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# Dynamic Interactions of Chromatin-Related Mesenchymal Modulator, a Chromodomain Helicase-DNA-Binding Protein, with Promoters in Osteoprogenitors

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**Key Words.** Differentiation • CHD • Promoters • Progenitors

## ABSTRACT

**The newly identified protein chromatin-related mesenchymal modulator (CReMM) is expressed by marrow stromal progenitors in vivo and ex vivo. CReMM belongs to a recently identified subgroup of chromodomain helicase-DNA-binding proteins composed of multiple domains including chromodomains, SNF2/ATPase, helicase-C domain, SANT, and A/T-hook-DNA binding domain. Chromatin immunoprecipitation assay was applied to follow the dynamics of CReMM binding to A/T-rich regions on promoters of genes that play a role in osteoblast maturation. CReMM interaction with BMP4 and biglycan promoters in the marrow stromal cells was challenged with transforming growth factor-$\beta$. Treatment with 17$\beta$-estradiol enhanced the binding to estrogen receptor and abolished binding to the prolactin receptor promoters; CReMM interaction with osteocalcin promoter was identified constantly. CReMM binding to the analyzed endogenous promoters suggests its direct role in the transcriptional program activated during osteogenic cell differentiation, which may be a useful tool for following the molecular mechanism of the "stemness" of mesenchymal cells.** STEM CELLS *2006;24:1288–1293*

## INTRODUCTION

The fate of mesenchymal stem cells during differentiation is determined by a cascade of events that depends on the timed and coordinated readout of the compacted DNA in the nucleus. Nucleosomes restrict the accession of transcription machinery to specific regulatory regions of the DNA. Therefore, only a relatively small number of genes are read and used, with the rest remaining repressed. Various regulatory elements act as binding sites for distinct factors to bind to the promoters, resulting in gene-specific transcription in cells and tissues. The transcription regulation of differentiating skeletal cells from mesenchymal precursors is a complex process, involving multiple factors. The challenge is to understand how regulatory signals affect gene expression in a promoter-dependent manner through the cooperation of multiprotein complexes that regulate chromatin remodeling. Other levels affecting the cellular and molecular regulation of stem cells include cell-cell and extracellular matrix interactions that switch on the regulatory mechanisms integrated at different hierarchical levels. Regulation of transcript turnover and translational control are an integral part of cell- and tissue-specific gene expression. Variety of post-translational modification processes are known to affect the stability and three-dimensional structure of proteins and consequently their functions.

Systemic hormones and growth factors regulate the skeleton physiology [1–8], and their effect is transduced by cellular transcription machinery to regulate the differentiation of mesenchymal stem cells that have the potential to become chondrocytes, osteoblasts, and adipocytes [3, 9]. The factors affecting the cell differentiation are translated at the epigenetic level via chromatin modification and remodeling, which becomes an important issue for understanding of stem cell biology. Attention is paid to the interplay between chromatin-remodeling factors, integrins, extracellular matrix ligands, and cytoskeleton associates that orchestrate the cell type-specific differentiation programs. Chromatin modification and remodeling activities play a crucial role in stem cell differentiation by regulating the intrinsic state of responsiveness of a cell. Although the reorganization of chromatin structures is clearly part of this process, we understand little regarding the fundamental mechanisms involved and their roles in transcription regulation [10–13]. Several chromatin remodeling complexes have been described for the promotion of transcription by the alteration of chromatin structure via an ATP-dependent mechanism [14–21]. In

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addition, the epigenetic regulation that changes the chromatin configuration has been linked directly to gene transcription [22, 23].

