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Binding of Two Nuclear Factors to a Novel Silencer Element in Human Dentin Matrix Protein 1 (DMP1) Promoter Regulates the Cell Type-Specific DMP1 Gene Expression

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Abstract DMP1 is an acidic phosphorylated protein with the spatial and temporal expression that is largely restricted to bone and tooth tissues. The biological function of DMP1 is associated with biomineralization of bone, cartilage and tooth development. To study the cell-specific expression of DMP1, a 2,512 bp upstream segment of the human gene was isolated and characterized. A series of progressive deletions of the human DMP1 5′ flanking sequence were ligated to the luciferase reporter gene, and their promoter activities examined in transfected human osteoblast-like (MG-63) and dental pulp (HDP-D) cells that express DMP1 and hepatic (HepG2) and uterine (HeLa) cells lacking DMP1 expression. A critical *cis*-regulatory element located between nt −150 and −63 was found to act as a specific silencer responsible for the negative regulation of DMP1 in HepG2 and HeLa cells. The transcriptional activity of this element in MG-63 and HDP-D cells had a 5–7-fold increase than that observed in HepG2 and HeLa cells. Electrophoretic mobility shift assays (EMSAs) showed that a 6-bp DNA sequence in this element was bound by two nuclear factors that are expressed at high levels in HepG2 and HeLa versus MG-63 and HDP-D cells. Competitive assays by EMSAs suggest that the 6-bp core DNA sequence, AG(T/C)C(A/G)C, is a novel DNA–protein binding site and conserved with high identity in reported DMP1 promoters for all species. Furthermore, point mutations of the core sequence caused a marked increase of DMP1 promoter activity in HepG2 and HeLa cells. We speculate that this silencing *cis*-element may play a critical role in the regulation of DMP1 cell-specific expression. J. Cell. Biochem. 92: 332–350, 2004.

Key words: DMP1; silencer element; transcription factors; gene regulation

Mineralized tissues such as bone, cartilage and dentin have many characteristics in common, especially those related to extracellular matrix (ECM)-mediated mineralization events. Both osteoblasts and odontoblasts synthesize and secrete many common ECM components consisting of collagenous and non-collagenous proteins [Linde and Goldberg, 1993; Zeichner-David et al., 1995; Butler, 1998]. The major common non-collagenous ECM proteins of bone and dentin include osteonectin (OSN), bone sialoprotein (BSP), osteopontin (OPN), osteocalcin (OC), matrix extracellular phosphoglycoprotein (MEPE), dentin sialophosphoprotein (DSPP) and dentin matrix protein 1 (DMP1). It is believed that mineralization is initiated by phosphorylated ECM proteins located within collagen gap zones that bind to calcium and phosphate ions in an appropriate conformation to nucleate formation of hydroxyapatite crystals [Linde and Goldberg, 1993; Zeichner-David et al., 1995; Butler, 1998].

DMP1 is an acidic phosphorylated protein identified as a novel cDNA from a rat pulp

Abbreviations used: DMP1, dentin matrix protein 1; ECM, extracellular matrix; PCR, polymerase chain reaction; Luc, luciferase; EMSA, electrophoretic mobility shift assay; nt, nucleotide; oligo, oligodeoxynucleotide.

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library [George et al., 1993]. George et al. [1993] initially reported that rat DMP1 cDNA contains 5 exons and 4 introns but later studies have shown in mouse and human two isoform one containing a novel exon [Hirst et al., 1997; MacDougall et al., 1998]. This small exon consists of a 45-bp segment encoding 15 amino acids representing an alternatively spliced segment [MacDougall et al., 1998]. More extensive studies have identified that the rat DMP1 full-length cDNA has the same structure as the mouse and human cDNAs [Feng et al., 1998a; Thotakura et al., 2000a]. The genomic structure of different species (6 exons and 5 introns) shares common features such as exon 1 containing untranslated sequences, the ATG start site in exon 2 and large last exon 6. Like other ECM proteins, DMP1 contains an arginine-glycine-aspartic acid (RGD) sequence [George et al., 1993; Hirst et al., 1997; MacDougall et al., 1998]. This RGD domain, related to cell attachment, has been shown to be functional [Yamada, 1991; Kulkarni et al., 2000; Verderio et al., 2003]. In situ hybridization and immunohistochemical analyses have indicated that DMP1 is expressed in odontoblasts, ameloblasts, osteoblasts, osteocytes, cementoblasts, and chondrocytes [George et al., 1994; D'Souza et al., 1997; Hirst et al., 1997; MacDougall et al., 1998; Feng et al., 2000; Toyosawa et al., 2001]. Therefore, DMP1 is an ECM protein expressed in diverse cells of bone and tooth tissues.

Human and mouse DMP1 genes have been mapped to chromosome 4q21 and 5q21, respectively [George et al., 1994; Aplin et al., 1995; Aplin et al., 1999; MacDougall et al., 1999]. This region of human chromosome 4 contains a dentin/bone gene locus that overlaps loci for genetic diseases including dentinogenesis imperfecta type II (DGI-II), type III (DGI-III), and dentin dysplasia type II (DD-II) [Dean et al., 1997; MacDougall et al., 1997; Thotakura et al., 2000b; Toyosawa et al., 2001]. Thus, DMP1 has been considered a potential candidate gene.

DMP1 has been reported to play a key role in mineralizing process observed in bone and tooth and being a potential signaling molecule for dentinogenesis [Luan and MacDougall, 2001; Narayanan et al., 2001; Toyosawa et al., 2001]. Incubation of mouse antisense DMP1 oligos with developing mouse mandibles at embryonic day 12 in tissue culture media results in inhibition of endogenous mouse DMP1 gene expression and abnormal morphologies of dentin and enamel compared to control groups [Luan and MacDougall, 2001]. In addition, overexpression of DMP1 induces differentiation of mouse embryonic mesenchymal cells to odontoblast-like cells and increases mineralization of these cells [Narayanan et al., 2001]. Recently, Feng et al. [2002] have reported that homologous DMP1 knock-out mice show abnormalities of skeleton and tooth development at postnatal stages as well as reduce mineral density in bone, dentin, and enamel [Abbas et al., 2003].

Although the human DMP1 gene structure has been determined [Hirst et al., 1997], the upstream promoter sequence, the transcription initiation site, and transcription factors involved in its regulation remain unknown. In particular DMP1 transcription in a tissue-specific manner is obscure. In this present study, we analyzed the promoter elements involved in the transcriptional regulation of the DMP1 gene expression in bone and tooth cell types. Initially, we cloned the 5′ flanking region of the human DMP1 gene and identified transcription initiation sites. Immunohistochemical analysis showed that DMP1 expression was highly restricted in the human osteoblast-like (MG-63) and dental pulp (HDP-D) cell lines, but not in the human uterine (HeLa) and hepatic (HepG2) cell lines. By using transient transfection of reporter plasmids bearing progressive deletions of the human DMP1 promoter into MG-63 and HDP-D as well as HeLa and HepG2 cell lines, we demonstrated varied cell-specific promoter activities in the different cell lines tested. Furthermore, an element in the proximal promoter region responsible for negative regulation of the human DMP1 gene in HeLa and HepG2 cells but not in MG-63 and HDP-D cells was identified. Electrophoretic mobility shift assays (EMSAs) revealed that a 6-bp DNA sequence in this element was bound by two nuclear factors that are expressed abundantly in HeLa and HepG2 cells but at much lower levels in MG-63 and HDP-D cells. A series of competitive assays by EMSAs indicated that this 6-bp DNA sequence is a novel DNA–protein binding site that is conserved with all species tested. Thus, this element interacting with two cell-specific nuclear factors may play an important role in controlling tissue-specific expression of DMP1 gene.

MATERIALS AND METHODS

Cell Lines

Human osteosarcoma (MG-63, osteoblast-like), uterine (HeLa), and hepatic (HepG2) carcinoma cell lines were obtained from the American Type Culture Collection (Rockville, MD). An immortalized human dental pulp cell line (HDP-D) that expresses DMP1 was established using the method of MacDougall et al. [1995]. MG-63, HeLa, and HepG2 cells were cultured in minimal essential medium (MEM) at 37°C under 5% CO_2. HDP-D cells were grown at 33°C in alpha-minimal essential medium (α-MEM) containing 50 μg/ml ascorbic acid, 10 mM Na β-glycerophosphate. All of the cells were grown in media supplemented with 10% fetal calf serum and 100 U/ml of penicillin/streptomycin.

Cloning of the 5′ Flanking Region of the Human DMP1 Gene and Generation of DMP1 Promoter Constructs

