The CCAAT Enhancer-binding Protein (C/EBP)β and Nrf1 Interact to Regulate Dentin Sialophosphoprotein (DSPP) Gene Expression during Odontoblast Differentiation

Terminal differentiation of odontoblasts, the principal cells in dentin formation, proceeds by synthesis of type I collagen and noncollagenous proteins. DSP and DPP are specific markers for terminally differentiated odontoblasts and are encoded by a single gene DSPP (dentin sialophosphoprotein). In an attempt to understand the molecular mechanisms required for tissue-specific expression of the DSPP gene, we have identified a novel interaction between two bZIP transcription factors, Nrf1 and the CCAAT enhancer-binding protein (C/EBP)β. This interaction was confirmed by both immunoprecipitation and chromatin immunoprecipitation assays. In undifferentiated odontoblasts, Nrf1 and C/EBPβ repress DSPP promoter activity individually and synergistically by cooperatively interacting with each other. This mutual interaction is facilitated by the bZIP domains in both the proteins. The repression domain in both Nrf1 and C/EBPβ was determined, and deletion of this domain abolished transcriptional repression. In fully differentiated odontoblasts, the loss of interaction between Nrf1 and C/EBPβ results in an increased DSPP transcription. Further, this interaction was found to be dependent on phosphorylation at Ser599 of Nrf1. Thus, the physical interaction between Nrf1 and C/EBPβ provide a novel mechanism for the transcriptional regulation of DSPP in odontoblasts.

Odontogenesis is regulated by several molecular determinants that are expressed in a temporal and spatial manner. Various signaling pathways trigger the induction of different transcription factors and DNA-binding proteins onto specific DNA regulatory regions facilitating transcriptional activation of odontoblast-specific genes that are required for dentin mineralization. The major proteins synthesized by fully differentiated odontoblasts are type I collagen and two major noncollagenous proteins (NCPs), namely dentin phosphophoryn (DPP) and dentin sialoprotein (DSP) (1).

DSP and DPP are specific markers for terminally differentiated odontoblasts and are encoded by a single gene DSPP located on mouse chromosome 5 (2, 3) and human chromosome 4 (4). Published reports have established that the DSPP gene encodes for a single transcript containing DSP at the 5'-end and DPP at the 3'-end of the transcript. Various reports have suggested that the holoprotein might be cleaved by specific enzymes into DSP and DPP (5). However, the compound protein DSPP has not been isolated yet as a complete entity. Mutations in the DSPP gene have been identified to be associated with the inherited genetic disorder, dentinogenesis imperfecta types I and II (6, 7). Recently, a single mutation in the signal peptide of the DSP protein was responsible for the genetic disorder dentine dysplasia type II (8). Further, the DSPP knock-out mice exhibits dentinogenesis imperfecta type III phenotype. Thus DSPP is a key molecule for normal dentinogenesis (9).

To determine the transcriptional regulation of DSPP gene we have cloned and characterized the rat DSP promoter and identified a repression domain between −700 and −400 bp. Further analysis of this region identified two binding elements, which have 90% homology to the DNA binding sites for two bZIP transcription factors Nrf1 and the CCAAT enhancer-binding protein (C/EBP)β. The bZIP transcription factors constitute an important class of eukaryotic DNA-binding protein in which dimerization is mediated through their coiled-coil regions. In an attempt to understand the molecular mechanisms behind the tissue-specific expression of DSPP gene, we have utilized the promoter sequence of DSPP and analyzed the direct function of Nrf1 and C/EBPβ on DSPP transcription.

Nrf1, also known as TCF11/LCR-F1 belong to the common CNC (cap’n’ collar) family (10) and plays an important role during development (11, 12). Even though Nrf1 is related to the hematopoietic specific transcription factor, p45NF-E2, it was shown to be different. However, it can bind to the same recognition sequence as that of p45NF-E2, either as a homodimer or as a heterodimer with other bZIP proteins like small Maf transcription factors (13–15). The specificity of Nrf1 in transcriptional regulation relies on post-translational modifications and its interaction with other co-factors. Nrf1 can modulate the antioxidant response element of stress related genes by cooperatively interacting with transcription factors such as AP1 proteins (c-Jun, JunB, and JunD) (16). Under normal conditions Nrf1 exists as a complex with Keap1 (inhibitor) and during stress Nrf1 dissociates from Keap1 and becomes active.
and thus acts as a dominant-negative inhibitor. Thus, C/EBP (LIP), which lacks most of the trans-activation domain of LAP, has been reported to be an activator and liver-enriched inhibitory protein (LAP), which is normally repressed in cells other than hepatocytes. In transfection assays, the repressor domain deletion was carried out by sequence-verified and cloned into CMV-3XFLAG tag vector (Sigma) for overexpression studies. The repressor domain deletion was carried out by sequence-verified and cloned into pGL3basic vector at NheI site for Nrf1 expression plasmid was a kind gift from Dr. Kolstø (27). All members share homology at the bZIP domain and are multifunctional proteins that exhibit a diverse set of cellular responses like differentiation, inflammatory response, liver regeneration, metabolism etc (23). The target genes of the C/EBP family members are diverse and various studies have shown that C/EBP can modulate the expression of various matrix genes critical for osteoblast differentiation such as pro-α1 and -α2 type I collagen, matrix Gla protein, and osteocalcin. In most cases C/EBPβ can form a homodimer and bind to its recognition sequence; however C/EBPβ can also intraheterodimerize with other members of the family (23) as well as with a variety of bZIP proteins such as CREB (24), C/ATF (25), and AP1 (26). C/EBPβ has two major isoforms, liver-enriched activator protein (LAP), which is normally reported to be an activator and liver-enriched inhibitory protein (LIP), which lacks most of the trans-activation domain of LAP and thus acts as a dominant-negative inhibitor. Thus, C/EBPβ can directly activate or inhibit target gene expression.