Chromatin-related mesenchymal modulator (CReMM) protein is a chromodomain helicase-DNA-binding (CHD) family member that is expressed by osteoprogenitors both in vivo and in vitro was described by us recently [24–26]. We demonstrated the expression of CReMM in the bone formation regions but not in cartilage and the engagement of CReMM with the bone-related promoters in periosteum cells or at endochondral ossification areas in mice in vivo [26]. The protein discloses multiple domains that relate its activity to the control of transcription regulation. In this study, we followed the dynamic changes in the occupancy of specific endogenous promoters in primary bone marrow stromal cells that posses an osteogenic potential. Specific interaction between partial CReMM peptide with DNA elements on the amplified promoters was demonstrated by electrophoresis mobility shift assay (EMSA). CReMM interaction with A/T-rich regions on promoters appeared to be sensitive to treatment with transforming growth factor-$\beta$ (TGF$\beta$) and 17$\beta$-estradiol, factors known to affect early stages of osteoblastic differentiation. These results suggest that CReMM has a potential role in the transcription regulation of osteogenic cells.

## Materials and Methods

### In Vitro Culture

Human bone marrow stromal cell (MSC) culture was previously described in detail [27, 28]. Cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, http://www.invitrogen.com) with the addition of 10% heat-inactivated fetal calf serum (Gibco). When indicated, MSCs were treated with $10^{-8}$ M 17$\beta$-estradiol (17$\beta$-E2; Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) or with 5 ng/ml TGF$\beta$ (Collaborative Research, Bedford, MA) for 24 hours in culture prior to analysis.

### Antibodies

Anti-CReMM [24], anti-estrogen receptor (anti-ER) (Upstate Biotechnology, http://www.upstatebiotech.com) and anti-pc-Jun (sc-822, Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com) were applied for the chromatin immunoprecipitation (ChIP) analysis.

### Chromatin Immunoprecipitation

The ChIP technique is based on formaldehyde fixation of DNA-protein complexes (http://www.upstatebiotech.com). After fixation, the chromatin fraction from MSCs was sheared, and CReMM-specific fragments were precipitated with anti-CReMM antibody. In some experiments, ChIP was performed with anti-ER and anti-pc-Jun. The ChIP was performed using CReMM-specific antibody to analyze 1) the precipitated protein from the DNA-protein complex, which was separated on SDS-polyacrylamide gel electrophoresis (PAGE) gel and verified by Western blot for CReMM; and 2) DNA purified from the isolated protein-DNA immunocomplex, which was analyzed for CReMM interactions with individual promoters. DNA was isolated upon reversal of the formaldehyde cross-linking and was used as a template for polymerase chain reaction (PCR) amplification with primers specific to the selected promoters. Promoters were chosen based on data available from the gene bank and analyzed using bioinformatics tools, including analysis for A/T-rich stretches as described previously (http://www.opd.tau.ac.il) [29]. We focused on promoters for BMP4, prolactin receptor (PRLR), ER, osteocalcin (OC) and biglycan. ChIP analysis was performed with cells obtained from several donors ($n$ = 5–9).

### Immunoprecipitation, SDS-PAGE Gels, and Western Blot Analysis

These assays were performed according to the standard protocols (http://www.protocol-online.net). Briefly, immunoprecipitation was performed with CReMM antibody incubated overnight with protein A immobilized on Sepharose CL-4B (Pfizer Global R&D, Cambridge, MA, http://www.pfizerrtc.com). The immunocomplexes were separated on 6.5% SDS-PAGE gel for 2 hours and then transferred to the nitrocellulose blots and probed with primary antibody to CReMM. Incubation with the primary antibody for 1 hour was followed by incubation with secondary antibody goat anti-rabbit biotin IgG (DAKO, Glostrup, Denmark, http://www.dako.com) and extravidin-peroxidase (Sigma-Aldrich) for detection with chemiluminescent substrate (Pierce, Rockford, IL, http://www.piercenet.com).

### PCR Amplification

The isolated DNA from ChIP protein-DNA complex was used as template for PCR amplification of selected promoter regions with Taq polymerase (Takara, Otsu, Japan, http://www.takara.co.jp) and specific primers (Table 1). The amplified immunoprecipitated DNA was compared with DNA from corresponding input chromatin fraction, and each PCR was performed at least twice. The reaction products were separated by electrophoresis in 1% agarose gels (SeaKem GTG FMC; Cambrex BioScience Rockland, Inc., Rockland, ME, http://www.cambrex.com) in Tris Borate EDTA (TBE) buffer. The amplified DNA fragments were stained by ethidium bromide, and their optical density was measured using Bio Imaging System (BIS 202D).