A human DMP1 genomic DNA was isolated from a human genomic DNA library (Stratagen, La Jolla, CA) and amplified by PCR using primers based on human DMP1 cDNA sequences [Hirst et al., 1997] and Human Genome Sequence (GenBank Accession No.: 11419275). These primers were used for PCR as follows: sense, $_{-2512\text{ to }-2492}$ 5′-CTGCTCCTATGGGATTTAACC-3′; sense, $_{-1191\text{ to }-1171}$ 5′-GCCTGAATAAATTGGGCACTC-3′; sense, $_{-656\text{ to }-635}$ 5′-CGGGGAATGATTAAGTGTTAAG-3′; antisense, $_{-591\text{ to }-611}$ 5′-CCCTTAAGTAGCTATTGTGTT-3′; antisense, $_{-130\text{ to }-150}$ 5′-GTACACCACACTTCCTCCAG-3′; antisense, $_{+83\text{ to }+63}$ 5′-CCCTCCGTAGTTCACAAAGAA-3′. The PCR reaction performed using *Pfu* DNA Polymerase (Promega, Madison, WI) was 4 min at 94°C for 1 cycle, 1 min at 94°C, 40 s at 58° or 56°C and 5 min at 72°C for 32 cycles followed by 7 min at 72°C. The amplified PCR fragments were first cloned into the pCR-II vector (Invitrogen, Carlsbad, CA). The DNA sequence in both directions was determined using a model 377 DNA sequencer (PE Applied Biosystems, Foster City, CA). The amplified fragments were released with *Hind*III enzyme on the 3′ end and various restriction enzymes on the 5′ end to produce seven different sized constructs, and then recloned into pGL3 luc basic expression vector (Promega). The orientation of all the inserts with respect to the pGL3 luc vector was verified by DNA sequencing. The pGL3luc −2512 to +83 plasmid consisted of pGL3luc vector with a ∼2.6 kb *Xho*I and *Hind*III fragment of the human DMP1 gene (p2512-luc), The pGL3luc −1656 to +83 plasmid was similarly generated from a 1,739 bp *Nhe*I and *Hind*III fragment (p1656-luc), the pGL3luc −1187 to +83 plasmid from a 1,270 bp *Sac*I and *Hind*III fragment (p1187-luc), the pGL3luc −656 to +83 plasmid from a 739 bp Smal and *Hind*III fragment (p656-luc), the pGL3luc −227 to + 83 plasmid from a 310 bp *Bgl*II and *Hind*III fragment (p227-luc), the pGL3 luc −150 to +83 plasmid from a 233 bp *Pvu*II and *Hind*III fragment (p150-luc), the pGL3 luc −63 to +83 plasmid from a 146 bp *Pst*I and *Hind*III fragment (p63-luc). All the various plasmid constructs contain part of the non-coding exon 1 region. The identity percentage of genomic DNA sequence among human, rat and mouse DMP1 genes was determined using the Pairwise Blast Program. Site-specific mutagenesis was created by using the QuickChange Site-Directed Mutagenesis Kit (Stragene, La Jolla, CA) on mutant constructs 150 and 656 bp of the human DMP1 promoter upstream of a luc reporter gene. Mutant oligos for the individual sites (mutant bases in lowercase) are as follows: mutant 4, 5′-CCTTGTGATGTCAcTCAgCCCCAGCCTGAGG-3′ and 5′-CCTCAGGCTGGGGcTGAgTGACATCACAAGG-3′ as well as mutant 6, 5′-CCTTGTGATGTCAGcCttCCCCAGCCTGAGG-3′ and 5′-CCTCAGGCTGGGGaaGgCTGACATCACAAGG-3′ (Table II). Potential TATA boxes and protein–DNA binding sites were determined using computer database programs such as http://www.genomatix.de/cgi-bin/eldorado/main.pl, http://bimas.dcrt.nih.gov/molbio/signal/ and http://www.itba.mi.cnr.it/webgene/.

Primer Extension Mapping of the Human DMP1 Transcriptional Start Site

Total RNA from MG-63 cells was isolated using RNA STAT-60 kit (Tel-Test, Inc., Friendswood, TX), treated with DNaseI (Promega), and purified with the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA). The transcriptional start site was mapped by primer extension using a 21 synthetic oligo complementary to nt 41–21 upstream of the DMP1 ATG start codon (5′-CCCTCCGTAGTTCACAAAGAA-3′). The primer extension assay was performed using the Promega primer extension kit according to

instructions with annealing reaction carried out at 55°C. Resulting products were electrophoresed on an 8% denaturing urea polyacrylamide gel and autoradiographed. The primer DNA sequences were used for size markers.

DNA Transfection and Luciferase Assay

For each transfection experiment, MG-63, HDP-D, HeLa, and HepG2 cells were seeded at 1×10^5 cells/35 mm diameter dishes and transiently transfected 18 h later with the various reporter plasmids or an empty pGL-3 basic plasmid as a control and pRL-TR (Promega), using the LipofectAMINE Plus reagent (GIBCO-BRL, Grand Island, NY) as specified by the manufacturer. The transfected cells were harvested with passive lysis buffer (Promega) 48 h after the start of transfection. Luc activity was measured with the Promega Dual-Luc reporter assay system as indicated by the manufacturer. In this dual luc system, DMP1 promoter fragments were linked to the *firefly* luc gene while the co-transfected *renilla* luc gene (pRL-TR) was driven by the SV 40 vector promoter. The promoter activity was calculated by the ratio of *firefly*/*renilla* luc for each construct with the value obtained from the control group was taken as one-fold. The fold increase in the luc activity was calculated by dividing the individual value by the control group value. The data show the mean ± SE from at least three separate experiments performed in triplicate.

Preparation of Nuclear Extract

The MG-63, HDP-D, HeLa, and HepG2 cells were grown as previously outlined and harvested at confluency. Nuclear extracts were prepared using the method of Dignam et al. [1983]. Briefly, 1×10^8 cells were washed twice with 10 ml of ice-cold phosphate buffer saline (PBS) and collected by centrifuging at 250*g* for 5 min. The cell pellet was resuspended in 500 μl of ice-cold NP-40 lysis buffer (10 mM Tris, pH 7.4, 3 mM $MgCl_2$, 10 mM NaCl, 0.5% NP-40) by gentle pipetting and incubated on ice for 10 min. The nuclei pellet was collected by centrifuging for 1 min and resuspended in 500 μl of ice-cold hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl). The pellet was again collected by centrifuging at 3,000*g* for 1 min, and resuspended in 500 μl of ice-cold extraction buffer (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 420 mM KCl, 0.2 mM EDTA, 20% glycerol), and vigorously rocked at 4°C for 30 min followed by microcentrifugation for 5 min. Aliquots of supernatant containing nuclear proteins were quick-frozen in a dry ice-ethanol bath and stored in −80°C. The protein concentrations were determined using a Bradford [1976] assay.

Oligonucleotides and Electrophoretic Mobility Shift Assay

Several double-stranded oligos covering an element between nt −150 and −63 in the human DMP1 promoter and a duplex oligo corresponding to the consensus binding site for CBF/NF-Y (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used in EMSA. The oligos are as follows: DMP1, −150 to −126 5′-CTGGAGGAAGTGTGGGTGTACCGGG-3′, −130 to −105 5′-CCGGGGTCCCGGTGACACCTTGTGAT-3′, −108 to −81 5′-TGATGTCAGTCACCCCCAGCCTGAGGTT-3′, −86 to −63 5′-GAGGTTGATTGGCTATGAAGAGCT-3′, and CBF/NF-Y consensus sequence, 5′-AGACCGTACGTGATTGGTTAATCTCTT-3′. Other consensus binding sites of transcription factors (Table I) were also purchased from Santa Cruz Biotechnology, Inc. For the EMSA, the double-stranded oligos were labeled with [Γ-^{32}P] ATP and T4 polynucleotide kinase as well as purified on a 15% polyacrylamide gel. EMSA was performed as described [Chen et al., 1997]. Briefly, 5 μg of nuclear extracts from MG-63, HDP-D, HeLa, and HepG2 cell lines were preincubated with in 20 μl reaction containing 10 mM Tris-HCl,

TABLE I. EMSAs Were Performed With the Oligonucleotide Duplexes (Only the 5′ to 3′ Strand of the Duplexes) Listed Below

Site I	−108	5′-TGATGTCAGTCACCCCCAGCCTGAGGTT-3′	−81
AP-1		5′-CGCTTGATGAGTCAGCCGGAA-3′	
SP-1		5′-ATTCGATCGGGGCGGGGCGAGC-3′	
CBF		5′-AGACCGTACGTGATTGGTTAATCTCTT-3′	
C/EBP		5′-TGCAGATTGCGCAATCTCCA-3′	
TCF		5′-GTAGGGCACCCTTTGAACTCTCCC-3′	
AP-2		5′-GATCGAACTGACCGCCCGCGGCCCGT-3′	
NF-κB		5′-AGTTGAGGGGACTTTCCCAGGC-3′	

TABLE II. Oligonucleotides were used for EMSA

Oligo position		Sequence		Competition
Site I				
−108/−81	5′	TGATGTC**AGTCAC**CCCCAGCCTGAGGTT	3′	+
Site I–L				
−108/−95	5′	TGATGTC**AGTCA**CC	3′	+
Site I–R				
−99/−81	5′	TCACCCCCAGCCTGAGGTT	3′	−
−101/−88	5′	AGTCACCCCCAGCC	3′	+
−113/−98	5′	CCTTGTGATGTCAGTC	3′	−
−115/−102	5′	CACCTTGTGATGTC	3′	−
−106/−90	5′	ATGTC**AGTCAC**CCCCAG	3′	+
Mutant 1	5′	----------g----------	3′	−
Mutant 2	5′	----------g----------	3′	−
Mutant 3	5′	----------g----------	3′	+
Mutant 4	5′	---------c---g----------	3′	−
Mutant 5	5′	----------t-a----------	3′	−
Mutant 6	5′	----------c-tt----------	3′	−
Mutant 7	5′	--------t--a----------	3′	−

Sequence identity is indicated by dashes. Substitution mutations are represented in lowercases. Core sequence is present in boldface.

pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, and 2 μg of poly (dI-dC). After 5 min at room temperature, 20 fmol of ^{32}P-end-labeled oligo-duplex probe were added, and incubation was 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 0.5× Tris-boric acid–EDTA (TBE) buffer, electrophoresed, dried, and exposed to X-ray film.

Immunohistochemical Analysis

Cells were grown on glass slide culture wells (Fisher Scientific, Naperville, IL) in growth medium for 3 days. The cells were washed with cold PBS three times and fixed in 50% methanol/ethanol for 10 min at room temperature. The cells were pretreated with 3% H_2O_2 (DAKO Corporation, Carpinteria, CA) for 10 min at room temperature and washed with Tris-buffered saline three times. The cells were treated with serum-blocking solution at room temperature for 40 min, followed by incubation with a specific DMP1 polyclonal antibody (kindly provided by Dr. Larry Fisher, NIDCR) at a dilution of 1:50 in Tris-buffered saline overnight at 4°C. Subsequently, the cells were rinsed with Tris-buffered saline and incubated for 40 min with labeled goat anti-rabbit IgG conjugated to peroxidase anti-peroxidase (PAP) (DAKO) at a 1:100 dilution. Excessive secondary antibody was removed by washing the cells three times with Tris-buffered solution. The cells were stained with DAB (0.4% 3-3′-tetrachloride diaminobenzidine oxidization) for 5 min at room temperature to visualize the immunoreaction. Negative controls for immunostaining were performed by substituting the primary antibody with normal rabbit serum (DAKO).

