Transcriptional Regulation of Dentin Matrix Protein 1 by JunB and p300 during Osteoblast Differentiation*

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Dentin matrix protein 1 (DMP1) is an acidic noncollagenous protein localized specifically in the mineralized matrix of bone and dentin. Expression analyses demonstrate that DMP1 is differentially regulated in osteoblasts and odontoblasts. Earlier we have reported on the transcriptional regulation of DMP1 by c-Fos and c-Jun (AP-1) transcription factors. Results from earlier study indicate that c-Fos and c-Jun play an important role in early osteoblast differentiation, whereas they do not have a significant effect on the terminally differentiated osteoblasts. In this paper, we demonstrate a regulatory mechanism by which JunB transcriptionally controls the expression of DMP1 during osteoblast differentiation. The cooperative interaction of JunB with p300 has been shown to dramatically modulate the DMP1 promoter activity during mineralization. Immunoprecipitation and chromatin immunoprecipitation analysis demonstrate the interaction of JunB and p300 in vivo. Further, phosphorylation of JunB at Ser-79 was found to be essential for its interaction with p300. Intrinsic histone acetyltransferase activity of p300 also plays a critical role in regulating DMP1 gene expression.

Osteoblasts and odontoblasts, the principal cells responsible for bone and dentin formation, synthesize extracellular matrix molecules that are responsible for mineralized matrix formation. Dentin matrix protein 1 (DMP1) is an acidic noncollagenous protein localized specifically in the mineralized matrix of bone and dentin. Expression analyses demonstrate that DMP1 is differentially regulated in osteoblasts and odontoblasts. During osteoblast differentiation, DMP1 is constitutively expressed, whereas in differentiating odontoblasts the expression is temporally and spatially regulated. In our earlier studies, we have demonstrated that blocking DMP1 expression inhibits osteoblast differentiation. Therefore, identifying factors that are involved in regulating DMP1 expression during osteoblast differentiation will be beneficial in deciphering the relationship between DMP1 expression and osteoblast differentiation.

Transcriptional regulations by the AP-1 family of transcription factors are known to be mediated by extracellular signals (2–4). The AP-1 transcription factor is composed of a mixture of homo- and heterodimers formed between Jun, Fos, and ATF families of proteins. AP-1 factors have been a paradigm for transcription factors that are involved in several cellular functions such as apoptosis (5), differentiation (2, 6, 7), proliferation, and transformation. Published studies demonstrate that AP-1 transcription factors regulate the expression of a myriad of genes in a variety of tissues and cell types.

Jun family proteins, which include c-Jun, JunB, and JunD, can form a homodimer or heterodimer with Fos family members. c-Jun knockout mice embryos die at midgestation, due to hepatic failure, indicating that neither JunB nor JunD functionally can replace c-Jun (8, 9). Similarly, mice lacking specific members of the Fos and ATF families are viable and fertile, although the adults show specific defects in distinct tissues, implying that only a subset of AP-1 target genes is affected in these mutants (10–13). Specific roles for AP-1 in skeletal development have been identified mainly through knockout studies in mice (14, 15). The c-Fos knockout shows a phenotype similar to osteopetrosis due to a complete block in osteoclast differentiation (16). On the other hand, transgenic mice overexpressing c-Fos develop osteosarcomas due to excessive differentiation of osteoblast (17). Transgenic mice overexpressing Fra-1, a splice variant of FosB, develop osteosclerosis caused by accelerated differentiation of osteoprogenitors into mature osteoblasts (18, 19). All of these results suggest that although AP-1 transcription factors have high homology between family members, each of these proteins possesses specific intrinsic function, and therefore they cannot be substituted by other members.

The transcriptional cofactor p300 is a large regulatory nuclear phosphoprotein with multiple functional domains. Several conserved motifs have been identified in p300; these include a bromodomain, a glutamine-rich (Q-rich) region, and three cysteine-histidine (CH-rich) regions (CH1, CH2, and CH3). The bromodomain is found to play an important role in protein-protein interactions and the association of histones with chromatin (20–23). The Gln-rich region, which is located at the C terminus of the protein, has features similar to those of the glutamine-rich transcriptional activation domains found in a number of transcriptional activators (24, 25). The CH3 region is the site of interaction with many different factors, which include the adenovirus E1A protein, the coactivator...
PCAF (26), RNA polymerase-containing complexes, and TFIIB (27). In addition, other regions in p300 serve as binding sites for a variety of DNA-binding transcriptional activator proteins. p300 has acetyltransferase activity and possesses a region of similarity to members of the GCN5-related N-AT family of proteins (28, 29). 