### Electrophoresis Mobility Shift Assay

EMSA was performed with recombinant protein (rP) (2332–2481 amino acids) and $^{32}$P-labeled PCR product of specific promoter region. Binding reaction with 1 $\mu$g of rP and 100,000 cpm of $^{32}$P-labeled probe was carried in 20 $\mu$l of binding buffer (26 mM Hepes [pH = 7.9], 2 mM $MgCl_2$, 40 mM KCl, 1 mM dithiothreitol, and 0.25% bovine serum albumin). PCR products were used as probes for EMSA in the presence or absence of distamycin A (Sigma-Aldrich) [30], which competed with the binding between A/T-rich probes and the rP. The samples were incubated for 15 minutes at room temperature prior to loading in 5% native polyacrylamide gel in 0.25 M TBE. The gels were run for 40 minutes at room temperature in TBE buffer and then were dried and autoradiographed using X-OMAT AR (Kodak).

## Results

CReMM is a chromatin remodeling protein, related to the CHD family, expressed by osteoprogenitors in bone section in vivo and by cultured stromal cells derived from the bone marrow. We have shown that CReMM disclosed both A/T-hook-like DNA binding and ATPase activity [24]. The functional domains of

**Table 1.** Primers for chromatin immunoprecipitation analysis

| Gene | Accession number | Primers | PCR product (bp) | % A/T |
|---|---|---|---|---|
| PRLR | 4886757 | F, TAGAAGTTTGCGCTTTGGGT<br>R, GGGCCACATATCTTTCATTG | 312 | 62 |
| ERα | 35159 | F, CGCATGATATACTTCACCTATTTTT<br>R, TTGGGCTAGGATATGCAGAA | 204 | 75 |
| BMP4 | 3850194 | F-GCTAAAGGAGCACAATGCCT<br>R- CCCCAAAAGGAGGACAAAAT | 145 | 67 |
| Biglycan | 3025471 | F, AGAAGGGGACACTACGGGAC<br>R, CCTCGGACATGAGAACCACT | 207 | 39 |
| OC | 10443354 | F, AGGCTGCCTTTGGTGACTC<br>R, TTATACCCTCTGGGCTGTGC | 497 | 38 |

Abbreviations: bp, base pairs; ER, estrogen receptor; F, forward; OC, osteocalcin; PCR, polymerase chain reaction; PRLR, prolactin receptor; R, reverse.

CReMM suggest the protein's potential for binding to the A/T-rich promoters of genes that play a role in osteogenic differentiation [24, 26]. The A/T-rich elements are embedded in promoters and play a role in the regulation of gene transcription by binding A-T-hook proteins. We identified A/T-rich regions for promoters of PRLR, ER, BMP4, biglycan, and OC genes (Figs. 1, 2; Table 1). We employed ChIP to investigate the association of CReMM with these promoters in ex vivo MSC cultures. The specificity of immunoprecipitated chromatin fraction was verified by SDS-PAGE and Western blot with anti-CReMM antibody (Fig. 1E). PCR analysis of the purified DNA from the immunoprecipitated chromatin fraction revealed that CReMM was associated with OC promoter (Fig. 1F) and PRLR (Fig. 2B) but was not present on the BMP4, biglycan, or ER promoters (Fig. 1F). Treatment of MSCs with 17$\beta$-E2 led to de novo interaction of CReMM with the ER promoter (Fig. 2F) and disappearance from the promoter of PRLR (Fig. 2B, lane 3). In contrast, stimulation of cells with TGF$\beta$ enabled CReMM binding to the BMP4 and biglycan promoters (Fig. 1F). The OC promoter was occupied constitutively by the protein, and this interaction was not affected upon challenging of MSCs with either 17$\beta$-E2 or TGF$\beta$. Amplified promoter regions from BMP4 and ER genes were used as probes for EMSA analysis with partial CReMM recombinant protein fragment containing the DNA binding domain (rP, residues 2332–2481 amino acids). The formation of the specific CReMM-DNA complex indicates the capability of binding to the A/T-rich region in analyzed promoters (Fig. 1G, lanes 2, 5), which was abolished in the presence of distamycin A (Fig. 1G, lanes 3, 6).