RESULTS

Cloning and Characterization of the 5′ Flanking Region of the Human DMP1 Gene

A PCR-based approach was used to clone the 5′ flanking region of the human DMP1 gene from a human genomic DNA library. Amplified PCR fragments were subcloned into pCR-II vector. The longest fragment, 2,512 bp, was used to identify potential DNA response elements located within the upstream region of exon 1 (Fig. 1A). Analysis of this sequence revealed TATA and CAAT boxes. The TATA box is located at 46 bp upstream of the major transcriptional start site with three potential CAAT boxes found at nt −76, −289, and −435 (Fig. 1A). The consensus DNA–protein binding sequences for several known transcription factors involved in controlling gene expression related to bone and tooth matrix proteins were contained within the DMP1 promoter sequence [Franceschi, 1999; Karsenty, 1999; Lian et al., 1999]. These include two OSE-2 binding sites for Runx2/Cbfa1 at −2425 and −183 bp. Several binding sites for the homeobox gene family MSX were found at −1361, −1165, −1117, −929, and −667 bp. Potential binding sites for additional well characterized transcription factors were


A
primer 1
Tcf-1
PEA-3
ctgctcctatgggatttaaccTTCATAGTTTTTTGCTGTTTGTTTTATATGAAAGTGGTTTTCCTGTACTTTTAGAAAGA -2433
Cbfa1/OSE2
AATGTGGTTCAGGGTAACTTTCAAACTTCACACGGCTCCCTCTTCTGTTGCTTTCGTGTTATGTTAAAAAAAAATAGCAG -2353
Tcf-1
CBF/NF-Y
CTTTCTAAGATTTCTTGGATCCATTCCCCTCTCCCATATTTATCGTGTGTCTTCTCAATTGTCTATATCCTGTGCAATTT -2273
TTATTCCACTCCCAGAAGTTTCTCCTGTATGTGAGTCTGTCTTAGAAAGCAGCTGTAGTGGCTTAGAATCCTTAGGGGCT -2193
C/EBP
AP-1
AGACTGGTCTAGATCCTTCAGCTGTGCTATGAACCATTGGCACCCATCAGCAATAATTCTTGACTGCTTTGGGTTCTGCT -2113
GTTCTCATGTTCATCAGATTTCCTGCTGCTTTCCTCCGCTCACTCCTGCATAGACTCTCTAGGTCTTGTGGCAGTTGTGG -2033
AP-1
SRF
ACTTTGTCCTTAGTCACCTTGGAGTTAGTGGATATAACTTGTCATAAGGGGTGGTAACTAATCAAAAACTGCCTCAACGT -1953
TTCCTCTGCTCCCTATACATGTGCATCCTGATCTGACACCAAAGGGTAGAGAGTATTTCTCCTTATGAATCTGAGATCTC -1873
YY-1
Sp1
CTGGTGATTGATTGACCAACAGAATATGGAAAAATGATATTCTAGGAACTCTTGGCCCCCAGCCTTCAGGTTGTGAGAAG -1793
Oct-1
GCCAAGCCAAGGAGAGGCCATGTAAAGAACTAAAGTGCTCAGAATTACAGCTCCAGCTGAGCTCTCAGGTAACAACTATG -1713
Sp1
AAAGTGGCATCTGAAATGTTCTGCCCCAACTGAGTCTTCAGATGACTACAATGCTAGCTGTGAGAGATAAGAAAATGGTA -1633
Oct-1
C/EBP
GTTGTTTTAAAATGCAAAGTATTGAAGTGGTTTTTGATATAGCAATAGATAACAAAAATGCCTAGTTTTATTGTAAATGT -1553
SEF4
TATCCACAGTTTTCAATTTTGTTATCTAATTGCTCCTCTGTTTTTATGTAGGGATTTGAAGAGAGTCTAAAATAATGTCT -1473
Sp1
AP-1
TGTAATGCAATCTGCCCAAATCTGCCCAGAATCCAATTCTGTCATAAGATTATAAGGTTCTTCTAGCATAGATCTCAGTT -1393
Msx-1
Ubp-1
AP-2
AAGACCCATGAAACCATCAGAGAGTGTAATTCAGAACCAGAGCAAATCACAGGGAATTTATAGCTTCCCCATATGGACTT -1313
PEA3
TGGCTTTCTAATCAACCCTAAATGAAAATAGACATCTCTTTCCTCATTGTCTGCACCACCCTCCCCCGCATATTATAGTT -1233
primer 2
Msx-1
CACACTAAATATGTTGATAATCCATATATCTGACATTAGTTgcctgaataaattgggcactcTAATTTTCTAGATCCATG -1153
Msx-1
TTAGGAGCATCAGCTCAATTTTTTTTTAACAATTAAAGCATTTTTTTAAAAGTTACAGTGAGTTATGCAGATACTGTAAT -1073
CBF
CAAGTTAATAGTTTGGCTAATTTTCAAGCCAATCCACTCAGTCATTCAAAGAGTACTGAGTGATTATTAAGAACTGTTCT -993
Msx-1
AGGTATCCAGGCACTGATTATAGATCAGTAAACAAGCAAACATCCCTACTCTTAAGGAAATTAAGGTCCAGCAGCTGATA -913
TTCTCTAAATTCTTGTTCAGTGGCCATCCTGCCGTCACTTGAACACGCTCCTGGGAAATTGCCAAACCCATTTCCAGCTC -833
Tcf/Lef
CCTACAACTTTTAGTACGCTTCCTCGAAAATGTTTTGGTAAATATCATTTTATCCATTTCATTACATTTTCGGTGGTGGC -753
GATA-1
ACAGAGTCCCTTCAAGATTAGTTTCCTTGGATGCTACCCAGCTAGTATTCCCCTTAATCCAATTCTGATTTCAAAGAATC -673
Msx-1
Sp1
primer 3
Pit-1
primer 4
ATTAAAATGTGGCAAAcggggaatgattaagtgttaagTGTTAGATCTAAATTTCTTGAATaacacaatagctacttaag -593
CP-2
AP-2
ggGGGTACTTTAGTGGACGCTTTATTAGATTAGTAGGAGTTTGGGAACTTTGTGTGTATATGGGGGTGGGGAGATGAGAG -513
CAAT box
GAAAAGGAGGTGAGTAGTAGAGGTTGTTAAAAGAGGAAAAAGGAAGAAATAAAAGCCCATATTCTCAAAACCAACAATTT -433
Tcf-1
AAGGAACTCTAGTATTTTAGTAGCTTTCTCCATGTACAACCTGGAAATGTACATTAACAGGAAACTTTCTACCCACCCCT -353
Sp1
AP-2
CAAT box
GCCTCCGCCTTTCCCATCCTTGGGTCTGCTGGGATCTGTCGGTGGGTAGAAGACATTGTCATTGTTCATAAGACTGCCTG -273
AATGTGAATGACATATATTTAGAAATCAAAATAAAGTTTTGGGAAGATCTTCAATTTAATGGAAACTAGTCTAGTCATTT -193
Cbfa1/OSE2
Tcf-2α
primer 5
GGGAACCACAAGAGCTTTTTAGGGAAAATGTGCCTCTGCCAGctggaggaagtgtgggtgtacCGGGGTCCCGGTGACAC -113
AP-1
AP-2
C/EBP
CAAT box
TATA box
CTTGTGATGTCAGTCACCCCCAGCCTGAGGTTGATTGGCTATGAAGAGCTGCAGCAGAGTTTAAATTCCTGCACCATCAG -33
+1
AGAAGTGTCCAGAATATCAACACAAGAGTGGCTtcattgggcatagatttcctctttgagaacatcaacctgattttgag +47
primer 6
acttttgaaaaaattctttgtgaactacggagggtagaggtatcacacccaactATG

Fig. 1.

