c-myc Introns Element-binding Proteins Are Required for 1,25-Dihydroxyvitamin D₃ Regulation of c-myc during HL-60 Cell Differentiation and the Involvement of HOXB4

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1,25-Dihydroxyvitamin D₃ (1,25-(OH)₂D₃) suppresses c-myc expression during differentiation of HL-60 cells along the monocytic pathway by blocking transcriptional elongation at the first exon/intron border of the c-myc gene. In the present study, the physiological relevance of three putative regulatory protein binding sites found within a 280-base pair region in intron 1 of the c-myc gene was explored. HL-60 promyelocytic leukemia cells were transiently transfected with three different c-myc promoter constructs cloned upstream of a chloramphenicol acetyltransferase (CAT) reporter gene. With the wild-type c-myc promoter construct (pMPCAT), which contains MIE1, MIE2, and MIE3 binding sites, 1,25-(OH)₂D₃ was able to decrease CAT activity by 45.4 ± 7.9% (mean ± S.E., n = 8). The ability of 1,25-(OH)₂D₃ to inhibit CAT activity was significantly decreased to 18.5 ± 4.3% (59.3% reversal, p < 0.02) when examined with a MIE1 deletion construct (pMPCAT-MIE1). Moreover, 1,25-(OH)₂D₃ was completely ineffective at suppressing CAT activity in cells transfected with pMPCAT-287, a construct without MIE1, MIE2, and MIE3 binding sites (−6.5 ± 10.9%, p < 0.002). MIE1- and MIE2-binding proteins induced by 1,25-(OH)₂D₃ had similar gel shift mobilities, while MIE3-binding proteins migrated differently. Furthermore, chelerythrine chloride, a selective protein kinase C (PKC) inhibitor, and a PKC activator sphinganine, were shown to block 1,25-(OH)₂D₃ inhibition of c-myc transcription. Sphinganine, a PKC inhibitor that acts on the regulatory domain of PKC, also prevents 1,25-(OH)₂D₃ down-regulation of c-myc expression (8). These results provide evidence that PKC plays a critical role in 1,25-(OH)₂D₃ inhibition of c-myc transcription.

Zajac-Kaye and Levens (16, 17) identified several protein binding sites located at intron 1 of the c-myc gene. They termed these protein binding sites MIE1, MIE2, and MIE3. Furthermore, they reported that mutations/deletions in MIE1, MIE2, and/or MIE3 are associated with uncontrolled c-myc expression in numerous Burkitt’s lymphoma cell lines (18). Recently, we reported that 1,25-(OH)₂D₃ significantly enhances the binding of two nuclear proteins to MIE1 in HL-60 cells (19). In the present study, MIE1-, MIE2-, and MIE3-binding proteins were demonstrated to be required and may act in concert to regulate c-myc during HL-60 cell differentiation. HOXB4 was identified to be the major MIE1-binding protein. Moreover, HOXB4 protein levels were increased by 1,25-(OH)₂D₃ prior to monocytic differentiation of HL-60 cells.

EXPERIMENTAL PROCEDURES

Cell Culture—HL-60 promyelocytic leukemia cells were obtained from American Type Culture Collection and cultured in RPMI 1640 medium supplemented with 10% horse serum, 50 units/ml penicillin G, and 50 µg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere with 5% CO₂. Cells used in this study were from passage 14–45.

Plasmids—The c-myc plasmids were generously provided by Dr.

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1,25-(OH)₂D₃ Regulation of c-myc

Maria Zajac-Kaye. pMPCAT contains a 3.2-kilobase HindIII-SstI fragment of c-myc cloned upstream of the chloramphenicol acetyltransferase (CAT) gene (18). This c-myc fragment includes 2328 bp upstream of exon 1, exon 1, and the first 387 bp of intron 1, which contains the MIE1, MIE2, and MIE3 sequences. pMPCAT-287 was constructed by deleting 195 bp of CAPS-SstI fragment (bp 2981–3268). The MIE1 sequence was deleted from pMPCAT by polymerase chain reaction methodology using overlapping oligonucleotides to form pMPCAT-MIE1 (deletion of bp 3001–3020). A luciferase expression vector (pGL3) (Promega) was used to normalize for transfection efficiency. The concentration and quality of all vectors used for transfections were confirmed by agarose gel electrophoresis.