We analyzed the interactions of CReMM with a promoter of PRLR that is regulated by 17$\beta$-E2 (Fig. 2). 17$\beta$-E2 binds to its receptors and transduces the signals through binding either to the promoters directly or in cooperation with the AP-1 transcription factor complex. In addition to the A/T rich region, the PRLR promoter contains ER-response elements and AP-1 binding sites. We applied ChIP with antibodies for CReMM, ER, and *p*-cJun to analyze the interactions of these regulatory factors with the PRLR promoter in the presence or absence of 17$\beta$-E2. The ChIP analysis showed occupancy of the PRLR promoter by all three proteins in untreated cultures (Fig. 2B). In contrast, the binding of CReMM, ER, and *p*-cJun to the PRLR promoter was abolished in cells treated with 17$\beta$-E2 (Fig. 2B).

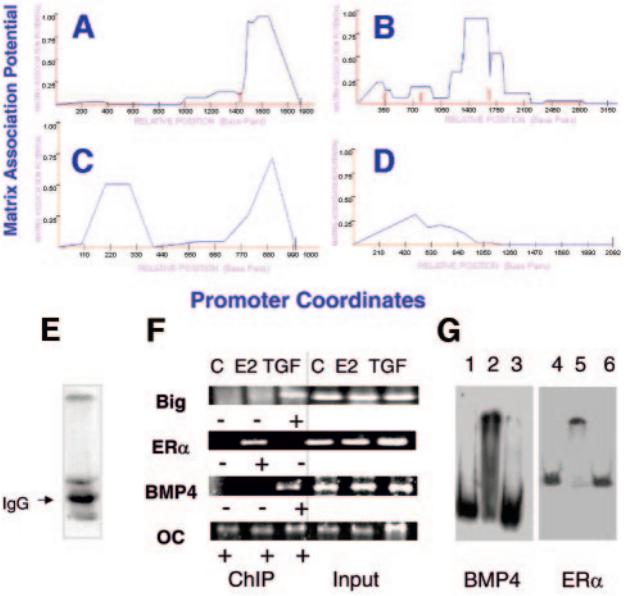


**Figure 1.** A/T-rich analysis of promoter regions of ERα **(A)**, BMP4 **(B)**, biglycan **(C)**, and OC **(D)** genes. Graphs present the relative position (in base pairs) of the matrix association potential region upstream to the transcription start site. **(E):** Western blot analysis of chromatin-related mesenchymal modulator (CreMM) protein immunoprecipitated from chromatin fraction with anti-CReMM antibody. **(F):** ChIP assay of CReMM binding to different promoters of ER, BMP4, byglican, and OC genes amplified by polymerase chain reaction (PCR) from C, E2, or TGF. DNA quality was analyzed in corresponding input chromatin fraction. **(G):** PCR products amplified from ChIP samples were used as probes for electrophoresis mobility shift assay analysis to BMP4 (lanes 1–3) and ER (lanes 4–6) promoter probes. Lanes 1 and 4 correspond to radiolabeled probes; lanes 2 and 5 correspond to hybrid of probe with recombinant protein (rp) CReMM; and lanes 3 and 6 demonstrate abolishment of hybridization with rpCReMM in the presence of distamycin A. Abbreviations: C, untreated cells; ChIP, chromatin immunoprecipitation; E2, 17$\beta$-E2; ER, estrogen receptor; OC, osteocalcin; TGF, transforming growth factor $\beta$-treated.