Earlier we have reported on the transcriptional regulation of DMP1 by c-Fos and c-Jun (AP-1) transcription factors. Results from this study indicate that c-Fos and c-Jun play an important role in early osteoblast differentiation, whereas they do not have a significant effect on the terminally differentiated osteoblasts (30). In this paper, we demonstrate a regulatory mechanism by which JunB transcriptionally controls the expression of DMP1 during osteoblast differentiation. The cooperative interaction of JunB with p300 has been shown to dramatically modulate the DMP1 promoter activity during mineralization. Further, phosphorylation of JunB at Ser-79 was found to be essential for interacting with p300. Intrinsic histone acetyltransferase (HAT) activity of p300 also plays a critical role in regulating DMP1 gene expression.

**MATERIALS AND METHODS**

**Plasmids**—A 3-kb genomic clone containing the 5' upstream regulatory sequences for the rat DMP1 gene was isolated and characterized (31). PCR-mediated serial deletions were made and cloned into the promoterless luciferase reporter plasmid construct. The expression plasmids for p300 and p300 deletion constructs including p300-AT were generous gifts from Dr. Thimmappaya (Northwestern University, Chicago, IL). The expression vector containing p38 kinase and p38 kinase dominant negative constructs were kind gifts from Dr. Philipp E. Scherer (32).

**Cell Culture and Transfections**—The preosteoblast cell line MC3T3-E1 was cultured as described earlier (31). Briefly, the cells were cultured in Dulbecco's modified Eagle's medium/F-12 medium containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% fungizone. For transient transfections, 5 g of plasmid DNA was mixed with 100 ng of poly(dG-dC) along with 100 ng of poly(dG-dC) and DMP1 promoter as the template. This sequence resides between positions -32P/ATP. Protein-DNA interactions were performed for 45 min in the presence of 0.1 mM MgCl2, 20% 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 oligonucleotides. The specificity of the protein-DNA complexes was demonstrated by supershift experiments, which were performed as described above except that the nuclear extracts were preincubated for 30 min at 4 °C with JunB antibody.

**Site-directed Mutagenesis**—Site-directed mutagenesis was performed using the site-directed mutagenesis kit (Promega) according to the manufacturer's instructions. Two oligonucleotides were designed to replace the conserved phosphorylation sites on JunB, namely Ser-79 to Ala and Thr-299 to Ala using the primers (Ser-79, GACAGGGG-CAGCGCCGCGACCTACGCCTC; Thr-299, AAGGGAAAGGCGCCG-CCTGCTGAAAGCGG) (codon changes are underlined). Incorporation of the mutation was confirmed by DNA sequence analysis. 

**Western Blot and Immunoprecipitation Assays**—Western blot analysis was performed as described by Towbin et al. (33). Cell lysates were subjected to immunoprecipitation by incubating with primary antibody for 3 h at 4 °C. Protein A-agarose was added to the antibody-antigen complex. The beads were washed with PBS containing 10 mM NaCl. The beads were resuspended in 1× SDS-PAGE buffer, and proteins were denatured and resolved on SDS-PAGE followed by Western blotting to analyze the bound complex proteins.

**Expression of GST Fusion Proteins**—Expression of GST fusion proteins in Escherichia coli was induced by adding 0.5 mM isopropylthio-galactoside for 3 h to the bacterial culture in the exponential phase of growth. The bacteria were centrifuged, resuspended in EBC buffer (120 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5% (v/v) NP-40, and complete protease inhibitor mixture (Pierce)), lysed using a sonicator, and centrifuged to remove bacterial debris. Soluble proteins were purified by glutathione-Sepharose beads. The GST fusion proteins were then used for a pull-down assay.

**RNA and Northern Blot Analysis**—Total RNA was isolated from cultured cells at log phase with the use of TriZol reagent (Invitrogen). RNA gel electrophoresis and Northern blotting were performed as described by Sambrook et al. (34). Twenty micrograms of total RNA was resolved on a 0.8% agarose gel containing formaldehyde. The RNA was transferred to a Hybond nylon membrane (Amersham Biosciences). The membrane was prehybridized with the use of PerfectHyb-Plus (Sigma) and probed with randomly labeled (Decaprime kit; Ambion, Austin, TX) probes, washed, and developed overnight.