DNA Transfection and Reporter Gene Assays—HL-60 cells (6 × 10⁶) were transiently transfected with 20 μg of plasmid DNA and 2 μg of pGL3 by electroporation using a Gene Pulser II Electroporator (Bio-Rad) at 270 V and 975 microfarads. After a 24 h recovery period, the transfected cells were divided equally into two treatment groups. Subsequently, cells were treated with vehicle or 100 nM 1,25-(OH)₂D₃ for 48 h. Cells were freeze/thawed for three cycles, and cell debris was pelleted by microcentrifugation. Protein concentration of the supernatants was assayed by the Bradford method (20). The amount of cell extract and the incubation time used in the CAT assay were optimized so that the CAT activity measured was linear. The percent of CAT conversion was quantified on a Beckman LS8100 counter and normalized to the percent conversion of the pMPCAT transfection. This relative CAT expression value was then corrected for transfection efficiency by dividing it by the ratio of luciferase expression between pMPCAT-MIE1 or pMPCAT-287 and pMPCAT transfection. Luciferase activity was measured according to the Luciferase Assay System (Promega).

Preparation of Oligonucleotides—Oligonucleotides were synthesized by the DNA Synthesis Core Facility at the University of Michigan. The oligonucleotides were synthesized on Applied Biosystems automated DNA synthesizers (Forest City, CA), employing β-cyanothymosinphosphoramidite chemistry on controlled pore glass support. MIE1 recognition sequence: 5′-AGAGTTAGTTGAATTGTTAGCGG-3′; MIE2 recognition sequence: 5′-CTTATGAAATATACTGCGC-3′; MIE3 recognition sequence: 5′-CTCCCGGCGCCGACATCTGTTATGTG-3′.

Gel Shift Assay—Nuclear protein extracts were prepared as described previously (19). Nuclear proteins (2 μg) were incubated with 10,000 cpm (0.1 ng) of 32P-labeled MIE1, MIE2, or MIE3 probe and 100 ng of poly(dI-C) in a buffer containing 20 mM HEPES (pH 7.9), 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol (DTT) 30 min at 25 °C. Protein-DNA complexes were separated from free DNA probe by electrophoresis on high ionic strength 4% polyacrylamide gel (acrylamide:bisacrylamide, 29:1) containing 50 mM Tris base (pH 8.5), and 2 mM EDTA. Thereafter, gels were dried and visualized by autoradiography.

Purification of a MIE1-binding Protein by Biotin/Streptavidin Affinity System—Nuclear extract (500 μg) from 1,25-(OH)₂D₃-treated HL-60 cells was incubated with 20 ng of biotin-labeled MIE1 double-stranded DNA probe containing 25% of MIE1 binding sites (pMPCAT) and 200 mM HEPES (pH 7.9), 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT for 30 min at 25 °C. Subsequently, the binding reaction mixture was incubated with 5 μl molar excess of streptavidin-agarose on a rotating wheel for 2 h. The resin was centrifuged at 14,000 rpm for 30 s, and the supernatant (flow-through) was removed. The pellet was washed with binding buffer two times and resuspended in elution buffer (12% glycerol, 20 mM Tris-Cl (pH 6.8), 1 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM DTT). After incubating on a rotating wheel for 20 min, the resin was centrifuged at 14,000 rpm for 30 s, and the supernatant containing the MIE1-binding proteins was collected.

Protein Sequencing—For N-terminal protein sequencing, gels were transferred to a polyvinylidene difluoride membrane in buffer containing 10 mM CAPS (pH 11) for 4 h at 70 V. The membrane was stained with Coomassie Blue, and the desired protein band was excised. Samples were sequenced using a Protein PI 2090E Integrated Micro-sequencing System. Protein sequence analysis was performed by the Protein and Carbohydrate Structure Facility at the University of Michigan.

