription by various trans-acting factors that interact with cis-acting elements in the promoter regions of these genes, some acting as enhancers and others as repressors (24, 25). In fact, previous deletion studies have identified positive and negative regulatory regions in mouse and rat DSPP gene promoters using mouse odontoblast and dental papillae cells (13, 21, 57). However, until now, transcription factors binding to these elements have not been described. To study the tissue-specific transcription events that control odontoblastic differentiation and matrix formation, we have embarked on the systematic analysis of the mouse DSPP gene promoter. In this study, we show that the first 241 base pairs of this promoter contain a critical region that strongly down- and up-regulates promoter activity in odontoblasts using stable transfection experiments in odontoblasts, expanding previous transient transfection studies (13). Using protein-DNA binding assays, we focused on a 49-bp element, the B region, located between nt –123 and –74 upstream of the DSPP transcription start site, showing that this region contains two transcription factor-binding sites, DE1 and DE2. Competition EMSA analyses demonstrated that DE1 is

**TABLE III**

| Gene promoter | Source/Reference |
|---------------|------------------|
| OC           | GenBankTM AF071079 |
| COL1A1       | GenBankTM AC093906.3. |

A oe, osteocalcin; bsp, bone sialoprotein; dmp1, dentin matrix protein 1; amel1, amelogenin; opn, osteopontin; colIA1, a 1 collagen type I.

A Sequence identity is underlined.
an inverted CBF binding site and acts as an enhancer element, whereas DE2 is a novel protein-DNA binding site bound by DF2, which functions as a repressor for DSPP gene expression in odontoblasts.

DF2 expression was found to be inversely expressed in relation to DSPP, whereas CBF was steadily expressed. In vivo studies have shown that transcription levels of DSPP are increased during tooth differentiation, and high DSPP expression coincides with dentin matrix formation and mineralization (2, 53). Our study showed that DSPP protein levels were high during odontoblastic differentiation and mineralization, whereas expression levels of DF2 were declining (Fig. 7). Furthermore, HeLa cells lacking DSPP expression expressed high levels of DF2. The biological activity of the mutant DSPP gene promoters lacking the DF2 binding site resulted in a 4.6-fold increase of promoter activity in odontoblasts and a 14-fold increase in HeLa cells over that of the wild type (Fig. 5). Therefore, levels of DSPP expression in cells are likely to be partially determined by DF2. Additionally, we also observed that when upstream promoter regions of the mouse DSPP gene were included, promoter activities were changed in MO6-G3 cells (Fig. 1). This finding suggests that other transcription factors contribute to regulation of the DSPP gene expression. Similar results are found with the dentin matrix protein 1 gene promoter regions (58). Thus, these factors, DF2, CBF, and possibly other factors can potentially interact with basal transcription factors such as the TFIID complex to dictate the final rate of the DSPP transcription in cells (59).

CBF subunits have been shown to regulate collagenous and noncollagenous gene expression including type I collagen, bone sialoglycoprotein, and osteocalcin by CBF binding sites identified in the proximal promoters of these genes (37–40). Interestingly, like the promoters of several dentin/bone genes such as dentin matrix protein 1, osteocalcin, and bone sialoglycoprotein (39, 40, 58, 60), the mouse DSPP gene promoter also contains an inverted TATA motif and a CBF binding site near its transcriptional start site. Our analysis showed that the inverted CBF binding site is also present at the same location in rat and human DSPP promoters (Fig. 3C). These observations suggest that CBF plays the same role in activating expression of the DSPP gene in these three species as well as for other dentin/bone genes (36–39).

Effects of CBF and DF2 on regulation of the DSPP gene expression were observed in our transfection experiments in odontoblasts. Although double mutations of both CBF and DF2 binding sites partially increased promoter activity (∼2-fold) as compared with that of the wild type construct p241wt (Fig. 5), the mechanisms of possible interactions are not known.

To our knowledge, we have for the first time identified a
novel negative element DE2 in the proximal promoter of mouse DSPP and defined the core DE2 element sequence through an extensive mutagenesis approach. This core sequence showed no strong homology to any known binding sites of identified transcription factor families and is highly conserved in the promoters of human and rat DSPP. Strikingly, the identified core sequence is also present in the promoters of other bone/tooth ECM genes. The ubiquitous existence of the core DE2 element across species and in other bone/tooth gene promoters suggests that it may also be involved in regulating these genes. In addition, differences in the binding affinity of the core DE2 element sequence were observed related to different bone/tooth tissues (Fig. 6). Dent et al. (61) reported that an octamer-binding transcription factor, Oct-2, is able to bind to its octamer motif in the promoters of the herpes simplex virus and other genes with use of EMSAs, but there are differences in the binding affinity. The same evidences are found in the consensus DNA sequences of ethylene response factor (62) and transcription factors (63, 64). These studies indicate that the binding affinity of DF2 to its core sequence in those bone/tooth genes is potentially modulated by the sequences surrounding the core DE2 element.

DF2 expression varied during odontoblastic differentiation and mineralization as well as within mineralizing versus non-mineralizing cells such as MO6-G3 and HeLa. Changes in the expression levels of the osteoblast-specific transcription factor, Cbfal/Runx2, with osteoblastic and odontoblastic differentiation have been reported. Cbfal/Runx2 is highly expressed in undifferentiated osteochondro progenitor cells but down-regulated in differentiating nonhypertrophic chondrocytes (49). Moreover, D’Souza et al. (48) reported that Cbfal/Runx2 is down-regulated in mouse differentiated odontoblasts. With identification of the molecular mass of DF2, cloning of DF2 will be initiated to further characterize its role in controlling the tissue-specific and spatial-temporal expression of DSPP in mineralizing tissues.