**Isolation of Nuclear Extracts**—Nuclear extracts from MC3T3E1 cells were isolated for electrophoretic mobility shift assay. Briefly, cells were washed twice with PBS and incubated for 20 min at 4 °C in 10 mM HEPES buffer (pH 7.5) containing 10 mM KCl, 0.1 mM EDTA, 0.1% Nonidet P-40, 1 mM dithiothreitol, and 1× protease mixture (Pierce). The cell lysates were subjected to centrifugation for 5 min at 5000 × g. The cell pellet was further incubated for 30 min at 4 °C with 50 mM HEPES buffer (pH 7.5) containing 420 mM KCl, 0.1 mM EDTA, 5 mM MgCl2, 20% glycerol, 1 mM dithiothreitol, 2 mM phenethylsulfonyl fluoride, and other protease inhibitors. After the lysis of the nuclei, the lysates were centrifuged at 14,000 × g for 30 min to collect the nuclear extract.

**Electrophoretic Mobility Shift Assay**—Nuclear extracts from MC3T3E1 cells were prepared as described before. Oligonucleotides (AAGAG-AATAAGTTTATGGTCATTTTCTTTCTATTATTG) were synthesized, annealed, and labeled using T4 polynucleotide kinase in the presence of [γ-32P]ATP. Protein-DNA interactions were performed for 45 min in 0.1 mM Tris (pH 8.0), 60 mM KCl, 5 mM MgCl2, 20% 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 oligonucleotides. The specificity of the protein-DNA complexes was demonstrated by supershift experiments, which were performed as described above except that the nuclear extracts were preincubated for 30 min at 4 °C with JunB antibody.

**ChIP assay**—The dual luciferase assay system was used in all experiments and was obtained from Promega and used in an automated luminometer from Dynex as reported earlier (31). Briefly, 50 μg of the lysate (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 was normalized by dividing the measurement for the firefly luciferase activity by Renilla luciferase activity.

**Induction of Mineralization**—Cells were induced to produce a mineralized matrix as described earlier (1). 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). DMP1 promoter (CBP) assay—CBP assay kit obtained from Upstate Biotechnology was used in this experiment. Briefly, MC3T3-E1 cells were transfected with plasmid containing the DMP1 promoter. The DNA-protein complex was cross-linked with 1% formaldehyde for 10 min at 37 °C. The cells were washed with PBS containing protease inhibitor mixture and scraped into a conical flask. The cells were lysed with SDS-sample buffer (provided in the kit) for 10 min. The DNA was recovered with phenol/chloroform and diluted 10
DMP1 is in the region between 1950 and 1940 bp upstream with respect to the transcriptional start site. To determine if the transcriptional activity of DMP1 would be mediated by JunB, transfection assays were performed using MC3T3-E1 cells cotransfected with JunB and p300 in combination with the DMP1 promoter. The mutated promoter was analyzed by co-transfections with JunB and p300. Serial deletion constructs were made at the 5′-end of the DMP1 promoter and cloned into pGL3-basic vector to investigate the activity of the JunB binding site. These deletions were co-transfected with JunB and p300. The transfections contained an internal control of Renilla luciferase vector. The value (ratio between firefly and Renilla luciferase) for the full-length DMP1 promoter was taken as 1-fold (−3 kb). The individual value for each of the constructs was divided by the values obtained for the full-length DMP1 promoter (−3 kb) to calculate the fold increase in the luciferase activity. All transfections were carried out in triplicate; the mean value was taken with appropriate S.E. The region between −2000 and −1500 bp has JunB binding activity. Reporter transfections were carried out in triplicate and repeated three times to obtain a mean value. Thus, the region between −2000 and −1500 bp is required to execute the JunB- and p300-mediated activation of the DMP1 promoter. Next, a computer program (Matinspector) was used to disrupt the JunB binding site on the DMP1 promoter, using the designed oligonucleotide AATGAATTTTcGaCATTTTCTTTC (lowercase indicates the modified base) and DMP1 promoter as the template. This sequence resides within the region −1950 to −1940 of the DMP1 promoter. The mutated promoter was then analyzed by co-transfections with JunB and p300. All transfections were carried out in triplicate; the mean value was taken with appropriate S.E.