Western Blot Analysis—Equal amounts of nuclear protein from each condition were run on a 10% polyacrylamide gel, and proteins were subsequently transferred onto Immobilon-P (Millipore). The membrane was blocked with buffer containing 10 mM Tris (pH 7.4), 0.1% Tween 20, and 2% bovine serum albumin. It was then probed for 2 h with a HOKB4 antibody (Berkeley Antibody Co.), then washed three times with blocking buffer and incubated for 1 h with a secondary antibody conjugated with horseradish peroxidase (Sigma). Then, the membrane was washed five times with Tween-TBS (10 mM Tris and 0.2% Tween 20, pH 7.4). Finally, the membrane was developed using enhanced chemiluminescence (NEN Life Science Products) and exposed to x-ray film.

Statistical Analysis—Statistical analysis was performed using two-tailed Student’s t test.

**Results**

Effect of 1,25-(OH)₂D₃ on c-myc Promoter Activity in Transiently Transfected HL-60 Cells—We showed previously that 1,25-(OH)₂D₃ increases the binding of a novel 32-kDa doublet protein to the MIE1 of the c-myc gene (19). Since the time course of this increase in protein binding was similar to that of 1,25-(OH)₂D₃ inhibition of c-myc transcription, MIE1-binding proteins were suggested to be responsible for blocking c-myc transcription. In the present study, a CAT reporter gene system was utilized to determine whether the MIE-binding proteins play a functional role in 1,25-(OH)₂D₃ regulation of c-myc. The wild-type c-myc construct (pMPCAT) contained 2.3 kilobases upstream of exon1, exon1, and the first 387 bp of intron 1, which contains the MIE1, MIE2, and MIE3 binding sites. pMPCAT-MIE1 was constructed by deleting MIE1 (bp 3001–3020) from pMPCAT. pMPCAT-287 was constructed from pMPCAT by deleting a HinPI-SstI fragment (bp 2981–3268), which removed the MIE1, MIE2, and MIE3 binding sites. HL-60 cells were transiently transfected with pMPCAT, pMPCAT-MIE1, or pMPCAT-287 by electroporation. In all experiments pGL3, a luciferase expression vector, was cotransfected to normalize for transfection efficiency. After a 24-h recovery period, transfected cells were divided equally into two treatment groups. One-half was treated with vehicle, the other was treated with 100 nM 1,25-(OH)₂D₃ for 48 h. It should be noted that these transfected cells differentiated normally in response to 1,25-(OH)₂D₃ as determined by nitro blue tetrazolium dye reduction and nonspecific esterase activity (data not shown). As shown in Fig. 1, 1,25-(OH)₂D₃ inhibited CAT activity by 45.4 ± 7.9% (mean ± S.E., n = 8) in cells transfected with the wild-type c-myc construct (pMPCAT). With the MIE1 deletion construct (pMPCAT-MIE1), 1,25-(OH)₂D₃ was only able to suppress CAT activity by 18.5 ± 4.3% (mean ± S.E., n = 8); this 59.3% reversal was statistically significant (p < 0.02). Deletion of MIE1, MIE2, and MIE3 binding sites (pMPCAT-287) completely reversed 1,25-(OH)₂D₃’s ability to inhibit CAT activity (~6.5 ± 10.9% inhibition by 1,25-(OH)₂D₃, mean ± S.E., p < 0.002). These transfection results indicate that MIE2- and/or MIE3-binding proteins are acting cooperatively with the MIE1-binding proteins to block c-myc transcription.