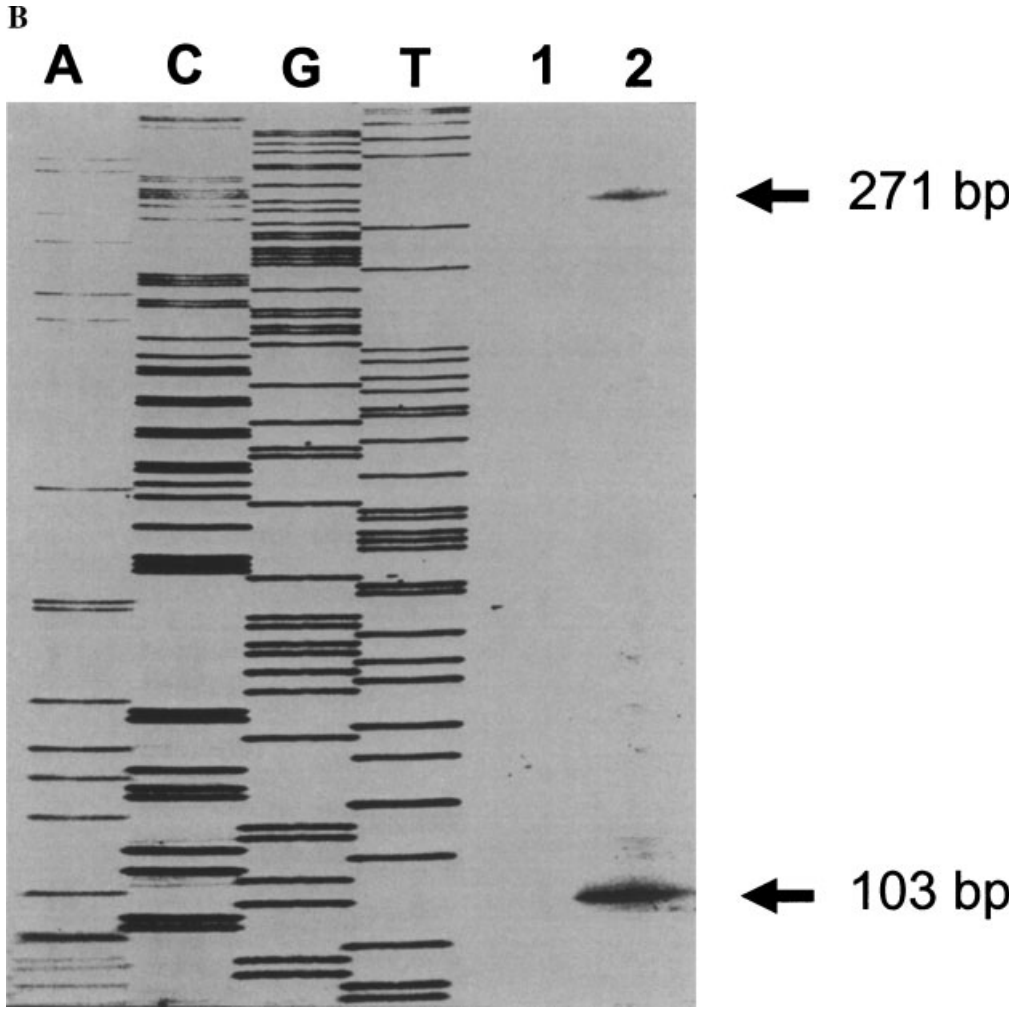


Fig. 1. Determination of DNA sequence of the 5′ promoter region of the human DMP1 gene and its transcription initiation site. **A**: The DNA sequence of the ~2.6-kb flanking region and part of exon 1 are shown. Nucleotides are numbered on the right with +1 corresponding to the transcription start site. Arrow (major) and arrowhead (minor) indicate the two transcriptional start sites. The potential response elements of TATA and CAAT boxes, NF-Y/CBF-CAATT box, Cbfa1/OSE2, Msx, Tcf/Lef, Pea-3, C/EBP, SRF, AP-1, SP-1, YY-1, Oct-1 are underlined and labeled above. The locations of primers, 1–6, are underlined, labeled, and orientations indicated with arrows above their locations in the sequences. These primers were used to generate various promoter constructs. Primer 6 was also used for the primer extension studies to determine the transcription start site. **B**: The γ-^{32}P-end-labeled primer was annealed to 50 μg of total RNAs isolated from human MG-63 cells. The primer–RNA complex was precipitated and reverse-transcripted. The cDNA was added by stop solution. Yeast tRNA was used as a negative control. A sequencing reaction was performed using the Sequencase version 2.0 kit as described by the manufacturer (Biochemical Corporation, Cleveland, OH). The control single-stranded DNA in the kit was used with its sequencing primer. All samples were denatured at 95°C for 3 min and cooled on ice, and 5 μl of the sample were resolved on an 8% acrylamide/urea gel. **Lanes A**, **C**, **G**, **T** represent the sequencing reaction corresponding to dideoxy A, C, G, T, respectively. **Lane 1** shows the negative control using yeast tRNA. **Lane 2** represents the cDNA products from MG-63 total RNA with arrows indicating the transcriptional extended fragments.

also present including SP1, C/EBP, Tcf/ Lef, AP-1, and AP-2.

Primer Extension Mapping of the Transcriptional Start Site for the Human DMP1 Gene

The transcriptional initiation site was mapped by primer extension using a 21-synthetic antisense oligo probe complementary to nt 41 to 21 upstream of the human DMP1 ATG start codon (Fig. 1A) using RNA isolated from human MG-63 cells. As shown in Figure 1B, two extended fragments were obtained with the human MG-63 cell RNA, as opposed to yeast tRNA. These major and minor bands were seen at 103 and 271 bp upstream of the ATG start codon, respectively.

DMP1 Expression in Bone and Tooth Cell Lines

Previous in situ hybridization analysis has shown that DMP1 is expressed in bone, cartilage, and tooth tissues [George et al., 1994; D'Souza et al., 1997; MacDougall et al., 1998; Toyosawa et al., 2001]. To determine that DMP1 is expressed in human bone and tooth cell lines, a specific polyclonal DMP1 antibody was used for immunohistochemical analysis showing the intracellular localization of DMP1 present within the human MG-63 and HDP-D cells (Fig. 2A,B) as opposed to the human HeLa and HepG2 cells (Fig. 2C,D) and experimental control (Fig. 2E–H). This study further demonstrated that DMP1 expression is highly restricted to the bone and tooth tissues.

Human DMP1 Promoter Activity in MG-63 and HDP-D Versus HeLa and HepG2 Cell Lines

To determine elements in the human DMP1 promoter that are cell type-specific, we gener-

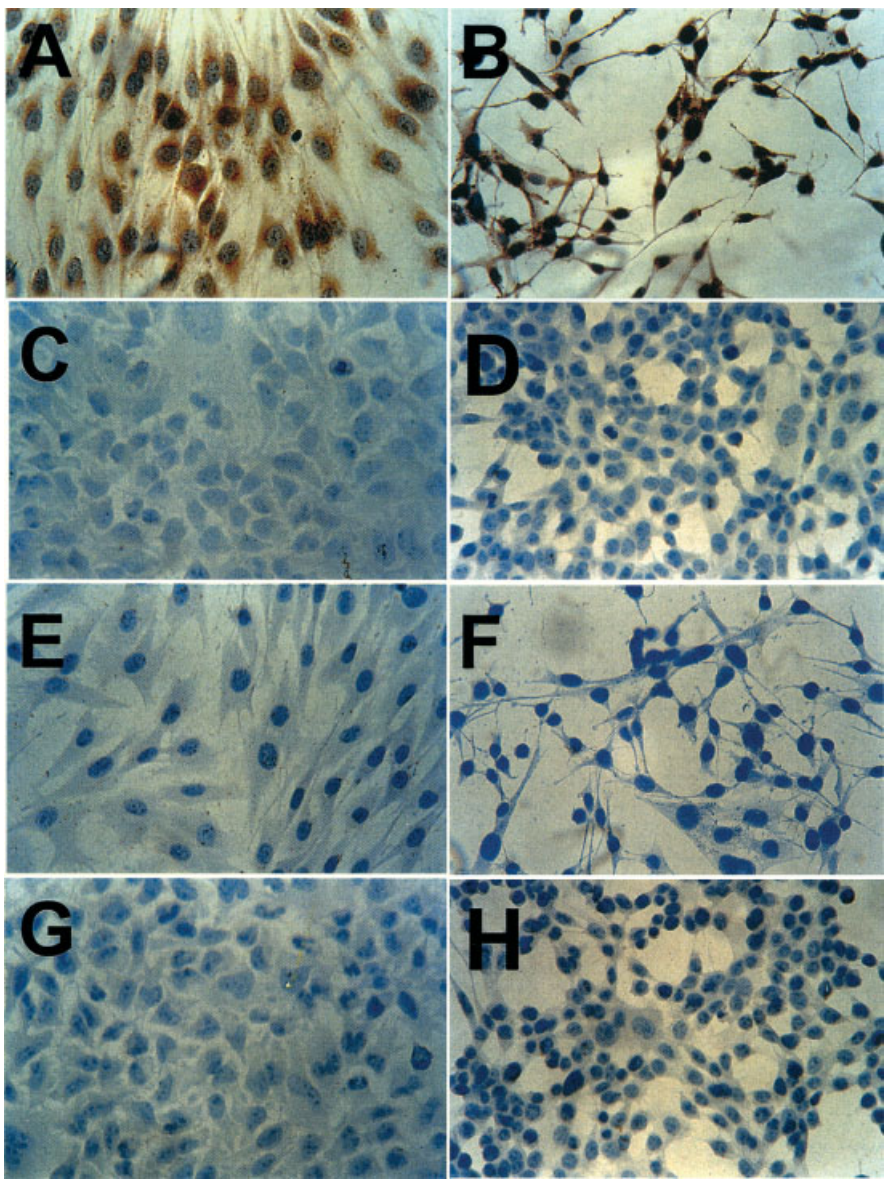


Fig. 2. Immunohistochemical analysis of DMP1 expression in human MG-63 and HDP-D as well as HeLa and HepG2 cells. Positive immunostaining (brown reaction) was seen within MG-63 (**A**) and HDP-D (**B**), but not HeLa (**C**) and HepG2 (**D**) cells. Negative control using pre-immuno serum was shown for MG-63 (**E**), HDP-D (**F**), HeLa (**G**), and HepG2 (**H**) cell lines, respectively. Magnification 40×.

ated seven chimeric DMP1 luc reporter gene constructs (Fig. 3A). Four human cell lines were used for the transient transfection assays: two MG-63 and HDP-D cells with DMP1 expression and two HeLa and HepG2 cells lacking DMP1 expression. Results showed that different transcriptional activities for the various constructs were obtained with the four cell lines (Fig. 3B,C). Potential enhancer and repressor regions were identified within the ~2.6 kb DMP1 promoter. However, these regions varied among the four cell lines. In MG-63 and HDP-D cells (Fig. 3B), enhancer regions were observed in p150-luc between nt −150 and +83 as well as in p2512-luc between nt −2,512 and −1,657 compared to p1656-luc construct. Regarding repressor regions, one was located within p227-luc between nt −227 and +83 and another observed in p1656-luc between nt −1,656 and −1,188 compared to p1187-luc. Analysis of promoter activity in HeLa and HepG2 cells demonstrated no significant variations (Fig. 3C). Moreover, promoter activity in p150-luc demonstrated a 8–12-fold increase over that of p63-luc between nt −63 and +83 and showed the greatest one of all the seven reporter constructs in the bone and tooth cell lines as well as had 5–7-fold higher in HDP-D and MG-63 than that of HeLa and HepG2 cells. These data suggest that this element between nt −150 and −63 acts as a silencer in HeLa and HepG2 cells.