Odontoblast-specific gene transcriptions are regulated by highly sophisticated synergistic interactions between transcription factors binding to specific regulatory sequences, with the basal transcriptional machinery. Both positive and negative regulatory mechanisms are required for transcriptional regulation of dentin matrix genes resulting in normal dentin formation. In this study we provide compelling evidence for the transcriptional repression of DSPP by association of C/EBPβ with Nrf1. The physical interaction between C/EBPβ and Nrf1 was both direct and physiologically relevant. This functional interaction was mediated through the bZIP domain. The efficiency of this interaction was found to be dependent on phosphorylation of Nrf1 at Ser599 by PKA. Further, a transcriptional repression domain has been mapped to the C terminus of Nrf1 and C/EBPβ, which is required for the transcriptional regulation of DSPP.

MATERIALS AND METHODS

Plasmid Constructs—A 1.7-kb fragment was amplified from rat genomic DNA to obtain the DSPP promoter. The promoter was sequence-verified and subcloned into pGL3basic (Promega) vector at NheI and SmaI. Nrf1 expression plasmid was a kind gift from Dr. Kolsto (27). The Nrf1 cDNA was further subcloned into the HA tag vector C/EBP plasmids (α, β, γ, and δ) were kind gifts from Dr. Friedman (Department of Oncology, Johns Hopkins University School of Medicine). DSPP promoter deletions were made using specific primers. Deletions constructs of C/EBPβ and Nrf1 were carried out by PCR with gene-specific primers containing EcoRI and EcoRV sites. The products were sequence-verified and cloned into CMV-3XFLAG tag vector (Sigma) for transfection assays. The repressor domain deletion was carried out by inserting a Sall site for C/EBPβ and SacI site for Nrf1. Glutathione S-transferase fusion protein constructs were made by cloning the PCR products generated with EcoRI and EcoRV sites and inserted into EcoRI and SmaI sites in PGEX4T-3 vector. The fusion proteins were expressed in Escherichia coli, BL21 (Inovotrogen) according to the suggested protocol from the manufacturer (Amersham Biosciences).

Cell Culture and Transfections—An odontoblast cell line developed by hTERT-mediated immortalization as reported earlier (28) was used in this study. The odontoblast cells were grown in Dulbecco’s modified Eagle’s/F12 medium containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% amphotericin B. MC3T3-E1 cells were cultured as described earlier (29). Transient transfections with reporter plasmids were performed with Superfect (Qiagen) as per the manufacturer’s protocol. Reporter transfections were carried out in triplicate and repeated thrice to ensure reproducibility.

Table 1

| Table 1 | Primers used for EMSA and site-directed mutagenesis |
|---------|--------------------------------------------------|
| Wild type | ATTAGGCTTTAAGAAAAACCCAAAACCTTTATGTCAGTGTTATTA |
| C/EBP mutation | ATTAGGCTTTAAGAAAAACCCAAAACCTTTATGTCAGTGTTATTA |
| Nrf1 mutation | ATTAGGCTTTAAGAAAAACCCAAAACCTTTATGTCAGTGTTATTA |
| C/EBP and Nrf1 mutation | ATTAGGCTTTAAGAAAAACCCAAAACCTTTATGTCAGTGTTATTA |