**Induction of MIE1-, MIE2-, and MIE3-binding Proteins by 1,25-(OH)₂D₃**—Since the cell transfection data shows that MIE2 and/or MIE3 binding sites are also involved in 1,25-(OH)₂D₃ regulation of c-myc, we examined if 1,25-(OH)₂D₃ can enhance nuclear protein binding to MIE2 and MIE3. HL-60 cells were treated with vehicle or 100 nM 1,25-(OH)₂D₃ for 48 h. Nuclear proteins were isolated and assayed for MIE1, MIE2, and MIE3 binding sites using EMSA (Fig. 2). Consistent with our previous study, 1,25-(OH)₂D₃ enhanced the binding to two nuclear proteins to the MIE1 site. MIE2-binding proteins migrated similarly to MIE1-binding proteins. This observation was not surprising, since the MIE2 binding site has sequence homology to the MIE1 binding site. Interestingly, MIE3-binding protein induced by 1,25-(OH)₂D₃ migrated differently. To determine the specificity of these MIE-binding proteins, competition experiments were performed (Fig. 3). MIE1 and MIE2 complexes were completely blocked by competition with unlabeled MIE1, MIE2, and MIE3 (Fig. 3A, lanes 2–4, and Fig. 3B, lanes 7–9). Different slower migrating MIE1 and MIE2 complexes were observed when unlabeled MIE1 or MIE2 was used as the competitor (Fig. 3A, lanes 2 and 3, and Fig. 3B, lanes 7 and 8). Interestingly, these slower MIE1 and MIE2 complexes
were not apparent when unlabeled MIE3 was used as a competitor, suggesting that this protein has greater affinity for MIE3 than MIE1 or MIE2. As shown in Fig. 3C, unlabeled MIE1 or MIE2 was not as effective as unlabeled MIE3 in displacing the binding of the MIE3 complexes. Moreover, the MIE1, MIE2, and MIE3 complexes were not displaced with a nonspecific competitor (OCT1, 100-fold excess).

**Effect of a PKC Activity Inhibitor and a PKCβ Antisense Oligonucleotide on 1,25-(OH)2D3 Induction of MIE1-, MIE2-, and MIE3-binding Proteins**—Our laboratory reported that phorbol 12-myristate 13-acetate mimicked the effects of 1,25-(OH)2D3 by inducing a similar increase in the binding of a 32-kDa doublet protein to MIE1 (19). Moreover, this report showed that dephosphorylation with alkaline phosphatase completely prevented the 32-kDa proteins to bind to MIE1. However, treatment of nuclear proteins with phosphatase is a crude method to determine the importance of protein phosphorylation. To further elucidate the role of protein phosphorylation and specifically PKC, HL-60 cells were treated with two different protocols to inhibit 1,25-(OH)2D3 mediated increases in PKCβ levels and activity. Chelerythrine chloride, a PKC activity inhibitor, and a PKCβ antisense oligonucleotide were used. Previously, our laboratory reported the activity and specificity of the PKCβ antisense used in this study (15). HL-60 cells were cotreated with 100 nM 1,25-(OH)2D3 and 3 μM chelerythrine chloride or 30 μM PKCβ antisense for 48 h. Nuclear extracts were isolated and assayed for MIE1, MIE2, and MIE3 binding using EMSA. As shown in Fig. 4A, 1,25-(OH)2D3 significantly increased the binding of two nuclear proteins to MIE1. Extract from cells cotreated with 1,25-(OH)2D3 and chelerythrine chloride exhibited a marked reduction in protein binding to MIE1. Likewise, a PKCβ antisense oligonucleotide was able to significantly suppress 1,25-(OH)2D3-induced binding of proteins to MIE1, while the PKCβ sense had no effect. Similar results were obtained using MIE2 (Fig. 4B) and MIE3 (Fig. 4C) oligonucleotides. These observations support the notion that protein phosphorylation via PKCβ is required for 1,25-(OH)2D3 regulation of c-myc during HL-60 cell differentiation.