Cell Type-Dependent Nuclear Extract Interaction With an Element Between nt −150 and −63

Based on the high promoter activity of the p150-luc in MG-63 and HDP-D cells, we initially focused on the identification of transcription factor(s) able to bind this *cis* element. For this purpose, several double-stranded oligos covering the entire element were synthesized. EMSA

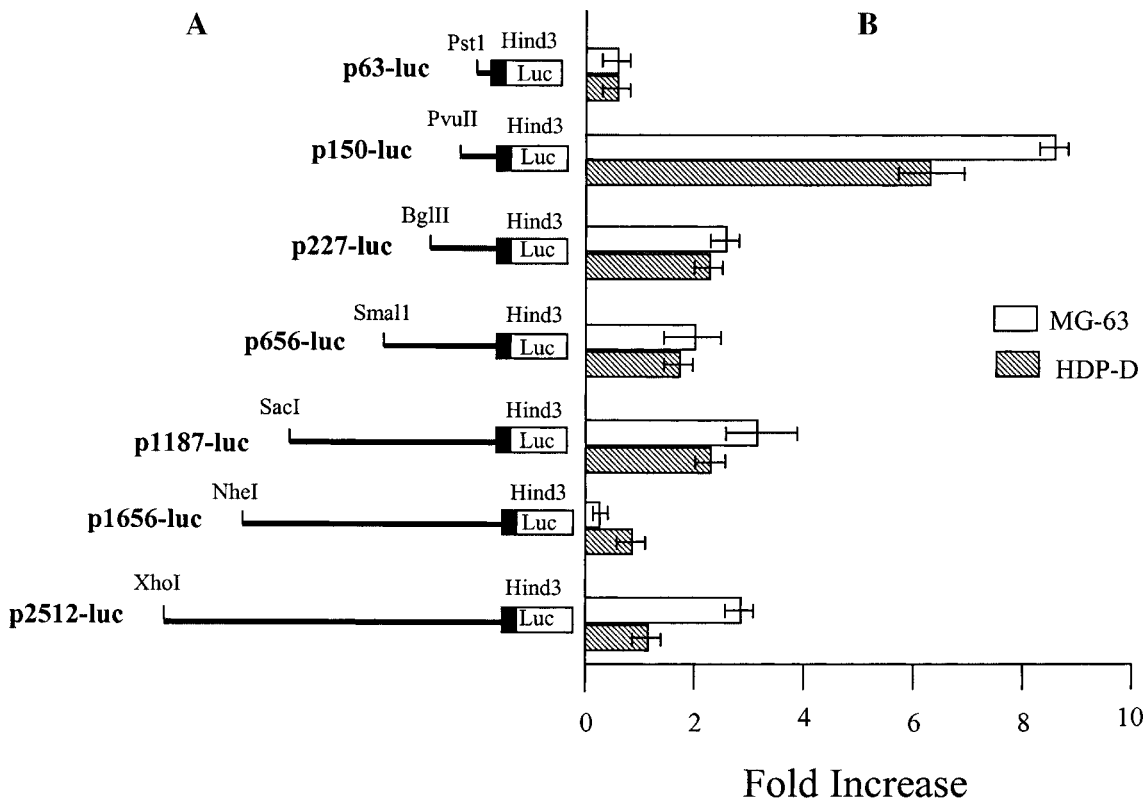


Fig. 3. Generation of seven human DMP1 5′ flanking luciferase constructs and promoter activity in four different cell lines. **A**: Schematic representation of the constructs used in the luc assay. Generation of promoter–luc DNA constructs was described in the "Materials and Methods." Results of the transfection experiment in MG-63 and HDP-D (**B**) as well as HeLa and HepG2 (**C**) cell lines. The cells were transfected with these seven DMP1 luc–DNA constructs (*firefly* luc) or an empty pGL3luc vector without DMP1 promoter as a control group and pRL-TK (*renilla* luc). Luc activity was determined after 48 h transfection. The value (ratio between *firefly* and *renilla* luc) obtained from the control group was taken as one-fold and fold increases calculated by dividing the individual value by the control group value. The fold increase was plotted as a graph. The data are the mean ± SE from three separate experiments.

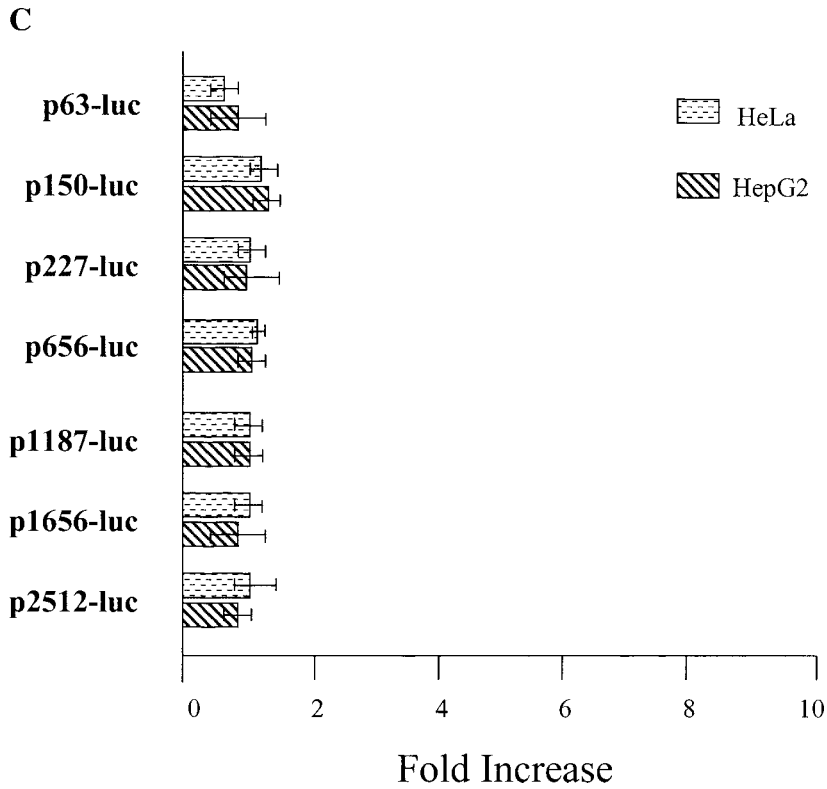


Fig. 3. (*Continued*)

was performed with these labeled probes and nuclear extracts from the four cell lines. We found that a 28-bp oligo between nt −108 and −81 was strongly bound by HeLa and HepG2 cell nuclear extracts, forming two protein–DNA complexes (Fig. 4A, lanes 2-3), whereas the two protein–DNA complexes were slightly detected in EMSA from this oligo with MG-63 and HDP-D cell nuclear extracts (Fig. 4A, lanes 4–5) using the same amount of nuclear extracts. This 28-bp sequence was termed site I. To further identify that the two nuclear factors binding to site I are cell type-dependent, a duplex oligo corresponding to the consensus binding site for CBF/NF-Y was used as a control in an EMSA. Importantly, using the same amount of nuclear extracts from all cell lines, the complex formed with the CBF/NF-Y binding site oligo did not show any significant differences (Fig. 4B, lanes 2–5). This indicates that the increased binding of HeLa and HepG2 cell nuclear proteins to site I represents a true cell type-specific phenomenon.

To further determine if site I is known or unknown DNA–protein binding sites, we used database search programs and found several potential known DNA–protein binding sites at site I such as AP1 and SP1 (Fig. 5A). To confirm whether site I is able to bind AP-1 and SP1 as well as other transcription factors, a series of double-stranded oligos were used as competitors for EMSA (Table I). Results showed that the two protein–DNA complexes derived from HeLa cell nuclear extracts with the site I probe were competed away only with the site I oligo (Fig. 5C, lane 3) but not with any of the other competitor oligos (lanes 4–10). These data strongly suggest that site I is not homologous to these DNA binding sites of known families of transcription factors. Furthermore, using a 100-fold excess of unlabeled competitor oligos that represented the left (site I–L between nt −108 and −95) or right (site I–R between nt −99 and −81) portions of the full site I element (Table II), only the full-length site I or site I–L oligos were able to compete off the specific protein–DNA complexes (Fig. 5D, lanes 2–3). Like the site I probe, the labeled double-stranded site I–L probe incubated with HeLa cell nuclear extracts was able to form the same

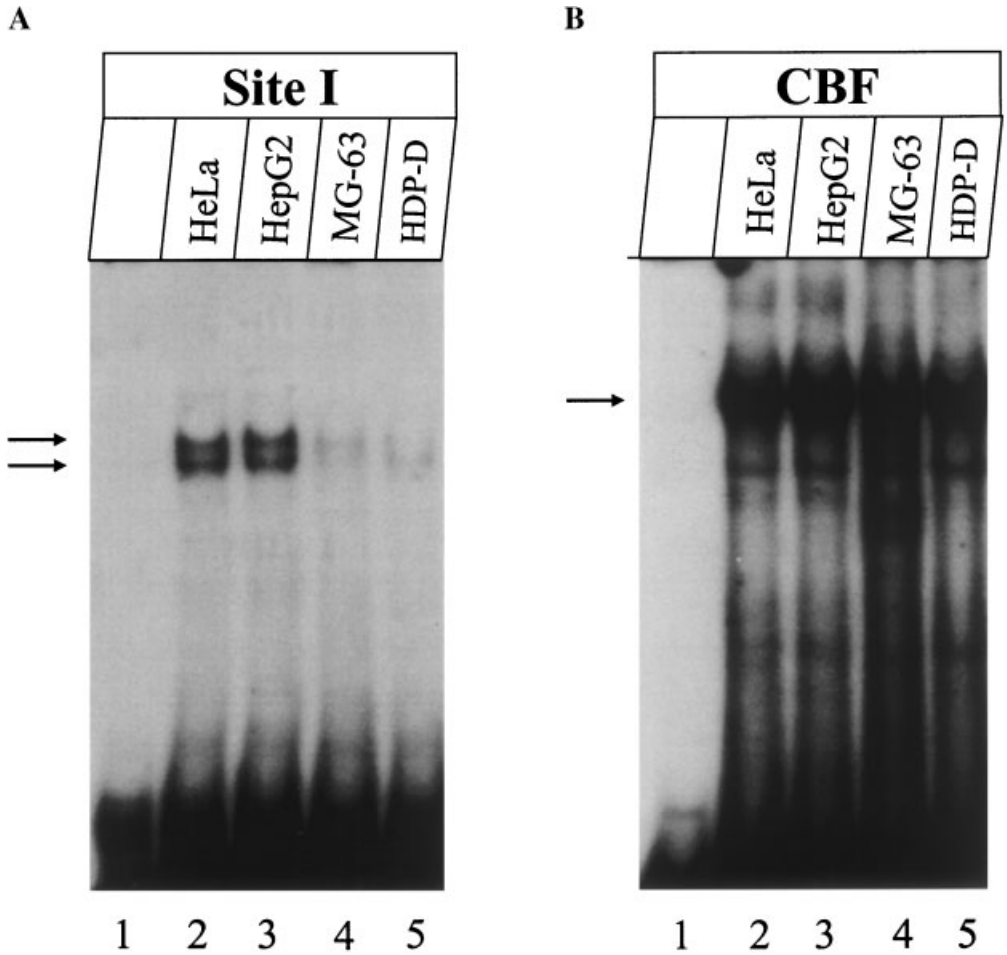


Fig. 4. Cell-dependent changes in the binding of nuclear proteins to the site I DNA element. The P^{32}-labeled probes from site I (**A**) and CBF/NF-Y (**B**) oligos were incubated at the same concentration (5 μg) of nuclear extracts from HeLa (**Lane 2**), HepG2 (**Lane 3**), MG-63 (**Lane 4**), HDP-D (**Lane 5**) cell lines. **Lane 1** is the control with free probes only. Arrows show the protein–DNA complexes formed.

pattern of the protein–DNA complexes, and these complexes could be abolished only with the site I–L and site I oligos (Fig. 5E, lanes 3–4), but not with sense- and antisense- single-stranded site I–L oligos (Fig. 5E, lanes 5–6). Therefore, the specific DNA–protein binding element was refined to a 14-bp DNA sequence between nt −108 and −95.