FIG. 1. A, DSPP promoter activity in osteoblast (MC3T3-E1) and odontoblast cells. Transient transfections were performed with luciferase reporter vector containing a ~1.7-kb 5′-upstream sequence of the DSPP gene in pGL3basic vector in the odontoblast cell line and osteoblast cell line (MC3T3-E1) using Superfect reagent. DSPP promoter activity was quantified in terms of the firefly luciferase activity. SV40-driven Renilla luciferase vector was used as an internal control. Protein lysates were made using passive lysis buffer. The protein concentration was estimated, and 50 μg of the lysate (100 μl) was dispensed into a 96-well microtiter plate and assayed for dual luciferase in an automated luminometer from Dynex. Average values obtained from three independent experiments after normalizing for transfection efficiency are shown with appropriate standard error. The ratio between firefly and Renilla luciferase was compared between osteoblasts and odontoblasts. B, repressor element is located between −700 and −400 bp of the DSPP promoter. Serial deletions were made by PCR and cloned into pGL3basic vector. Transient transfections were made as mentioned earlier. Luciferase assay results suggest the presence of a repressor element between −700 and −400 bp region. The value (ratio between firefly and Renilla luciferase) for the full-length DSPP promoter was taken as 100%. Transfections were carried out in triplicate. Average values obtained from three independent experiments after normalizing for transfection efficiency are shown with appropriate standard error.
obtain a mean value. All transfections contained an internal control vector pRL-SV40, which contains a Renilla luciferase gene driven by the SV40 promoter. Double transfections were made with equimolar concentrations of Nrf1 and C/EBPβ expression plasmids.

**Luciferase Assay**—The dual luciferase assay system was used in all experiments and was purchased from Promega and used in an automated luminometer from Dynex as reported earlier (30). Briefly, 50 µg (100 µl) was dispensed into a 96-well microtiter plate and placed in a luminometer. Luciferase activity was measured using Luciferase Assay Reagent II and Stop&Glo reagent. Variations in transfection efficiency were normalized by dividing the measurement for the firefly luciferase activity by that for the Renilla luciferase activity.

**Induction of Mineralization**—Mineralization of cells was induced as described earlier (30). Briefly cells grown to 80–90% confluence were treated with β-glycerophosphate (10 mM) and ascorbic acid (100 µg/ml) in the presence of dexamethasone (10 nM).

**Antibodies, Immunoprecipitation, and Western Blotting**—Mouse monoclonal C/EBPβ (SC-7962) antibody and rabbit polyclonal Nrf1 antibody (SC-13031) was purchased from Santa Cruz Biotechnology. Monoclonal 3XFLAG antibody was purchased from Sigma. Western blot antibody (SC-13031) was purchased from Santa Cruz Biotechnology. For Western blots, antibodies were used at 1:1000 dilutions and membranes were probed with labeled cDNAs followed by autoradiography.

**Mobility Shift Assay**—Electrophoretic mobility shift assay (EMSA) was carried out to monitor the protein binding sites on the DSPP promoter. Nuclear extracts prepared from odontoblasts were used for EMSA as described earlier (31). Briefly, oligos were synthesized as listed in Table I. The oligos were annealed and labeled using T4 polynucleotide kinase in the presence of [γ-32P]ATP. Protein-DNA interactions were performed for 45 min in 4 mM Tris (pH 8.0) containing 60 mM KCl, 5 mM MgCl₂, 4% glycerol, and 100 ng of poly (dG-dC) along with 100 µg/ml bovine serum albumin. Typically 10 µg of nuclear proteins were incubated with 5 fmol of labeled oligos. Supershift experiments were carried out as described above except that the nuclear extracts were preincubated for 30 min at 4 °C with respective antibodies.

**Site-directed Mutagenesis**—Site-directed mutagenesis was carried out to mutate the Nrf1 and C/EBPβ binding sites (listed in Table I) using the site-directed mutagenesis kit (Promega). A mutation at the PKA phosphorylation site of Nrf1 was also carried out in a similar way.

The oligos used were CTCAGAAGGAAGCGCCAGAGGAGGGCC (lowercase and underlined were mutated) replacing Ser409 to Ala.

**Chromatin Immunoprecipitation (CHIP) Assay**—CHIP assay was performed to demonstrate the in vivo interaction of C/EBPβ and Nrf1 to their respective responsive elements using the ChIP assay kit obtained from Upstate USA, Inc. Briefly odontoblasts were transfected with plasmid containing (~700 to ~400 bp) of the DSPP promoter sequence. The DNA-protein complex was cross-linked with 1% formaldehyde for 10 min at 37°C. The cells were washed with phosphate-buffered saline containing protease inhibitors mixture and scraped into a conical tube. The cells were lysed with SDS-lysis buffer (provided in the kit) for 10 min. The DNA was recovered with phenol/chloroform and diluted 10 times. This sample is referred to as the input for the experiment. Respective primary antibody was added to the DNA sample and incubated 12–14 h at 4°C. The antibody-complex was allowed to bind to protein A-agrose for 30 min at 4°C. The DNA bound to the immune complexes was extracted with phenol/chloroform followed by ethanol precipitation and were used as templates for PCR. Anti-acetyl H4 polyclonal antibody (Upstate Biotechnologies) was used as a control for the ChIP assay.

**Protein Kinase Inhibitors**—Three different kinase inhibitors were used. 100 µM H-89 (for PKA), 0.2 µM GF109203X (for protein kinase C), and 100 µM PD 98059 (for mitogen-activated protein kinase) were purchased from Biomol, Inc., and experiments were performed according to standard protocols.