**Identification of a MIE1-binding Protein**—To purify the MIE1-binding proteins, we utilized a biotin-streptavidin affinity system (Fig. 5A). Nuclear extract from 1,25-(OH)2D3-treated cells was incubated with biotin-labeled MIE1 double-stranded oligonucleotide. The incubation buffer, duration of incubation, and temperature were identical to the conditions used in the EMSA. The binding reaction was then incubated with a 5-fold molar excess of streptavidin-agarose on a rotating wheel. The reaction mixture was centrifuged, and the supernatant (FT) was removed. The biotin-streptavidin-agarose pellet was washed two times (W1 and W2) with binding buffer and resuspended in elution buffer. After incubating on a rotating

![Figure 1](image1.png)

**Fig. 1. Effect of 1,25-(OH)2D3 on c-myc promoter activity in transiently transfected HL-60 cells.** A schematic representations of plasmids used in the transfection experiments. The parent construct pMPCAT consists of a 3.2-kilobase HindIII-SstI fragment of the human c-myc gene cloned upstream of a CAT reporter gene. pMPCAT-MIE1 contains a deletion of the MIE1 binding site. pMPCAT-287 contains a 287-base pair HindIII-SstI deletion and is missing MIE1, MIE2, and MIE3 binding sites. B, CAT activity measured in transfected HL-60 cells. Cells were transiently transfected with pMPCAT, pMPCAT-MIE1, or pMPCAT-287 by electroporation. Cells were cotransfected with pGL3, a luciferase expressing vector, to control for transfection efficiency. After a 24-h recovery period, cells were divided into two equal groups and treated with vehicle or 100 nM 1,25-(OH)2D3 for 48 h. Values are normalized for transfection efficiency by adjusting for luciferase activity. Data are presented as the means ± S.E. of eight independent determinations. *, p < 0.02; **, p < 0.002.

![Figure 2](image2.png)

**Fig. 2. Identification of MIE1-, MIE2-, and MIE3-binding proteins induced by 1,25-(OH)2D3.** HL-60 cells were treated with vehicle or 100 nM 1,25-(OH)2D3 for 48 h. Nuclear proteins were assayed for MIE1, MIE2, and MIE3 binding using EMSA. MIE1 sequence: 5′-AGAGTATATGGGTAACGGG-3′; MIE2 sequence: 5′-CTTATGATATATTACGGC-3′; MIE3 sequence: 5′-CTCCCCGGCCGTCGGACATTCC-TGCTTTATTGT-3′. This figure is representative of five independent experiments.
wheel, the mixture was centrifuged, and the supernatant containing the MIE1-binding proteins was collected (E).

The collected fractions were dialyzed for 2 h against 50 volumes of 20 mM HEPES (pH 7.9), 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT. Fifty mg of nuclear protein from the starting material (SM) and the FT fraction along with the entire volume collected from the wash 1 (W1), wash 2 (W2), and elute (E) fractions were separated on a 10% SDS-PAGE and visualized by Coomassie blue staining. As shown in Fig. 5B, a protein around 32 kDa was selectively retained until eluted. Importantly, this elute fraction still maintained its capacity to bind to MIE1 (Fig. 6, lane 4). The retarded proteins from the elute fraction had an identical migration pattern as compared with the starting material (Fig. 6, lane 2 versus lane 4), indicating that the MIE1-binding proteins were still present in the elute fraction. The sequence of the 32-kDa MIE1-binding protein (QPE(X)G) was found to partially match (80% identity) with residues 49–53 (QPEAG) of the human HOXB4 protein. With this information, we determined whether the 32-kDa protein that was selectively retained by biotin-streptavidin purification was HOXB4. When nuclear protein extracts (1,25-(OH)2D3-treated) were subjected to purification using MIE1-biotin, a HOXB4 antibody was able to detect a 32-kDa protein from the starting material (SM) and elute (E) fractions but not from the flow-through (FT) and wash (W1 and W2) fractions (Fig. 6B). To further support the identification of the 32-kDa protein as HOXB4, supershift experiments were performed using a HOXB4 antibody (Fig. 7). Nuclear protein extracts used in the supershift experiments were subjected to biotin-streptavidin purification. Consistent with...
**Fig. 5.** Purification of a MIE1-binding protein by biotin-streptavidin affinity system. A, purification scheme for a MIE1-binding protein. Nuclear extract (500 μg) from 1,25-(OH)2D3 (100 nM for 48 h)-treated cells was incubated with biotin-labeled MIE1 double-stranded oligonucleotide in binding buffer for 30 min at 25 °C. Subsequently, the binding reaction was incubated with a 5-fold molar excess of streptavidin-agarose on a rotating wheel for 2 h. The reaction mixture was centrifuged at 14,000 rpm for 30 s, and the supernatant (FT) is removed. The pellet was washed two times with binding buffer (W1 and W2) and resuspended in elution buffer. After incubating on a rotating wheel for 20 min, the mixture was centrifuged at 14,000 rpm for 30 s, and the supernatant containing the MIE1-binding proteins was collected (E). B, SDS-PAGE analysis of fractions collected from the MIE1 biotin-streptavidin affinity system. Fifty (50) μg of nuclear protein from the starting material (SM) and the FT fraction along with the entire volume collected from the W1, W2, and E fractions were separated on a 10% SDS-PAGE and stained with Coomassie Blue. First lane: STD, protein standard; second lane: SM, starting material; third lane: FT, flow-through; fourth lane: W1, wash 1; fifth lane: W2, wash 2; sixth lane: E, elute. This figure is representative of three independent experiments.