Determination of Core Site I Sequence Interacting With Two Nuclear Factors

Definition of the consensus sequence within the 14 bp of site I will allow us to identify the element in other bone and tooth gene promoters. Therefore, the 14-bp probe was incubated with HeLa cell nuclear extracts and various DNA competitor oligos (Table II). As shown in Figure 6A, the binding site was narrowed to a 6-bp DNA sequence between nt −101 and −96. To identify the core sequence within site I that is required for the two nuclear factor binding, we mutagenized base pairs within the 6-bp DNA sequence and defined a 6-bp core site I sequence, AG(T/C)C(A/G)C (Fig. 6B and Table II). This core site I DNA sequence is not overt homologous to known transcription factor–DNA binding sites by the existing computer database programs. Moreover, the same results were confirmed when the labeled wild-type and mutant oligos as probes were incubated with HeLa cell nuclear extracts (Fig. 6C). Though the labeled mutant 2 probe with the nuclear extracts produced a lower retarded band (*an asterisk*), the binding was substantially

abolished with the unlabeled homologous oilgo, but not with the unlabeled wild-type site I oligo (data not shown). This indicates that the binding of the nuclear factor to the mutant 2 oligo is different from the wild-type site I oilgo.

Notably, the core site I sequence in the human DMP1 gene promoter is highly conserved with promoter of rat and mouse DMP1 genes present at same location (Fig. 5B). Based on EMSAs, it seems that binding of the two nuclear factors to

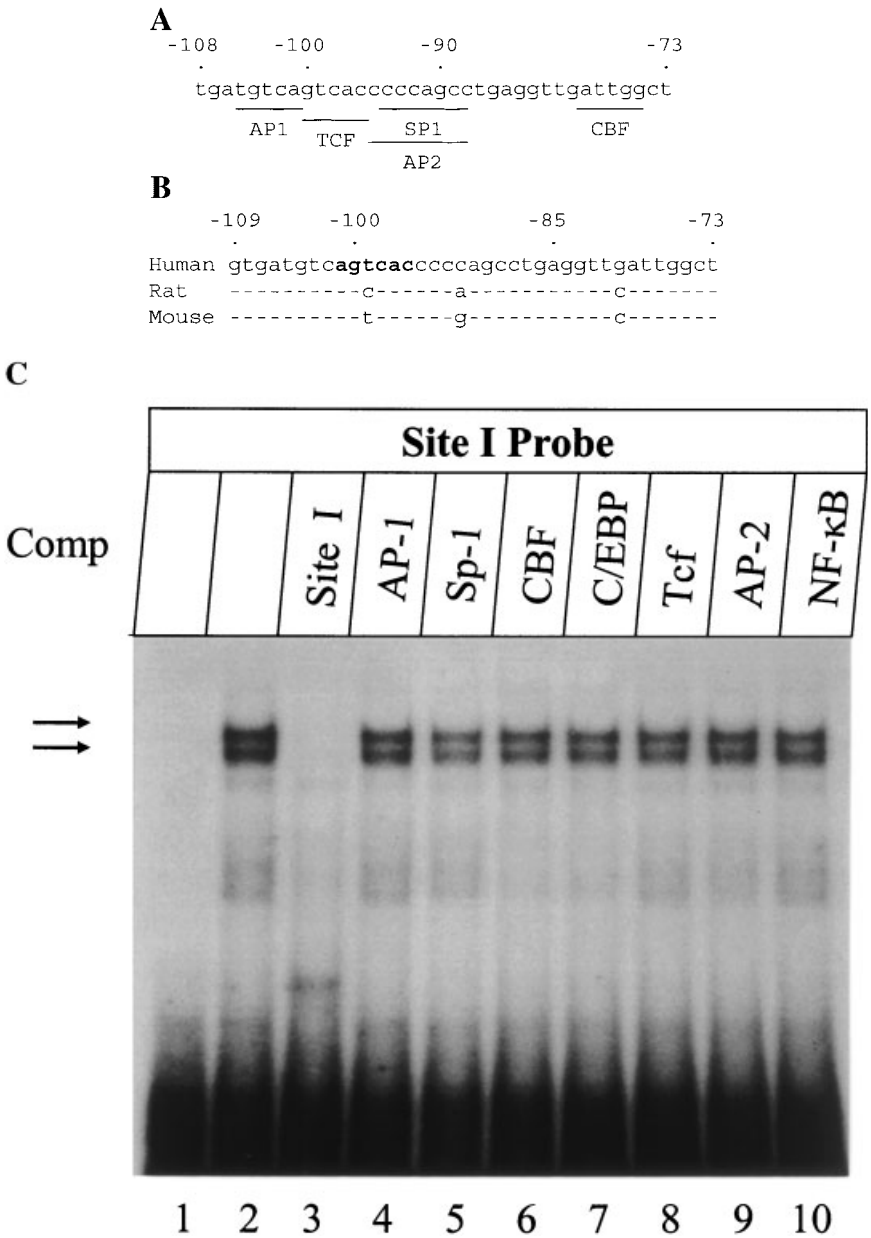


Fig. 5. Competition analyses of sites I and I–L by EMSA. **A**: The nucleotide sequences of the human DMP1 proximal promoter are shown from nt −108 to −73. Several potential protein–DNA binding sites are present in this element. **B**: Comparison of human, rat, and mouse DNA sequences from nucleotides −109 to −73. The core site I sequence is shown in boldface. DNA sequences that identical between the species are shown in dashes. **C** and **D** display the results of incubating ^{32}P-labeled double-stranded site I probe with 5 μg of HeLa cell nuclear extracts, either alone or a 100-fold excess of unlabeled competitors. The probes and competitors used are shown in Tables I and II. **E**: ^{32}P-labeled double-strand site I–L probe was incubated with 5 μg of HeLa cell nuclear extracts, either alone (**lane 2**) or a 100-fold excess of unlabeled site I–L (**lane 3**), site I (**lane 4**), sense- (**lane 5**) and antisense- single-stranded site I–L (**lane 6**) competitors. **Lane 1** shows a free probe control. S.s and a.s site I–L show sense- and antisense- single-strand site I–L oligos. Comp indicates competitor.

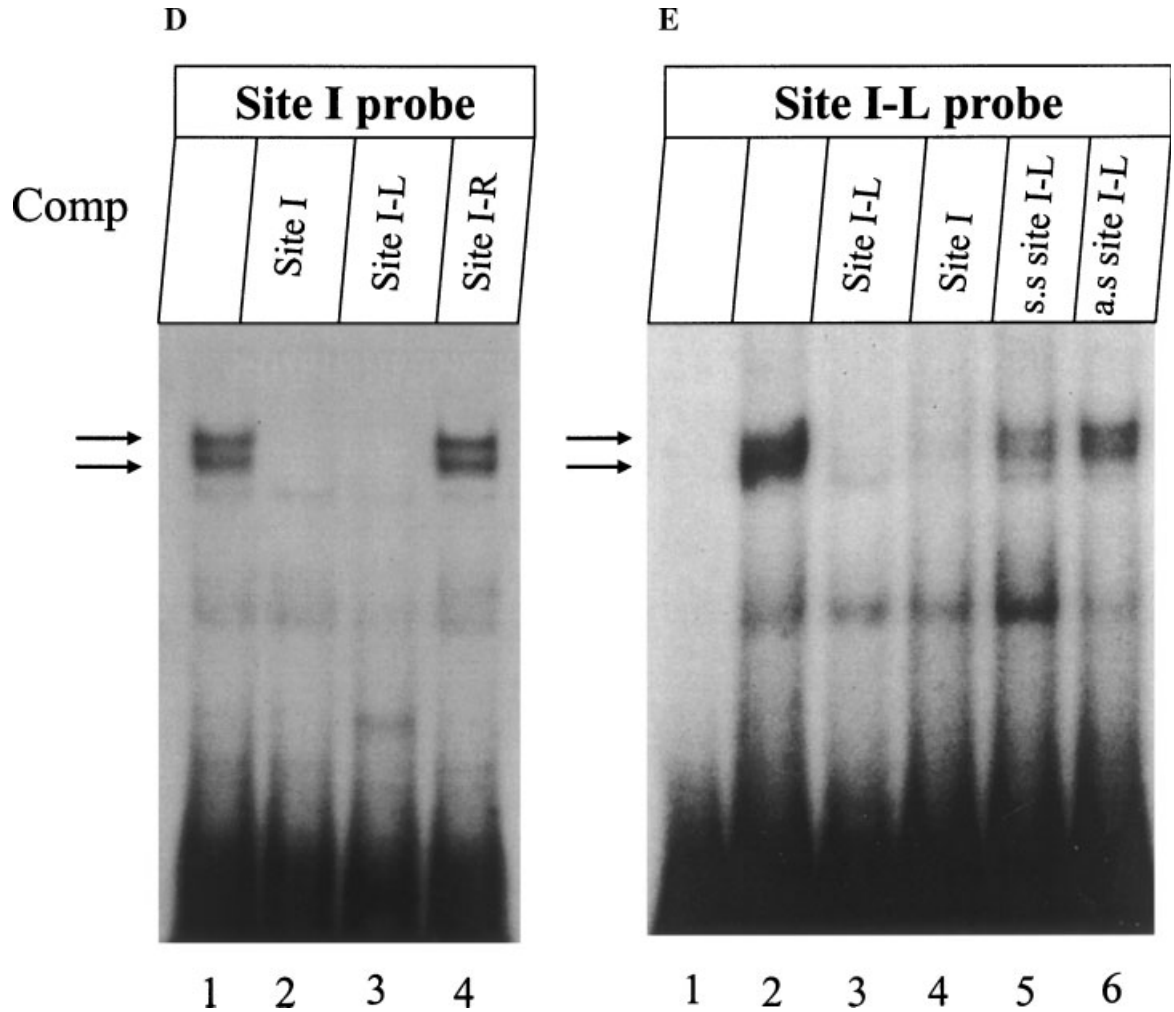


Fig. 5. (*Continued*)

the 6-bp consensus sequence is two subunits from the same transcription factor family. However, it needs to be further investigated.