**RESULTS**

**Cloning and Characterization of Rat DSPP Promoter**—Approximately 1.7 kb of a DNA fragment, 5′-upstream of DSPP cDNA was amplified using PCR and cloned into the luciferase reporter vector to assay for transcriptional activity. This 1.7-kb DNA fragment was referred to as the rat DSPP promoter and has 81% similarity to the mouse DSPP promoter (3). Transient transfection assays reveal that the promoter is active only in odontoblast cells and inactive in osteoblast cells (MC3T3-E1) (Fig 1A).

In order to identify the regulatory elements, serial deletions at the 5′-end were made by PCR and cloned into pGL3basic vector and transiently transfected into odontoblast cells. Luciferase assays of these constructs revealed (Fig. 1B) that an initial deletion of a ~1250 bp at the 5′-end of the DSPP promoter increases its transcriptional activity when compared with the full-length DSPP promoter (~1710 bp). Further a deletion of another ~300 bp (from ~700 bp to ~400 bp) increases the transcriptional activity with respect to the full-length DSPP promoter. This suggests the presence of repression elements within this 300-bp region. A 100-bp promoter...
fragment driving the luciferase gene showed greater activity than the full-length promoter sequence.

\( \text{C/EBP}\beta \) and Nrf1 Represses DSPP Promoter Activity—Analysis of the repression activity domain spanning between −700 and −400 bp upstream of the DSPP promoter revealed the presence of a C/EBP\( \beta \) binding site in close proximity to the Nrf1 binding site. Further, this domain is conserved in the rat, mouse, and human DSPP gene. Initial transfections with different C/EBP (α, β, γ, and δ) members clearly showed that only the C/EBP\( \beta \) expression plasmid can act as a repressor for the DSPP promoter (data not shown). We then defined the inhibitory role of C/EBP\( \beta \) on the transcriptional activity of the DSPP gene using a luciferase assay. CMV-driven C/EBP\( \beta \) expression plasmid was transfected with a luciferase reporter in odontoblasts. As shown in Fig. 2A, with an increase in concentration of C/EBP\( \beta \) DNA, a corresponding decrease in the DSPP promoter activity was observed. Approximately 50% reduction of DSPP promoter activity was observed with the expression of 10 µg of C/EBP\( \beta \) expression plasmid. Similarly, 50% reduction in DSPP promoter activity was observed with the expression of 5 µg of Nrf1 plasmid (Fig. 2B). Thus, both C/EBP\( \beta \) and Nrf1 possess intrinsic transcriptional repressor activity. In this study, higher concentrations of the plasmid DNA was used because of the poor transfection efficiency of the odontoblast cell line. Western blot analysis was performed to indicate the transfection efficiency using FLAG antibody (Fig. 2, C and D).

Nrf1 and C/EBP\( \beta \) Synergistically Repress the DSPP Promoter—The binding sites for Nrf1 and C/EBP\( \beta \) were observed to be in close proximity to each other (11-bp apart) and this prompted us to investigate their possible interactions. Results obtained from transient co-transfection with 1710 bp of the promoter region and overexpression of Nrf1 and C/EBP\( \beta \) demonstrated a dramatic reduction in the promoter activity to only 10% indicating a synergistic repressor activity (Fig. 3). This suggests that Nrf1 and C/EBP\( \beta \) act in concert to repress DSPP transcription possibly by functional interaction of these proteins. This result was further confirmed by Northern blot analysis of the DSPP gene after transfection with Nrf1 and C/EBP\( \beta \) (Fig. 3). The presence of an Nrf1- and C/EBP\( \beta \)-mediated repressor element between −700 and −400 bp of the DSPP promoter was identified by transient overexpression of Nrf1 and C/EBP\( \beta \) along with the DSPP promoter deletion constructs (Fig. 4). Results with the 700-bp construct highlight the potential for synergism between Nrf1 and C/EBP\( \beta \) to strongly suppress transcription (Fig. 4).

Association of Nrf1 and C/EBP\( \beta \)—To test whether Nrf1 and C/EBP\( \beta \) mutually interact with each other in vitro, cell extracts were prepared from odontoblasts, and the immunoprecipitation assay was carried out with a monoclonal C/EBP\( \beta \) antibody. The presence of Nrf1 in immunoprecipitates was detected by Western blot analysis using a polyclonal antibody against Nrf1 (Fig. 5, panel a). In a similar manner, immunoprecipitation with Nrf1 antibody followed by cross-blotting with C/EBP\( \beta \) antibody showed the presence of C/EBP\( \beta \) in the complex (Fig. 5, panel b). These results indicate that Nrf1 and C/EBP\( \beta \) co-exist as a complex in vitro. We have also explored the in vivo localization of Nrf1 and C/EBP\( \beta \) by immunostaining with specific antibodies followed by confocal microscopy on MC3T3-E1 cells. As expected, Nrf1 and C/EBP\( \beta \) colocalized within the nucleus of MC3T3-E1 cells (Supplementary Fig. 1).