**Fig. 6.** MIE1 binding was still observed with the elute fraction from 1,25-(OH)2D3-treated nuclear proteins. A HOXB4 antibody was able to completely block the binding of nuclear proteins to MIE1. The nonimmune serum had no effect. These results demonstrate that the 32-kDa HOXB4 protein is the major MIE1-binding protein.

**Effect of 1,25-(OH)2D3 on HOXB4 Protein Levels—**HL-60 cells were treated with vehicle or 100 nM 1,25-(OH)2D3 for 48 h. HOXB4 protein levels were determined by Western blot analysis (Fig. 8). HOXB4 has been characterized as a phosphoprotein with a molecular mass of 33 kDa (21). HOXB4 levels were significantly increased in cells treated with 1,25-(OH)2D3 by 125 ± 15% (n = 5). Interestingly, 1,25-(OH)2D3 also increased the levels of a 60-kDa protein that is recognized by the HOXB4 antibody by 95 ± 12% (n = 5). We do not know the relationship between the 60-kDa protein and the 32-kDa HOXB4.

**DISCUSSION**

The c-myc protooncogene has been shown to be important in the control of cellular proliferation and differentiation. Overexpressing c-myc in transgenic mice results in high incidences of widespread neoplasia (22, 23). Conversely, transgenic mice carrying the null mutations for c-myc die in utero between 9.5 and 10.5 days (24). Moreover, c-myc levels are elevated in both experimentally induced and naturally occurring tumors (25). Recently, c-myc has been reported to play a role in the regulation of telomerase activity. Several groups have shown that differentiation of HL-60 cells with retinoic acid or phorbolester 12-myristate 13-acetate results in a significant reduction of telomerase activity (26–28). Fujimoto and Takahashi (29) reported that a c-myc antisense oligonucleotide inhibits telomerase activity in three leukemia cell lines, HL-60, U937, and K562. Telomerase is a ribonucleoprotein complex that adds telomeric repeats onto the ends of chromosomes during the replicative phase of the cell cycle. Without telomerase activity cells will withdraw from the cell cycle and undergo cell death. Telomerase activity has been detected in a wide variety of human tumors, but only in a few normal somatic cells (30). Thus, differentiation of tumor cells is associated with decreased c-myc expression and telomerase activity.

Regulation of c-myc gene expression is complex and has been shown to involve several mechanisms, including changes in transcription initiation, transcription elongation, RNA stability, and RNA translation (4, 31). Previously, we reported that binding of a 32-kDa doublet protein to MIE1 may be a mechanism used by 1,25-(OH)2D3 to regulate c-myc transcription during HL-60 cell differentiation (19). The results from our cell transfection data indicate that MIE1-, MIE2-, and MIE3-binding proteins are required and may cooperate to down-regulate c-myc during 1,25-(OH)2D3 promotion of HL-60 cells (Fig. 1). In addition, we demonstrated that 1,25-(OH)2D3 induces nuclear proteins to bind to MIE2 and MIE3. As shown in Fig. 3, a 100-fold excess of unlabeled OCT1 was unable to displace the binding of specific nuclear proteins to MIE1, MIE2, and MIE3. Unlabeled MIE1 or MIE2 was able to completely displace the binding of two retarded bands to 32P-MIE1 and caused the appearance of a higher molecular weight MIE1 complex. This
Results and Discussion