Thus, the prediction of JunB binding site resides between −1950 and −1940 bp on the DMP1 promoter and is necessary for its activation.

Serine 79 Phosphorylation Is Important for JunB Activation—It is well known that JunB and p300 are nuclear phosphoproteins. In order to determine how phosphorylation regulates their function and thus mediate their activation of the DMP1 promoter, we examined the involvement of kinases responsible for phosphorylation of JunB and p300. The involvement of MAP and p38 kinases was determined by using specific inhibitors. The addition of MAP kinase inhibitor PD98509 to either JunB or p300 alone did not have any significant effect on
the reporter activity. On the other hand, the addition of p38 kinase inhibitor SB203580 to MC3T3 cells in the presence of JunB along with p300 reduced the DMP1 promoter activity dramatically (equal to the activity of the control) when compared with the reporter activity in the absence of the inhibitor (Fig. 3). These results clearly demonstrate that phosphorylation by p38 kinase is essential for the regulation of DMP1 transcription by JunB and p300.

In order to confirm the involvement of p38K on the DMP1 promoter activity mediated by JunB and p300, we used expression vectors containing p38K and dominant negative p38K, which were characterized earlier (32). Transfection results clearly demonstrate that p38K wild type in the presence of JunB along with p300 increased the transcriptional activity of the DMP1 promoter by about 2-fold compared with the transfection without p38K. Furthermore, p38K did not have any significant effect on the activation of DMP1 promoter by p300 alone (Fig. 4). These results demonstrate the important role of p38K in the activation of the DMP1 promoter. This was further confirmed by transfections with the dominant negative p38K in the presence of JunB and p300. Results demonstrate that dominant negative p38K along with JunB and p300 suppresses the DMP1 promoter activity significantly (~<1-fold) compared with the DMP1 promoter alone (Fig. 4). Thus, these results demonstrate that p38K stimulates JunB- and p300-mediated transcription of DMP1 possibly through direct phosphorylation events.

Analysis of the primary sequence of JunB revealed the presence of two potential phosphorylation sites, one at Ser-79 and another at Thr-299. To investigate the role of these potential amino acids on JunB activation by phosphorylation, mutations were made to replace these amino acids with Ala. Results demonstrate that the S79A mutation by itself reduced the transcriptional ability. Moreover, the increased DMP1 promoter activity observed with wild type JunB along with p300 was not observed when transfected with S79A-JunB along with p300. However, mutation at T299A on JunB did not have any effect on the JunB-mediated DMP1 promoter activity either alone or along with p300 (Fig. 5A). Further, p38K failed to activate the S79A-JunB, indicating that this Ser-79 residue is an important phosphorylation site for p38K. Thus, we conclude that phosphorylation of JunB at Ser-79 is necessary for the transcriptional activation of DMP1.

Interaction of JunB with p300—Since p300 can cooperate with JunB in the transcriptional activation of DMP1, we therefore determined whether these factors might interact with each other and exist as a complex in vivo. Immunoprecipitation analysis with JunB antibody and cross-blotting with p300 antibody clearly demonstrated the presence of p300 in the complex (~300 kDa). This result was further confirmed by immunoprecipitate analysis with p300 antibody and cross-blotting with JunB antibody (Fig. 6A). Thus, these results confirm the presence of p300 and JunB in an associated complex in vivo.

To investigate the association of p300 with the JunB mutations, immunoprecipitations were performed with monoclonal FLAG antibody to immunoprecipitate the complex. Immunopre-
p38 kinase phosphorylates Ser-79 in JunB. Primary sequence analysis of JunB indicated a potential phosphorylation site at Ser-79 and Thr-299 by p38 kinase. Ser-79 (M-I) and Thr-299 (M-II) were mutated using a site-directed mutagenesis kit and modified into Ala; further, a NotI restriction enzyme site was introduced to identify the mutation. These mutants were analyzed for their activity in the presence of p38K and also p300. The value (ratio between firefly and Renilla luciferase) for the full-length DMP1 promoter (~3 kb) was taken as 1-fold. DMP1 promoter activities in the presence of these mutants were compared with the control (full-length DMP1 promoter).