Binding of Nrf1 and C/EBP\( \beta \) on the DSPP Promoter—The direct interaction of Nrf1 and C/EBP\( \beta \) to their putative binding sites on the DSPP promoter was analyzed by EMSA. Protein components from nuclear extracts of odontoblasts formed a clear complex with oligonucleotides containing Nrf1 and C/EBP\( \beta \) binding sequences (Fig. 6A). The binding of Nrf1 and C/EBP\( \beta \) was confirmed by antibody-mediated supershift. Further, with Nrf1 and C/EBP\( \beta \) antibodies together, a slower migration of the total complex in the gel was observed (Fig. 6A). EMSA results from the mutated oligonucleotides showed that mutation at the Nrf1 binding site did not affect the binding of C/EBP\( \beta \), and mutation at the C/EBP\( \beta \) binding site had no effect on the binding of Nrf1 to the DSPP promoter. Taken together, these data demonstrate that the DNA binding property of Nrf1 is independent of the presence of C/EBP\( \beta \) to its binding site and vice versa.

Nrf1-mediated Repression of DSPP Promoter Requires C/EBP\( \beta \)—Mutations were made at the binding sites of Nrf1 and C/EBP\( \beta \) individually and in combination. These constructs were transfected and analyzed for their activities in the presence of either Nrf1 or C/EBP\( \beta \) expression plasmids, and the controls used for each assay contained the respective mutations. Transient transfections followed by luciferase assays demonstrated that mutation at the Nrf1 binding site increased the DSPP promoter activity to 168% compared with the 100% activity of the wild type (Fig. 6B). Nrf1 overexpression did not
and C/EBPβ binding sites increased the DSPP promoter activity to 245% compared with the wild type, and overexpression of either C/EBPβ or Nrfl did not have any effect on the double mutation constructs (Fig. 6B). The in vivo binding of C/EBPβ and Nrfl to their respective sites was confirmed by ChIP assay (Fig. 6C). Collectively, these results demonstrate that Nrfl requires the presence of C/EBPβ whereas C/EBPβ does not require Nrfl to repress the DSPP promoter activity.

Mapping the Repressor Domains in Both Nrfl and C/EBPβ—As Nrfl and C/EBPβ physically associate and functionally cooperate to repress DSPP promoter activity, we then sought to identify the regions in Nrfl and C/EBPβ that are involved in transcriptional repression. Nrfl and C/EBPβ deletions were cloned into the mammalian expression vector (pCMV-3XFLAG). Deletion constructs are shown schematically in Fig. 7. Odontoblasts were transiently transfected with the different constructs along with a luciferase reporter containing the DSPP promoter and assayed for luciferase activity. Results demonstrate that the full-length C/EBPβ deletion C1 and C2 did not substantially alter or relieve the C/EBPβ-mediated repression but C3 and C4 relieved this activity. Thus, the C-terminal region spanning residues 165–215 in C/EBPβ contained the potential repression elements (Fig. 7A). Similarly, data for Nrfl deletions in Fig. 7B demonstrate that the full-length Nrfl and deletions N1, N2, N3, and N4 did not alter Nrfl-mediated repression of DSPP but constructs N5 and N6 relieved this repression. Thus, residues 480–580 in Nrfl contained potential repression elements. Deletion of this repressor domain from C/EBPβ (residues 165–215) and Nrfl (residues 480–580) failed to repress the DSPP promoter activity. DNA binding property of the constructs without the repression domains were confirmed by ChIP assay (Fig. 7C).

Nrfl-C/EBPβ Association Is Mediated by Their Leucine Zipper Domain—Nrfl and C/EBPβ are bZIP proteins containing a leucine zipper domain at their C-terminal region that is essential for DNA binding and dimer formation. To map the Nrfl and C/EBPβ domain that is required for mutual interaction, we performed immunoprecipitation experiments using deletion constructs in pCMV-3XFLAG vectors. Deletions were made at the leucine zipper region of Nrfl (685–717 amino acids) and C/EBPβ (264–285 amino acids). Immunoprecipitation with FLAG antibody followed by cross-blotting with C/EBPβ or Nrfl antibody showed that deletion of the leucine zipper region of either Nrfl or C/EBPβ affected their physical interaction with each other (Fig. 8). Deletion of Nrfl showed that the 480–580...
region of the protein (Fig. 7) was necessary for its inhibitory activity and did not influence binding with C/EBPβ (Fig. 8). Thus, Nrf1 and C/EBPβ mutually interact with each other through their leucine zipper domain.