A streptavidin affinity system. A, EMSA analysis using 32P-MIE1. HL-60 cells were treated with vehicle or 100 nM 1,25-(OH)2D3 for 48 h. Nuclear proteins were extracted and designated as the starting material (SM). Half of the SM was further subjected to biotin-streptavidin purification and the elute (E) fraction was collected. MIE1 binding was determined with the SM and E fractions from vehicle and 1,25-(OH)2D3-treated cells. B, Western blot analysis for HOXB4. Nuclear extract from 1,25-(OH)2D3-treated cells were subjected to biotin-streptavidin purification. The fractions collected were separated on a 10% SDS-PAGE. SM, starting material; FT, flow through; W1, wash 1; W2, wash 2; E, elute. This figure is representative of three experiments.

Higher molecular weight MIE1 complex was visible in the absence of unlabeled MIE1 or MIE2 if the film was allowed to expose for a longer period of time (data not shown). These observations suggest that the higher molecular weight binding protein has a lower affinity for MIE1. The same results were obtained using 32P-MIE2, suggesting that the same proteins bind to MIE1 and MIE2. Moreover, unlabeled MIE3 was able to block the binding of nuclear proteins to MIE1 and MIE2 (Fig. 3A, lane 4). Unlabeled MIE3 was more effective at displacing nuclear proteins to bind to 32P-MIE3 than unlabeled MIE1 or MIE2 (Fig. 3C, lanes 12–14). It should be noted that the fastest migrating MIE3 band appears to bind with specificity to MIE3. Taken together, these results suggest that these 1,25-(OH)2D3-inducible proteins are capable of binding to MIE1, MIE2, or MIE3 with different specificities and affinities.

We and others (9–11) showed previously that 1,25-(OH)2D3 promotes the differentiation of HL-60 cells by increasing PKC levels. In addition, it was demonstrated that 1,25-(OH)2D3 must also provide the cofactors, calcium and phospholipids, to chronically activate PKC in order to promote HL-60 cell differentiation (32). Recently, we reported that PKCβ antisense oligonucleotides were able to block 1,25-(OH)2D3-mediated increases in PKCβ levels as well as 1,25-(OH)2D3 promotion of HL-60 cell differentiation (15). Using the same PKCβ antisense construct, MIE1, MIE2, and MIE3 complexes induced by 1,25-(OH)2D3 were completely abrogated (Fig. 4). Similarly, chelerythrine chloride, a selective PKC activity inhibitor, was able to significantly reduce the binding of MIE1, MIE2, and MIE3 to the nuclear proteins. These data suggest that c-myc regulatory proteins must be phosphorylated via PKCβ in order to bind to their respective MIE sites.

The results from the biotin-streptavidin purification showed that a 32-kDa protein was retained by biotin-MIE1. Previously, we reported that the predominant MIE1-binding protein is a 32-kDa doublet protein as determined by SouthWestern blot analysis. From this, we were interested in identifying the 32-kDa protein isolated from the biotin-streptavidin purification. The amino acid sequences obtained for the 32-kDa protein have similarities to the human HOXB4 protein. Also, the characteristics of HOXB4 was similar to our unknown 32-kDa MIE1-binding protein. HOXB4 and the 32-kDa MIE1-binding protein are phosphoproteins with similar molecular weights. Additionally, both proteins bind DNA and act as transcriptional regulators. To determine whether the 32-kDa MIE1-binding protein was HOXB4, a supershift EMSA was performed using a HOXB4 antibody. As shown in Fig. 7, a HOXB4 antibody was able to block the binding of nuclear proteins to MIE1. Most likely, the HOXB4 antibody is interacting with the MIE1 binding site on HOXB4 since addition of 32P-MIE1 prior to antibody incubation partially reverses the block in complex formation (data not shown). Moreover, a HOXB4 antibody immunodecorated the 32-kDa protein that was affinity purified using biotin-MIE1 (Fig. 6). Taken together, our results strongly support the identification of the 32-kDa MIE1-binding protein as HOXB4.