Precipitation with FLAG antibody and cross-blotting with p300 antibody showed that mutation at Ser-79 affected the interaction of JunB with p300. On the other hand, mutation at Thr-299 did not have any effect on the complex formation with p300 (Fig. 6B). The presence of JunB was confirmed with JunB antibody (Fig. 6B). Thus, these results provide further evidence that Ser-79 on JunB is required for the p38K phosphorylation and also for its interaction with p300.

To map the region within p300 that binds to JunB, various p300 deletions cloned into the GST fusion vector as reported earlier were used (35). A GST pull-down assay on MC3T3-E1 extracts and Western blotting with JunB antibody clearly demonstrate that JunB binds to the C-terminal region of p300 (Fig. 7).

**Binding of JunB to DMP1 Promoter**—The physical interaction of JunB protein to its binding site on DMP1 promoter was studied by mobility shift assay. We have identified an AP-1 site on the DMP1 promoter sequence (between −1950 and −1940 bp). Double-stranded, labeled oligonucleotides were used for electrophoretic mobility shift assay. Competition assay results in Fig. 8A demonstrate the specificity of the binding site. Fig. 8B shows the binding of wild type JunB (lanes 2 and 3), S79A-JunB (lanes 4 and 5), and T299A-JunB (lanes 6 and 7) to the labeled binding sites. Specific supershift in the presence of JunB-specific antibody was also observed. These results further confirmed that mutation at S79A on JunB did not have any effect on the DNA binding property of JunB. ChiP analysis with antibody against p300 further confirmed the endogenous JunB interactions with the DMP1 promoter (Fig. 8C), whereas the Ser-79 mutant failed to interact with p300 (data not shown).

**HAT Activity during Late Osteoblast Differentiation and Expression of DMP1**—The expression of DMP1 increased during osteoblast differentiation. Northern blot analysis of total RNA isolated from different days (corresponding to osteoblast differentiation) showed a linear progressive increase in DMP1 message (Fig. 9A). This result was further confirmed by luciferase activity driven by the DMP1 promoter during osteoblast differentiation (Fig. 9B). These results suggest that DMP1 transcription is activated during osteoblast differentiation.

Next we investigated the role of acetylation of histones on the chromatin of DMP1 promoter using HDAC inhibitors and assaying for DMP1 transcription. Trichostatin A and sodium butyrate are the most commonly used HDAC inhibitors. Treatment of cells with sodium butyrate (5 mM) or trichostatin A (100 ng/ml) clearly increased DMP1 transcription compared with untreated cells (Fig 10). These results indicate a potential role of HAT activity on DMP1 transcription.

HAT assay was then performed to correlate the transcriptional activity of DMP1 during osteoblast differentiation. Cells were cultured with the mineralization media, and extracts...
JunB increased the expression of DMP1 with wild type p300 and p300-AT constructs. The expression of DMP1 possesses no HAT activity (p300-AT). Stable cell lines were made by introducing a mutation in the HAT domain. This mutant p300 possesses HAT activity. Therefore, we addressed the question of whether the HAT domain in p300 plays an important role in JunB-mediated transcriptional activation of DMP1.

p300 has been widely reported by several research groups to possess HAT activity. Therefore, we addressed the question of whether the intrinsic HAT activity of p300 plays a role in the transcriptional activation of DMP1 by JunB and p300. To address this, we obtained an expression vector for p300 containing a mutation in the HAT domain. This mutant p300 possessed no HAT activity (p300-AT). Stable cell lines were made with wild type p300 and p300-AT constructs. The expression of DMP1 was analyzed by Northern blot. Transfections with JunB increased the expression of DMP1 by 3-fold in the stable cell line with the p300 wild type construct; on the other hand, JunB transfection did not have any effect on the stable cell line containing the p300-AT construct when compared with the control JunB (Fig. 12). These results clearly indicate that the HAT domain in p300 plays an important role in JunB-mediated transcriptional activation of DMP1.

**DISCUSSION**

DMP1 is a multifunctional protein essential in osteoblast differentiation and calcified tissue formation. Expression of antisense DMP1 in preosteoblastic cells failed to differentiate into mature osteoblasts (1). A recent report on DMP1 null mice shows sparse amounts of mineralized dentin (36). Thus, these studies suggest that the putative function of DMP1 may be crucial to the overall biomineralization process.