Core Site I Sequence Acts as a Silencer Element of DMP1 Promoter Activity in HeLa and HepG2 Cell Lines

To investigate the biological role of the core site I sequence in cell-specific activity, we used transient transfection assay of wild-type and mutant promoters in human MG-63 and HDP-D as well as HeLa and HepG2 cell lines. The wild-type DMP1 promoter segment encompassing −150 and +83 bp nucleotide residues (p150-luc) and its two variant forms were ligated to the firefly luc reporter gene and compared for their promoter function in the four cell lines. Two mutant reporter gene constructs were generated and point substitution mutations within the 6-bp core site I (mutant 4 and mutant 6) were shown in Table II. The results present in Figure 7 showed that point mutations within the 6-bp core site I preventing the two nuclear factor binding caused a slight increase in promoter activity in MG-63 and HDP-D cells. However, these mutations resulted in a 5–7-fold increase of DMP1 promoter activities in HeLa and HepG2 cells. We have repeated these experiments with other reporter gene construct of p656-luc. Similar results showed that the mutant promoters were more effective in driving the DMP1-luc reporter gene activity as compared to the wild type control in the HeLa and HepG2 cells (data not shown). From all of these results, we conclude that binding of these two factors to their cognate *cis*-element is likely involved in cell-specific regulation of DMP1 gene.

DISCUSSION

The spatial and temporal restriction of DMP1 gene expression in mineralizing tissues is a highly controlled process. Although the 5′ flanking regions of mouse and rat DMP1 gene were cloned [Feng et al., 1998a; Thotakura et al., 2000a], the mechanisms regulating cell and tissue type-specific expression of DMP1 gene have not been determined. Feng et al. [1998a] have characterized a 4-kb segment of the mouse DMP1 gene promoter performed transient transfection studies in mouse odontoblast (MO6-G3) and osteoblast (2T3) cell lines. This fragment of mouse DMP1 promoter is able to drive reporter gene activity in both odontoblastic and osteoblastic cells, suggesting that it contains a tissue-specific element for bone and tooth. However, the precise regulatory element(s) and *trans*-acting transcription factors have not been reported yet. For the rat DMP1 promoter, the regulatory function was investigated in Chinese hamster ovary (CHO) and mouse calvarial 3T-like cells (MCT3T3) [Thotakura et al., 2000a]. Although Thotakura et al. [2000a] have reported that a ~3-kb fragment of the rat DMP1 promoter is activated in MCT3T3 cells, but not in CHO cells, the

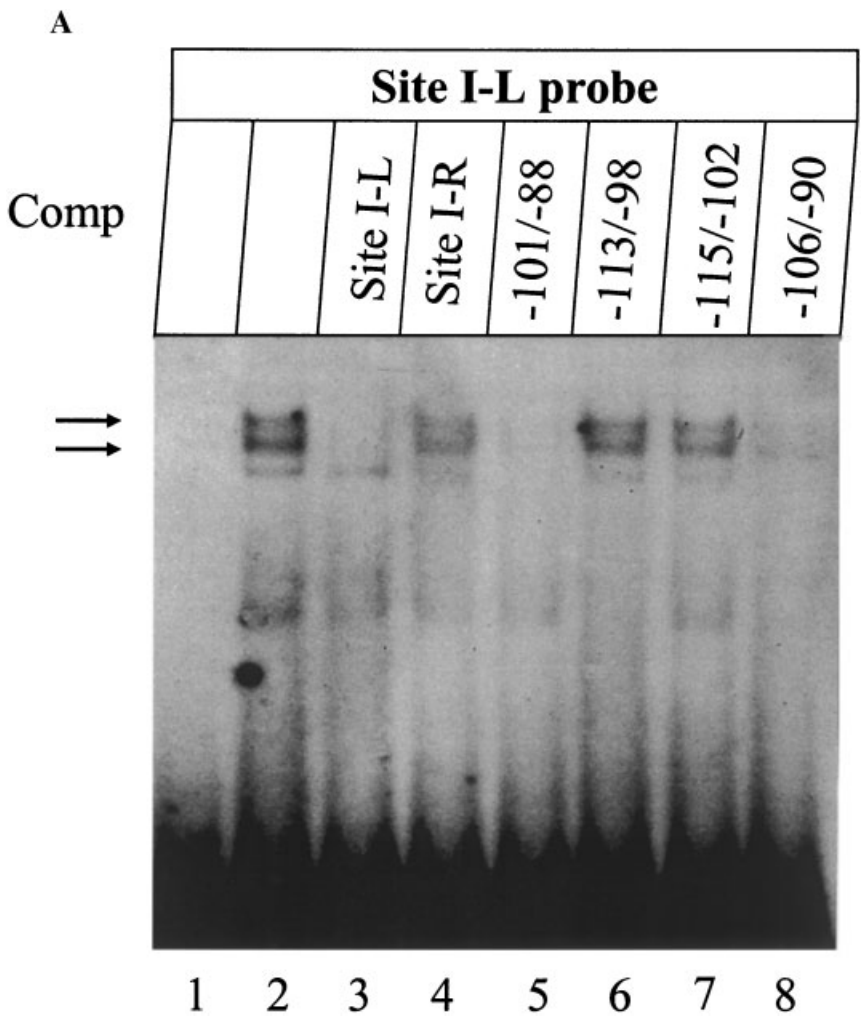
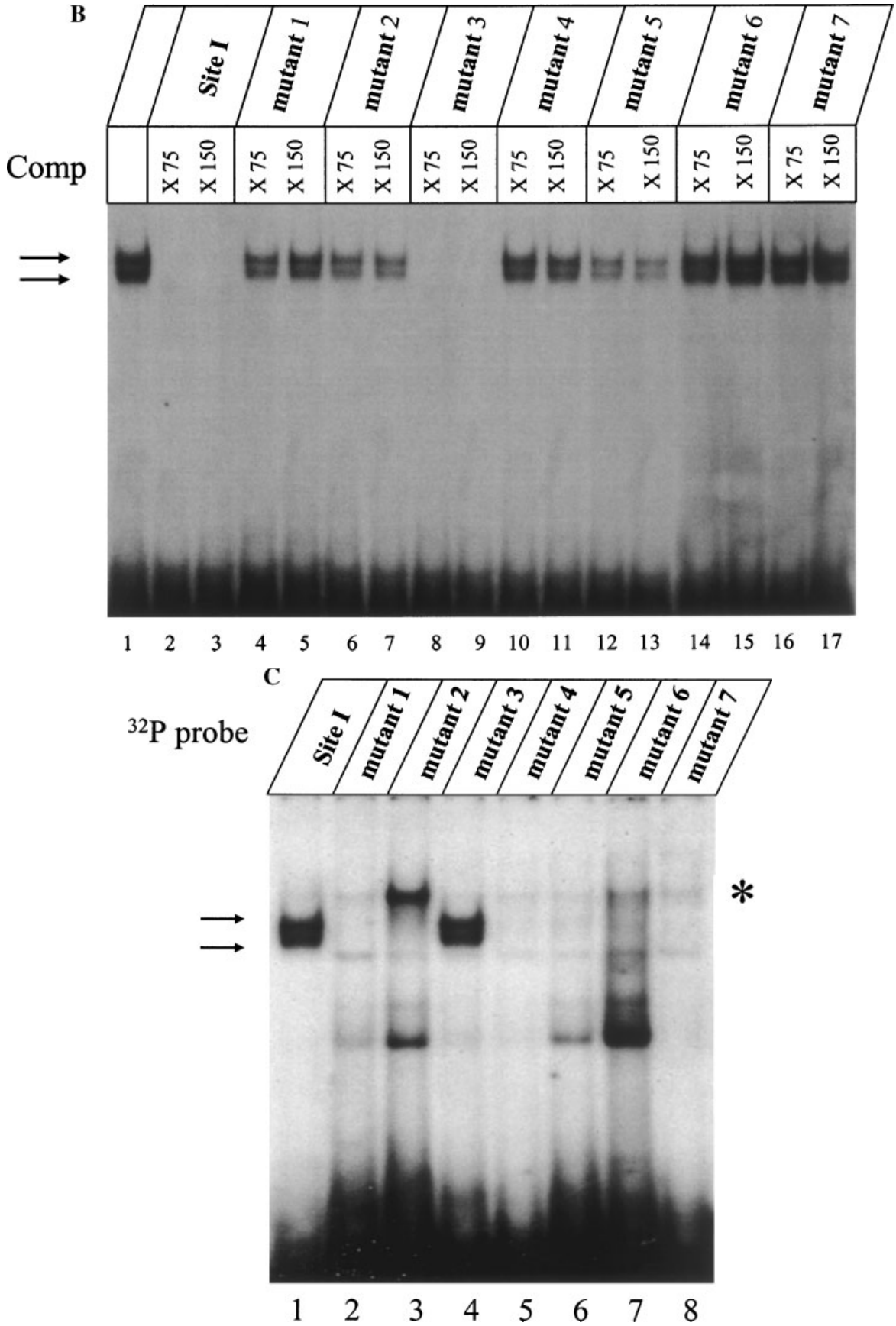


Fig. 6. Definition of core site I sequence bound by two transcription factors. **A**: EMSA was performed with HeLa cell nuclear extracts and the labeled site I–L oligo. Competition experiments were carried out with a 100-fold molar excess of unlabeled site I–L oligo (**lane 3**), or other competitor oligos (**lanes 4–8**) shown in Table II. **B**: EMSA was performed with the labeled site I oligo and HeLa cell nuclear extracts, either alone (**lane 1**), or 75- and 150-fold molar excesses of unlabeled wild type site I oligo (**lanes 2–3**) or mutant site I oligos (**lanes 4–17**). The presence (+) or absence (–) of competition is indicated in Table II. The core site I sequence is shown in boldface. **C**: Wild-type (**lane 1**) and mutant (**lanes 2–8**) site I oligos were analyzed by EMSA using HeLa cell nuclear extracts. The protein–DNA complexes are indicated by arrows or an asterisk.