**Role of Nrf1 and C/EBPβ on DSPP Expression during Mineralization**—Northern blot analysis has shown that odontoblasts undergoing mineralization in the presence of β-glycerophosphate and ascorbic acid triggered the expression of DSPP (28). In this study, we analyzed the DSPP promoter activity during the mineralization process. The DSPP promoter activity increased steadily during the mineralization process. We observed an increase of 400% on day 12 of mineralization (Fig. 9A). This data correlated well with the in situ hybridization experiments, where DSPP expression increased with the progressive differentiation of odontoblasts (Supplementary Fig. 2).

To examine the functional activity of Nrf1 and C/EBPβ during mineralization, Western blots were performed with specific antibodies against Nrf1 and C/EBPβ. Data in Supplementary Fig. 3 clearly demonstrate a marginal decrease of these proteins during differentiation of odontoblasts. Furthermore, transient transfections of C/EBPβ along with Nrf1 during mineralization showed a gradual loss of transcriptional repressor activity, however, the phosphorylation levels of the factors did not change during the differentiation of the odontoblasts (data not shown). In the absence of mineralization and under normal conditions a 90% reduction in the promoter activity was observed. However, on day 12 of mineralization, this robust transcriptional repressor activity of Nrf1 and C/EBPβ on DSPP was not observed (Fig. 9A). ChIP assay was performed at different days of mineralization to identify the binding of C/EBPβ and Nrf1 to their respective responsive elements. Results indicate that both C/EBPβ antibody and Nrf1 antibody failed to form chromatin complex with their responsive elements during mineralization (Fig. 9B). Thus, our findings suggest that the binding of Nrf1 and C/EBPβ to the DSPP promoter is directly
involved in the transcriptional repression of the DSPP gene during early odontoblast differentiation. With maturation of the odontoblasts, the Nrf1- and C/EBPβ-mediated repression of DSPP is relieved, leading to increased expression of DSPP.

Phosphorylation of Ser599 on Nrf1 Is Essential for C/EBPβ Interaction—Protein kinase A is known to have a profound effect on the expression of dentin and enamel genes (32). Treatment of odontoblasts with H-89, an inhibitor for protein kinase A, produced a profound effect on the expression of dentin and enamel genes (32). Treatments with H-89 decreased the expression of DSPP and EMD genes by 85% compared with the control which was 100% (Fig. 10A). Moreover, the repression of DSPP gene by C/EBPβ was reversed by this substitution (Fig. 11C).

**Phosphorylation of Ser599 on Nrf1**

**A** Schematic illustration of the deletions made to identify the repressor domain within the C/EBPβ protein. The constructs were amplified by PCR followed by the cloning into the CMV-FLAG vector. The repressor domain identified between 165 and 215 amino acids was deleted by introducing Sacl enzyme site. C/EBPβ deletion constructs were transfected along with pGL3-DSPP reporter vector. Luciferase assays were carried out as described earlier. The luciferase activity was compared with the control (pGL3-DSPP reporter, taken as 100%). FL represents the full-length construct whereas C1–C4 represents the C/EBPβ deletion constructs. Constructs were carried out in triplicate. Mean values were plotted with standard error. B, schematic illustration of the deletions made to identify the repressor domain within the Nrf1 protein. The constructs were amplified by PCR followed by the cloning into the CMV-FLAG vector. The repressor domain identified between 480 and 580 amino acids was deleted by introducing the Sacl enzyme site. The constructs were used in the experiments described below. Nrf1 deletion constructs were transfected along with pGL3-DSPP reporter vector. Luciferase assays were carried out as described earlier. The luciferase activity was compared with the control (pGL3-DSPP reporter, taken as 100%). FL represents the full-length construct whereas N1–N6 represents the Nrf1 deletion constructs. Transfections were carried out in triplicate. Mean values were plotted with standard error. C, ChIP assay. Expression plasmids (pCMV-3XFLAG) containing deletions between 165 and 215 amino acids (CΔRD) or between 480 and 580 amino acids (NΔRD) were co-transfected along with pGL3-DSPP reporter vector. Luciferase assays were carried out as described earlier. The luciferase activity was compared with the control (pGL3-DSPP reporter, taken as 100%). Lane C∆RD represents the C/EBPβ with deleted repressor region while NΔRD represents the Nrf1 with the deleted repressor region. The immunoprecipitated DNA was amplified by PCR using specific primers. Lane M represents the DNA marker. Acetyl-H4 polyclonal antibody was used as a control for the ChIP assay. T, represents the total input; H, represents the ChIP with H4 antibody.