In summary, the interactions of CReMM with various promoters of bone-related genes were studied in MSCs. According to our results, CReMM does not bind to the promoters of ER, BMP4, and biglycan genes in control MSCs. However, de novo binding to the ER promoter was achieved following 17$\beta$-E2 treatment of cells, and binding to the BMP4 and biglycan

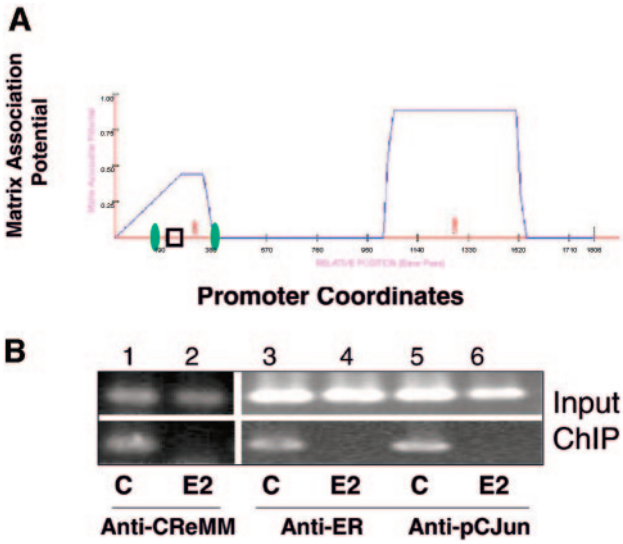


**Figure 2.** ChIP assay of CReMM binding to prolactin receptor promoter. **(A):** Bioinformatic analysis of A/T-rich regions, AP-1 (square), and ER-response elements (circle). Graph presents the relative position (in the base pairs) of the matrix association potential region upstream to the transcription start site. **(B):** DNA obtained from ChIP with anti-CReMM, anti-ER, and anti-p-cJUN from C and E2 cells. The DNA quality was analyzed in the corresponding input chromatin fraction. Abbreviations: C, untreated cells; ChIP, chromatin immunoprecipitation; CReMM, chromatin-related mesenchymal modulator; E2, 17$\beta$-E2 treated; ER, estrogen receptor.

promoters was stimulated by TGF$\beta$. The promoter of OC, a bone-matrix protein expressed in mature cells, was occupied by CReMM in all culture conditions. In untreated MSCs, we identified binding of ER, CReMM, and *p*-cJun to the PRLR promoter, which was negatively regulated by 17$\beta$-E2.

## DISCUSSION

CReMM is a new CHD-related protein that presumably functions as a chromatin modifying factor expressed by osteoprogenitors in vivo and by MSCs in vitro [24]. Recently, we demonstrated the engagement of CReMM with the bone-related promoters in vivo. CReMM is expressed by cells isolated from the periosteum and endochondral ossification areas and is not present in the cartilage of newborn mice [26]. CReMM distribution and function in vivo provides an attractive clue for the study of transcriptional regulation of the maturation of osteogenic cells in vitro. Expression of CReMM was studied in different in vitro models comparing mature and osteoprogenitors [25]. The level of CReMM mRNA was quantified in MSCs cultured from rat and human bone marrow. MSCs from 3-month-old rats expressed CReMM levels 7 times higher than cells harvested from 15-month-old rats, in agreement with reported age-related reduction in cell proliferation capacity and in osteoblastic commitment [25, 31]. CReMM expression in osteoprogenitors was 2.4 times higher than in mature osteoblasts derived from trabecular bone [25]. Furthermore, comparison of CReMM expression between clonal populations of MSCs revealed that it was 2.5 times higher in osteogenic than nonosteogenic MSC clonal sublines that were studied for their osteogenic potential in vivo [25, 32]. We demonstrated by immunostaining and enzyme-linked immunosorbent assay that CReMM expression precedes and partly overlaps with the increase in alkaline phosphatase [25]. The alkaline phosphatase expression was earlier correlated with the expression of osteopontin and osteocalcin that characterize osteoblast maturation [33]. Cellular and molecular parameters of cultured MSCs were extensively studied for their differentiation [9, 24–28, 32–34]. The multiple differentiation potential of MSCs makes them a powerful tool for future cell-therapy modalities. Accomplishment of such a task requires better characterization of molecular mechanisms that trigger the stemness potential of MSCs.