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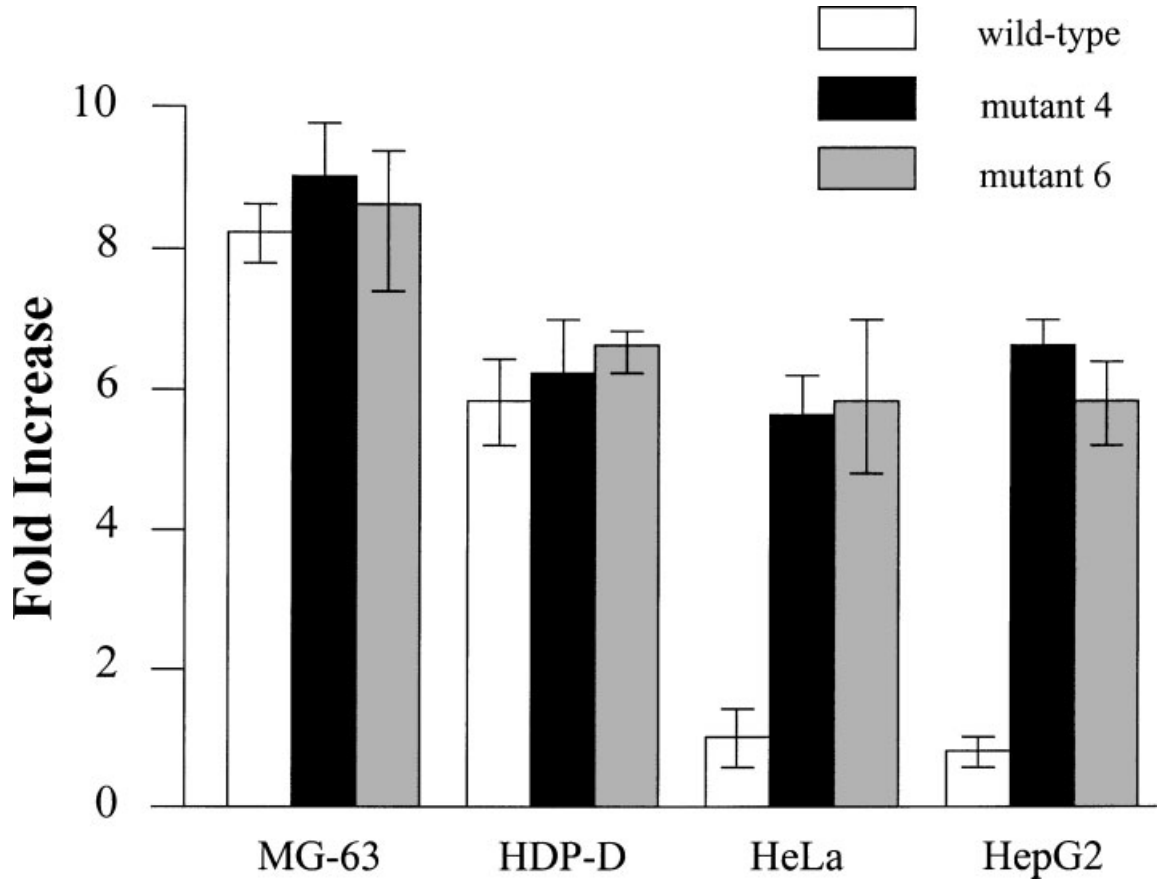

Fig. 7. Biological activity of the human DMP1 promoter with the wild-type and mutant core site I sequences in MG-63 and HDP-D as well as HeLa and HepG2 cells. The wild-type promoter–reporter construct contains −150 to +83 of the human DMP1 gene and the luc reporter gene. For two mutant constructs, the wild-type core site I sequence was substituted by mutant 4 and mutant 6 sequences shown in Table II. The cells were transfected with these wild-type and mutant DMP1 luc–DNA constructs. Luc activity was determined and the value obtained from the control group was taken as one-fold and fold increases calculated by dividing the individual value by the control group value. The fold increase was plotted as a graph. The data are the mean ± SE from three independent experiments.

region conferring this specialty has not been defined. Only several potential enhancer and repressor elements are identified within this ~3-kb rat promoter segment.

Differences in tissue-specific expression profiles of genes in dissimilar phenotypes are due, in part, to the regulation of transcription by *trans*-acting factors that interact with *cis*-acting elements within the promoter regions of these genes. To investigate the tissue-specific gene expression of human DMP1, we isolated and characterized the 5′ flanking region of the human DMP1 gene. Like other related dentin/bone matrix genes such as BSP and DSPP [Feng et al., 1998b; Kim and Sodek, 1999], DMP1 promoter contains an inverted TATA box found close to the major transcription initiation site. Moreover, an inverted CCAAT motif is also found at 76 bp upstream from the major transcription initiation site (Fig. 1A). A gene cluster at chromosome 4q21 in human and 5q21 in mouse such as BSP and DSPP contains the same orientation of the inverted CCAAT motif at proximal transcriptional start sites and this inverted CCAAT motif is functional [Feng et al., 1998b; Kim and Sodek, 1999; Chen and MacDougall, 2003]. In the higher eukaryotic promoters, the CCAAT motif is a widespread regulatory sequence between −60 and −110 relative to the start of transcription [Bi et al., 1997]. The functional inverted CCAAT motif is found at proximal promoters of other collagenous and non-collagenous genes such as OC and collagen type I genes and is essential for these

gene transcription activities [Towler and Rodan, 1994; Bi et al., 1997; Hu et al., 2002]. Furthermore, many other potential DNA–protein binding sites in the ~2.6-kb upstream region of the human DMP1 gene were identified (Fig. 1A). Some known transcription factors essential for tooth and bone tissue-specific regulation such as Cbfa1/Runx2, Msx, Tcf/Lef, C/EBP, AP-1, YY-1 also contained in the human DMP1 promoter [Franceschi, 1999; Karsenty, 1999]. Like many other gene promoters [Thotakura et al., 2000a; Kohler et al., 2001; Garuti et al., 2002], the human DMP1 gene contains two transcription initiation sites. It is interesting to speculate that the two promoters may regulate differentially the two isoforms. For instance, *Cbfa1*/*Runx2* gene gives rise to two transcriptional initiation sites [Stewart et al., 1997; Xiao et al., 1998; Drissi et al., 2000; Pozner et al., 2000]. Two major isoforms of Cbfa1/Runx 2 are generated by the two distinct promoters (P1 or P2, respectively). However, the functional role of these different transcriptional initiation sites in DMP1 gene related to expression of the two major DMP1 isoforms is not clear.

A series of human DMP1 promoter sequential deletion constructs were transfected into MG-63, HDP-D, HeLa, and HepG2 cell lines based on their positive and negative expression of DMP1, respectively. Luc assays revealed that enhancer and repressor elements were seen in MG-63 and HDP-D cells, but not responsive in HeLa and HepG2 cells. A critical *cis*-element between nt −150 and −63 had a 5–7-fold higher activity in MG-63 and HDP-D cell lines than that of HeLa and HepG2 cell lines. The data indicates that this element is involved in cell type-specific regulation of DMP1 expression in the MG-63 and HDP-D versus HeLa and HepG2 cells. We further demonstrated that this element acts as a silencer of the DMP1 gene expression in both HeLa and HepG2 cells.

Based on the transfection results we focused on the negative *cis*-regulatory elements between nt −150 and −63 examining its ability to bind specific nuclear proteins. EMSA results for the four cell lines studied suggest that this element plays a role in the cell type-specific protein–DNA interaction. Two nuclear factors found to interact with the 6-bp core site I DNA sequence within this element are abundantly expressed in HeLa and HepG2 versus MG-63 and HDP-D cells. A series of competitive analyses suggest that the 6-bp DNA sequence is a novel DNA-protein binding site. Notably, this core sequence alignment with the mouse, rat and human DMP1 promoters reveals a high conservation, suggesting a more generalized function for this element. Mutations of the core binding sequence caused a greater increase of DMP1 promoter activity in HeLa and HepG2 cell lines, but were slightly effective of promoter activity in MG-63 and HDP-D cell lines. The contributing role of these two nuclear proteins in the regulation of the DMP1 gene appears to be as transcription repressors. Whether the two nuclear factors are different subunits from the same family or interact with co-factor(s), forming different protein–DNA complexes need to be further investigated. These two nuclear factors and possibly other co-repressors may repress DMP1 gene expression in cells lacking DMP1 expression by recruiting histone deacetylase activity and/or interacting with the basal transcription factors to control the expression of cell type-specific DMP1 gene [Burke and Baniahmad, 2000; Willy et al., 2000; Lunyak et al., 2002]. These regulators may play critical roles in the spatial and temporal DMP1 gene repression in certain tissues such as liver, lung, and muscle.

Although these two protein–DNA complexes are the only transcription factors acting as repressors found to date that regulate DMP1 gene, many cell or tissue type-specific repressors have been reported [Chong et al., 1995; Breslin et al., 2002; Li et al., 2002; Lunyak et al., 2002]. For instance, a neuron-restrictive silencer factor is expressed in most non-neuronal tissues and undifferentiated neuronal progenitors. Because of its tissue distributions the function of this silencer transcription factor suppresses expression of neuronal-specific genes in non-neuronal tissues [Chong et al., 1995; Lunyak et al., 2002]. In a second example, the sequence-specific DNA binding protein AP-2 has dual functions and it is expressed at lower levels in liver and liver-derived hepatoma cell lines but at high levels in HeLa cells and in many other non-liver tissues [Ren and Liao, 2001]. AP-2 acts as a negative regulator repressing expression of certain liver-specific genes in non-hepatic cells. Transcription repressors and co-repressors can function as general negative regulators and contribute to cell differentiation [Burke and Baniahmad, 2000; Breslin et al., 2002]. Therefore, characterization of the two

transcription proteins would enhance our knowledge of their role in the mechanisms of controlling the cell or tissue type-specific and spatial-temporal DMP1 gene expression in future studies.

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