Previously, we had demonstrated that the members of AP-1 family (c-Jun and c-Fos) were involved in the transcriptional regulation of the DMP1 gene during early osteoblast differentiation. Moreover, these factors (c-Jun and c-Fos) were not required for DMP1 expression during late stages of osteoblast differentiation. This was also well supported by experimental results demonstrating a decrease in c-Jun expression during osteoblast differentiation (30). In this study, we have examined the ability of JunB to interact with p300 and stimulate the transcriptional activation of DMP1 promoter during late osteoblast differentiation and mineralization.

The transcription factor AP-1 regulates the expression of a myriad of genes in a variety of tissues and cell types. Evidence for specific functions of AP-1 subunits was shown for c-Jun and JunB, which act antagonistically to control cell transformation, differentiation, and expression of AP-1-dependent target genes (37–40). Loss of function approaches in mice led to the identification of specific, nonoverlapping functions of c-Jun and JunB in hepatogenesis and heart development (8, 41) and placentation (42). JunB knockout mice demonstrated that it is essential for placentation. Conditional knockout using CRE system showed that the mutant mice develop severe osteopenia along with reduced bone formation (43). In our studies, the JunB binding site on DMP1 was localized to the region between nucleotides containing the JunB binding sites from the DMP1 promoter region were synthesized, annealed, and labeled using T4 polynucleotide kinase in the presence of \(^{[\gamma-32P]}\)ATP. A competition assay was carried out with the unlabeled double-stranded oligonucleotides and the MC3T3 cell extract in order to show the specificity of the binding. Electrophoretic mobility shift assay was performed to characterize the binding of JunB to its site on the DMP1 promoter. End-labeled oligonucleotides were incubated with nuclear extracts (from MC3T3 cells or MC3T3 cells transfected with either Ser-79 mutant or Thr-299 mutant) 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. The specificity of the protein DNA complexes was demonstrated by supershift with JunB-specific antibody or FLAG antibody. Lane 1, free probe; lanes 2, 4, and 6, binding of wild type, Ser-79 (mutant-I), and Thr-299 (mutant-II), respectively. Lane 3 represents the supershift in the presence of JunB antibody. Lanes 5 and 7, supershift in the presence of FLAG antibody. C, ChIP assay was performed to demonstrate the in vivo binding of JunB to DMP1 promoter and with p300. MC3T3 cells were transfected with the DMP1 promoter. The protein-DNA complexes were cross-linked with formaldehyde. The DNA-protein complex was extracted, and this is referred to as input (I). JunB or p300 antibody was added, and the specific protein-DNA complexes were pulled down using protein A-agarose. DNA was extracted from the eluate and analyzed with gene-specific primers by PCR. J, ChIP with JunB antibody; P, ChIP with p300 antibody; M, DNA marker. ChIP assay confirms the association of JunB and p300 in MC3T3 cells.
cells were induced to differentiate into mature osteoblasts in the presence of ascorbic acid and β-glycerophosphate. Total RNA was extracted from these cells at different days and analyzed by Northern blot for the expression of DMP1. GAPDH was used as an internal control to normalize the RNA amount. B, MC3T3 cells were allowed to undergo differentiation as mentioned earlier. These cells were transfected with DMP1 promoter in pG3L-basic vector. A luciferase assay was carried out to monitor the promoter activity. The value (ratio between firefly and Renilla luciferase) at day 0 was taken as 100%. The percentage activation of the DMP1 promoter was calculated by comparing the value with day 0. All transfections were carried out in triplicate; the mean value was taken with appropriate S.E.

factors in order to activate the transcription of specific target genes of the transcription factors. Several AP-1 factors, such as c-Jun, c-Fos, etc., have been shown to interact specifically at the CH domains of p300. In this study, we have provided evidence for the functional cooperation between JunB and p300 in the activation of the DMP1 promoter. These associations were ascertained by immunoprecipitation assays and GST-fusion protein pull-down assays. Further analysis revealed that JunB can associate specifically with the C terminus region of p300. This result differs from an earlier observation that JunB can interact with both the c-terminus and the middle region of p300 in HeLa and U2OS cells (44). The plausible explanation is that during osteoblast differentiation, the interaction of p300 with JunB takes place only at the C terminus, leaving the N-terminal region free to interact with other additional transcription factors. Further, the in vivo interaction of JunB with p300 was ascertained by chromatin immunoprecipitation assays.