The homeobox (HOX) genes are a family of transcriptional regulators that contain a conserved 183-nucleotide sequence. This nucleotide sequence encodes a 61-amino acid domain, the homeomain, and includes a helix-turn-helix DNA binding motif (32, 33). Several investigators have examined the role of individual homeobox genes in cell lines and in primary hema-
topoietic tissues (34–36). From these studies, evidence exists to suggest the possibility of a correlation between expression of individual HOX genes and hematopoietic cell phenotype (36, 37). Studies utilizing human leukemia cells have demonstrated that cells representing various stages of hematopoietic differentiation exhibit different patterns of HOX gene expression (38). Furthermore, HOX genes have been involved in transcription events in certain leukemia cells, suggesting that mutant/nonfunctional forms of these proteins may be important in oncogenesis (39–41). Studies have shown that HOX proteins are able to bind to regulatory sites to control gene expression. HOX3C, HOX4C, HOX4D, and HOX4E are able to transactivate the HOX3D promoter in both HeLa and NIH3T3 cells (42). In addition, Zappavigna et al. (43) reported that HOX4C and HOX4D are able to transactivate the HOX4D promoter, whereas HOX4E is not. Relevant to our finding, Valergera et al. (44) demonstrated that the HOX 2.2 protein is able to bind to the second intron and the 3′ enhancer region of the β-globin gene, important regulatory sites for globin expression. Our studies indicate that HOXB4 is the major MIE1-binding protein. Thus, it is possible that HOXB4 is able to block transcription elongation and thereby negatively regulate c-myc expression during cell differentiation. Alternatively, it is possible that HOXB4 is heterodimerizing with a distinct MIE1-binding protein to form a cooperative MIE1 binding complex. Several studies have shown that HOX proteins heterodimerize with non-HOX proteins to form DNA binding complexes (45, 46). Shen et al. (46) reported that the Meis1-HOX heterodimeric complex dissociates at a much slower rate from its target DNA than Meis1 alone. Our data does not exclude any of these possibilities but indicates that HOXB4 is involved in the complex regulation of c-myc expression.

Several investigators have reported that modulation of HOX expression can affect myeloid differentiation. Overexpression of HOXB8 in a murine myeloid cell line inhibited the ability of interleukin-6 to promote differentiation (47). Lill et al. (48) demonstrated that overexpression of HOXB7 in HL-60 cells blocked granulocytic differentiation in response to retinoic acid or Me2SO, but did not potentiate monocytic differentiation promoted by 1,25-(OH)2D3. Moreover, they reported that overexpression of HOXB7, by itself, is not sufficient to promote monocytic differentiation, suggesting that other HOX proteins must be involved in this differentiative process. Recently, 1,25-(OH)2D3 has been shown to increase HOXB7 expression in HL-60 cells prior to monocytic differentiation (48). In the present study, HOXB4 protein levels in HL-60 cells were significantly increased upon exposure to 1,25-(OH)2D3. Interestingly, in differentiated HL-60 cells (1,25-(OH)2D3 for 72 h), HOXB4 protein levels were similar to untreated cells (data not shown). This observation suggests that HOXB4 is involved in the process of initiating differentiation but not in maintaining a mature cell phenotype.

In summary, these results demonstrate that MIE1, MIE2, and MIE3 binding sites are necessary for 1,25-(OH)2D3 down-regulation of c-myc. Also, nuclear proteins binding to MIE1, MIE2, and MIE3 are blocked by inhibition of PKC activity and decreased PKCβ levels. This study further reveals that a major MIE1-binding protein is HOXB4. Moreover, HOXB4 protein levels are regulated by 1,25-(OH)2D3 during HL-60 cell differentiation.

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