**B** Northern dot blot (Fig. 10A). We next analyzed the effect of H-89 on the phosphorylation of Nrf1. Immunoprecipitation with Nrf1 antibody and cross blotting with phosphoserine antibody clearly demonstrates that Nrf1 undergoes serine phosphorylation and in the presence of H-89 this phosphorylation is inhibited in odontoblasts (Fig. 11A). Nrf1 has a protein kinase A phosphorylation site at serine 599. In order to investigate the role of this phosphorylated serine on its interaction with C/EBPβ, we substituted serine 599 with alanine. This modification knocked off the C/EBPβ interaction as shown by the immunoprecipitation assay (Fig. 11B). These results imply that phosphorylation of serine 599 by protein kinase A is important for its interaction with C/EBPβ. Moreover, the repression of DSPP promoter activity observed with the wild-type Nrf1 protein was reversed by this substitution (Fig. 11C).

**Fig. 7.** A, schematic illustration of the deletions made to identify the repressor domain within the C/EBPβ protein. The constructs were amplified by PCR followed by the cloning into the CMV-FLAG vector. The repressor domain identified between 165 and 215 amino acids was deleted by introducing Sacl enzyme site. C/EBPβ deletion constructs were transfected along with pGL3-DSPP reporter vector. Luciferase assays were carried out as described earlier. The luciferase activity was compared with the control (pGL3-DSPP reporter, taken as 100%). FL represents the full-length construct while C1–C4 represents the C/EBPβ deletion constructs. Transfections were carried out in triplicate. Mean values were plotted with standard error. B, schematic illustration of the deletions made to identify the repressor domain within the Nrf1 protein. The constructs were amplified by PCR followed by the cloning into the CMV-FLAG vector. The repressor domain identified between 480 and 580 amino acids was deleted by introducing the Sacl enzyme site. The constructs were used in the experiments described below. Nrf1 deletion constructs were transfected along with pGL3-DSPP reporter vector. Luciferase assays were carried out as described earlier. The luciferase activity was compared with the control (pGL3-DSPP reporter, taken as 100%). FL represents the full-length construct whereas N1–N6 represents the Nrf1 deletion constructs. Transfections were carried out in triplicate. Mean values were plotted with standard error. C, ChIP assay. Expression plasmids (pCMV-3XFLAG) containing deletions between 165 and 215 amino acids (CΔRD) or between 480 and 580 amino acids (NΔRD) were co-transfected with the plasmid containing the DSPP promoter region between ~700 and ~400 bp. The protein-DNA complex was cross-linked with 1% formaldehyde, cells were lysed, and DNA was extracted with phenol:chloroform. This is the total input (T); ChIP was carried out with the FLAG antibody. Lane CΔRD represents the C/EBPβ with deleted repressor region while NΔRD represents the Nrf1 with the deleted repressor region. The immunoprecipitated DNA was amplified by PCR using specific primers. Lane M represents the DNA marker. Acetyl-H4 polyclonal antibody was used as a control for the ChIP assay. T, represents the total input; H, represents the ChIP with H4 antibody.

**Fig. 8.** Identification of Nrf1 and C/EBPβ interacting domains. Odontoblasts were transfected transiently with different deletion constructs of either Nrf1 or C/EBPβ. Immunoprecipitation was carried out for the total protein of the transfected cells with the FLAG antibody as described under “Materials and Methods.” The immune complexes were resolved on SDS-PAGE followed by Western blotting and probing with either Nrf1 antibody (panel A) or C/EBPβ antibody (panel B). FL represents the full-length protein, C1–C4 represents the C/EBPβ deletions and N1, N2, N3, and N6 represents the Nrf1 deletions. Deletions of bZIP domain of either Nrf1 (685–717 amino acids) or C/EBPβ (264–285 amino acids) was identified to be required for its interaction with each other. Deletions of Nrf1 showed that the 480–580 amino acids region of the protein (upstream of bZIP) is necessary for inhibitory activity, and this region does not influence its interaction with C/EBPβ.
Transcriptional repression, like transcriptional activation, has emerged as a common mechanism of transcriptional regulation (33). Transcription repression plays an important role in tissue specificity of gene expression and one such tissue-specific gene regulated by transcriptional repression is reported here. DSPP is a tooth-specific gene expressed by odontoblasts and its expression is spatially and temporally regulated during odontoblast differentiation. The expression of DSPP in terminally differentiated odontoblasts exceeds 15–20-fold compared with its expression in early differentiated odontoblasts. Initial characterization of the rat DSPP promoter indicates the presence of a repressor element between −700 and −400 bp. In this manuscript, we present evidence for a novel regulatory mechanism by which protein-protein interactions between members of the CNC-bZIP family, namely, Nrf1 and C/EBPβ, achieve and maintain repression of DSPP expression during early odontoblast differentiation.

Several lines of evidence support the transcriptional repression activity by both Nrf1 and C/EBPβ. They possess intrinsic repressor activity as demonstrated by transient transfection assays with the DSPP promoter. The synergistic suppression (to 10% promoter activity) suggests the possibility of interaction between Nrf1 and C/EBPβ as their DNA binding sites on the DSPP promoter are only 11 base pairs apart. By means of immunoprecipitation and ChIP assay we have clearly demonstrated that C/EBPβ associates and functionally cooperates with Nrf1 in vivo. Deletion analysis indicated that this inter-
action was mediated by the bZIP domain present in both Nrf1 and C/EBPβ.