AP-1 activity is dependent on the phosphorylation state of each heteropartner. Proteins of the Jun family can undergo phosphorylation by MAP kinases activated by a variety of stresses (45, 46). Activation of MAP kinases with receptor tyrosine kinase also regulated JunB/AP-1 and DNA synthesis in vascular smooth muscle cells (47). Hence, phosphorylation of JunB plays an important role in AP-1 activation. In this study, two potential phosphorylation sites were identified, and phosphorylation of JunB at Ser-79 played an important role in the transcriptional activation of DMP1. It is also noteworthy that phosphorylation of JunB at Ser-79 by p38 kinase is also essential for its interaction with p300. A recent study demonstrates that p38 MAP kinase is required for hypertrophic chondrocyte differentiation (48).

In a published study, JunB has been shown to be phosphorylated by Cdc2 kinase. It was observed that replacement of Ser-23 with Ala and Thr-150 with Ala reduced the phosphorylation state, indicating that these two residues were important phosphorylation sites for Cdc2 kinases (40). However, additional phosphorylation sites at Thr-102 and Thr-104 (JNK sites) have been reported by others (40). Besides, Ser-186 was also shown to be involved in the Cdc2-cyclin B phosphorylation pathway (50). The cyclin B-Cdc2 is a mitotic kinase complex (serine-threonine kinase), known to phosphorylate proteins, triggering their degradation (51, 52).

p300 is a multifunctional transcriptional coactivator that acts as an adapter for several transcription factors. It has several conserved regions with known functional domains (53). They are (a) the bromodomain, which is frequently found in mammalian HATs; (b) three CH-rich domains (CH1, CH2, and CH3); (c) a KIX domain; and (d) an ADA2 homology domain, which has similarity to a yeast transcriptional coactivator, Ada2p. The CH1, CH3, and KIX domains are likely to be important in mediating protein-protein interactions, and a number of cellular and viral proteins have been shown to bind to these regions. In this study, a GST pull-down assay on MC3T3-E1 cell extracts demonstrates that JunB binds to the C-terminal region of p300. p300 has an intrinsic HAT activity that may be critical for transcriptional activation (54, 55), which transfers an acetyl group to the ε-amino group of a lysine residue (reviewed in Ref. 56). p300 HAT activity is involved in chromatin remodeling (57–68).

The role of p300 in mineralized tissue formation is not well defined. In bone, osteoblast-specific transcription factor Cbfa1 controls the expression of osteocalcin (bone-specific gene). Cbfa1 has been shown to interact with p300 and stimulate the expression of osteocalcin in response to vitamin D3. Interest-
DMP1 binds to the AP-1 binding site of the JunB at the Ser-79 position. The phosphorylated JunB and p300 acetylates the core histones, leading to an increase in the expression of DMP1 during the osteoblast differentiation. The intrinsic HAT activity of p300 acetylates the core histones, leading to an increase in the expression of DMP1 during the osteoblast differentiation. So far, p300/CBP proteins have been shown to interact with a multitude of signal transduction pathways, and this complexity action of Sox9 with CBP/p300 has been reported recently (68).

Fig. 12. p300 HAT domain and DMP1 expression. p300 construct lacking HAT activity (p300-AT) was obtained from Dr. Thimmapaya (Northwestern University). Stable cell lines were constructed with wild type p300 and p300-AT. Transient transfection with JunB or p300 was carried out on these stable cell lines. Total RNA was extracted from these cell lines after transfection, and Northern blot was carried out to analyze the expression of DMP1. Lane 1, transfection of JunB alone in mock cells; lane 2, transfection of JunB in stable cell lines containing wild type p300; lane 3, mock cells containing the empty vector; lane 4, transfection of JunB in stable cell lines containing the p300-AT construct. GAPDH was used as an internal control. Densitometry analysis of the Northern result is shown as a bar diagram. The scan values were normalized with GAPDH values for loading errors.

In conclusion, we have demonstrated that during osteoblast differentiation, p38 kinase phosphorylates JunB at the Ser-79 position. The phosphorylated JunB binds to the AP-1 binding site of the DMP1 promoter. p300 interacts with JunB and the basal transcription machinery. The intrinsic HAT activity of p300 acetylates the core histones, leading to an increase in the expression of DMP1 during the osteoblast differentiation.

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Transcriptional Regulation of Dentin Matrix Protein 1 by JunB and p300 during Osteoblast Differentiation

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