The remodeling of chromatin structure facilitates the access of transcription factors to DNA by repositioning nucleosomes at the promoter region [22, 35]. Chromatin-remodeling proteins, such as SWI/SNF, Mi2/NURD, and ISWI, were shown to interact with sequence-specific DNA-binding factors [36–39], enabling the access of transcription factors to promoters and to the regulation of gene expression. Transcription regulation by these remodelers provides promoter targeting of hormone and growth factor receptors. It is still unclear under what circumstances chromatin-remodeling complexes are recruited to a specific promoter to provide tissue-selective gene transcription. A/T-rich elements are recognized for their role in the regulation of gene transcription by the binding of A/T-hook proteins and serving as an interface between transcription factors and chromatin-remodeling proteins. The ability of CReMM to recognize A/T-rich sequences on promoters is similar to that of HMG and other CHD and SNF2 proteins [14, 24]. In this study, we demonstrated by EMSA in vitro and by ChIP analysis in vivo that endogenous CReMM interacts with the A/T-rich regulatory regions on promoters of osteoblast-specific genes, including the OC, PRLR, ER, BMP4, and biglycan genes, which are expressed at various stages of osteoprogenitor cells differentiation. Estrogen and prolactin receptors are detected in the proliferating cells and are important in the early stages of osteoblast development [40–42]. BMP4 and biglycan are expressed with the beginning of matrix production, and osteocalcin is upregulated in mature osteoblasts [3, 8, 27, 40, 43–45].

Given that CReMM is expressed by osteoprogenitors, we suggest its role in transcriptional response to systemic hormones and growth factors. We treated cells with TGF$\beta$ or 17$\beta$-E2, which affect the cells at the proliferative stages. TGF$\beta$ induces cells at early stages of chondroblastic and osteoblastic differentiation but inhibits myogenesis, adipogenesis, and late-stage osteoblast differentiation [46]. 17$\beta$-E2 enhances bone formation through inhibitory effects on osteoblast apoptosis in vivo and stimulates the effects of osteoblast transcription factors, such as CBFA1 [47, 48]. CReMM interaction with the A/T-rich promoters appears to be a dynamic process that is subjected to regulation by either by 17$\beta$-E2 or TGF$\beta$. CReMM binding to ER, BMP4, and biglycan promoters is stimulated by the related modulation. The protein constitutively occupied PRLR and OC promoters in untreated cells. The PRLR gene is expressed in early mesenchymal cells, stimulates adipocyte and chondrocyte maturation, and is inhibited in mature osteoblast function [40, 41, 49]. Several transcriptional complexes have been analyzed for occupancy by the PRLR promoter during developmental transitions in different cell lineages, including the estrogen receptor and AP-1. Transcriptional factors, including ER and AP-1, affect the PRLR promoter during developmental transitions in different cell lineages and were demonstrated for these

factors' roles during osteoblast maturation [42, 50–53]. Our findings show that occupation of the PRLR promoter by CReMM, ER, and *p*-cJUN is modulated by 17$\alpha$-E2 in a similar way. The results suggest that CReMM, a chromatin remodeler, directly reorganizes chromatin that affects the binding of transcription factors.

In summary, CReMM is a new member of the CHD family that possesses a DNA-dependent ATPase activity and binds to A/T-rich regulatory regions of various promoters. It binds to promoters in a differential pattern modulated by TGF$\beta$ and 17$\beta$-E2. Demonstrated dynamic interactions of endogenous CReMM with the regulatory regions of promoters that are critical to osteoblastogenesis suggest a possible role of CReMM in the transcriptional program in osteoprogenitors and its possible influence on the direction of mesenchymal stem cell maturation. CReMM is a chromatin-remodeling protein with a putative role in the epigenetic control of particular genes along the osteogenesis pathway; it may be helpful in the identification of the cascade of regulatory factors in the commitment and differentiation of mesenchymal cells.

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## DISCLOSURES

The authors indicate no potential conflicts of interest.

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