An insight into the molecular mechanisms by which Nrf1 and C/EBPβ represses DSPP transcription was further obtained from mobility shift assays, which showed that both Nrf1 and C/EBPβ could bind independently to their respective domains on the DSPP promoter. Mutational studies suggest that the binding of C/EBPβ to its respective site is independent of Nrf1 binding to the DNA and vice versa. Results from transient transfections of DSPP promoter constructs with mutated Nrf1 and C/EBPβ sites indicate that functionally C/EBPβ could exert its repressor activity on the DSPP promoter containing the mutated Nrf1 site. In contrast, Nrf1 could not repress the promoter activity by binding itself to the DSPP promoter containing the mutated C/EBPβ site. The repressor domain in both Nrf1 and C/EBPβ was also identified, and the functionality of these domains was confirmed by deletion assays. Results presented in this study also indicate that phosphorylation of serine 599 in Nrf1 plays an important role in controlling its DNA binding activity with C/EBPβ. When Ser599 was replaced with Ala, the mutated Nrf1 protein failed to interact with C/EBPβ and failed to repress the DSPP promoter activity when compared with the wild-type Nrf1.

The emerging concept from our study is that Nrf1 and C/EBPβ have unique physiological role in regulating DSPP expression. During the early stages of odontoblast differentiation, expression of C/EBPβ and Nrf1 and their interaction to form a complex or their interaction with a co-repressor could repress DSPP promoter activity to its basal level. During odontoblast differentiation, there are two possible scenarios, one is the inability of Nrf1 or C/EBPβ to bind to their responsive elements on the DSPP promoter during mineralization and the second would be a possible disruption of the bigger repressor complex formed by Nrf1 and C/EBPβ by interacting with other common co-repressors. Thus abrogation of Nrf1 and C/EBPβ functional activity results in increased levels of DSPP expression during mineralization.

C/EBPβ is known to participate in the regulation of gene expression by functioning either as transcriptional activator or repressor. In mineralized tissues (such as bone and teeth), C/EBPβ has been shown to interact with Runx2 synergistically to enhance the transcription of osteocalcin gene expression (34). Furthermore, transgenic mice overexpressing dominant-negative form of C/EBPβ have osteopenia (35). These results indicate that during osteoblast differentiation C/EBPs in general may have a complicated mechanism by which gene expression is regulated. However the role of C/EBPβ on tooth-specific gene expression has not been addressed yet.

Nrf1 is a ubiquitous transcription factor. Gene knockout studies have clearly demonstrated the importance of this molecule during development. Nrf1 can form a homodimer as well as heterodimer with small Maf proteins.

Dual functions for DNA-binding proteins, namely activation and repression is an emerging mechanism of transcriptional regulation. The oncogene c-Myc acts as an activator for transcription of target genes. Myc forms a heterodimeric complex with Max (Myc-associated factor X) and binds to recognition sequences called E-boxes and represses transcription. c-Myc was shown to inhibit adipocyte differentiation by repressing the expression of C/EBPs (required for adipocyte differentiation) (36). Thus c-Myc has been reported to down-regulate several other genes (37). Two possible mechanisms have been proposed for this dual action: one suggesting that c-Myc could activate the transcription of transcriptional repressor genes thereby inhibiting the expression of target genes. The second hypothesis suggests that the Myc-Max complex bind to the DNA sequences at the core promoter region of the target genes thereby blocking the binding of the basal transcription machinery complex. However the signals that control the recruitment of these factors to either the core promoter region or to the E-boxes have not been completely understood.

Regulation of gene expression by cooperative binding of transcription factors constitutes an important mechanism to induce cellular differentiation. In summary, we have shown for the first time that the interplay between bZIP transcription factors Nrf1 and C/EBPβ can act as a transcriptional repressor for a dentin-specific gene DSPP. Thus the repressor effects of Nrf1 and C/EBPβ contribute to the tissue specificity of the DSPP gene during dentin formation. As the odontoblasts differentiate and become functional by actively synthesizing a mineralized matrix, these bZIP proteins failed to exert their inhibitory effect indicating that there are multiple
partners involved in the repression of the \textit{DSPP} gene. In the last few years several co-repressors have been identified in eukaryotes. Co-repressors are non-DNA-binding proteins, usually recruited by the DNA binding silencers of transcription. Co-repressors play an important role in maintaining the cellular phenotype in an undifferentiated state by repressing several important genes required for differentiation. A wide variety of transcription factors and their interactions with co-repressors were reviewed recently (38).

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The CCAAT Enhancer-binding Protein (C/EBP)β and Nrf1 Interact to Regulate Dentin Sialophosphoprotein (DSPP) Gene Expression during Odontoblast Differentiation

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