Pre-B Cell Leukemia Transcription Factor (PBX) Proteins Are Important Mediators for Retinoic Acid-dependent Endodermal and Neuronal Differentiation of Mouse Embryonal Carcinoma P19 Cells*

Pu Qin‡, Juliet M. Haberbusch§, Zhenping Zhang¶, Kenneth J. Soprano§¶, and Dianne R. Soprano§¶

From the Departments of ¶Microbiology and Immunology and §Biochemistry and the ¶Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

Pre-B cell leukemia transcription factors (PBXs) act as cofactors in the transcriptional regulation mediated by Homeobox proteins during embryonic development and cellular differentiation. PBX1 protein is expressed throughout murine embryonic development, and its deletion in mice disrupts chondrogenesis. PBX protein levels are also increased in mouse embryonal carcinoma P19 cells during retinoic acid (RA)-induced differentiation. To elucidate the role of PBX proteins in this process, we stably overexpressed PBX1b antisense mRNA in P19 cells (PBX1b-AS cells). PBX1b-AS cells did not differentiate to neuronal or endodermal cells following treatment with RA suggesting PBX proteins are required for both processes. Furthermore we demonstrated that PBX proteins regulate the RA-dependent induction in the mRNA levels of bone morphogenetic protein 4 (BMP4) and Decorin (DCN) in P19 cells using both PBX1b-AS cells and PBX1b ASI-1 small interfering RNA. Chromatin immunoprecipitation assays further demonstrated that PBX proteins directly bind to the promoter of Bmp4 and Dcn in vivo in a RA-dependent fashion. In addition, type I and type II BMP receptor mRNA levels were also increased in P19 cells following RA treatment; however, this was PBX-independent. Taken together these data demonstrate that PBX proteins are required for RA-induced differentiation of P19 cells and that PBX proteins regulate the expression of BMP4 and DCN during this differentiation process.

Vitamin A is an important nutrient for growth, differentiation, immune function, and embryonic development. In vivo, vitamin A is converted to its major biologically active form retinoic acid (RA) that binds to retinoic acid receptors (RARs) and retinoid X receptors to mediate its functions (for a review, see Ref. 1). RA regulates the expression of a number of Homeobox (Hox) genes during embryonic development (for reviews, see Refs. 2–4). HOX proteins act as transcription regulatory factors that bind to specific DNA sequences in the promoter of their target genes via the 61-amino acid homeodomain (5). These HOX proteins are closely associated with not only skeletal patterning but also the patterning of limb and craniofacial structures and the development of pharyngeal arches, hindbrain, and the reproductive tract (for a review, see Ref. 6).

Pre-B cell leukemia transcription factors (PBXs) are members of the three-amino acid loop extension superclass of Homeobox proteins that contain three extra conserved amino acid residues (PYP) between helix 2 and helix 3 of their homeodomains (7). PBX1 was originally identified in a chromosomal translocation t(1;19) found in pre-B cell leukemia in which the homeodomain of PBX1 was fused to the transactivation domain of E2A (8, 9). Later other subtypes of PBX including PBX2 and PBX3 were also identified and found to have high amino acid sequence homology to PBX1 (10). Alternative splicing generates multiple isoforms of PBX1 and PBX3 but not of PBX2.

PBX proteins and other members of the three-amino acid loop extension superclass of proteins including MEIS proteins are important cofactors for the transcriptional regulation mediated by HOX proteins. PBX and MEIS proteins can bind to a number of HOX proteins to form a dimeric complex or even trimeric complex with higher DNA binding affinity and specificity (11–17). These protein complexes regulate the expression of a number of genes including, EphA2, p21, Hoxb1, and Hoxb2 (15, 18–20).

PBX1b protein is expressed throughout murine embryonic development, and the functional inactivation of the Pbx1 gene is lethal in mice at embryonic day 15/16 (21, 22). The defects in these Pbx1 knock-out mice are associated with chondrogenesis suggesting an irreplaceable role for PBX1 in this process. Interestingly the skeletal malformations are restricted to the domains specified by HOX proteins that contain PBX interaction motifs suggesting the PBX-HOX complexes are regulating skeletal patterning. Furthermore our laboratory has demonstrated that a teratogenic dose of RA induced the mRNA and protein levels of all three PBX subtypes in the limb buds suggesting that PBX could be associated with RA-induced malformation in the limb buds (23). However, it is still unclear how PBX proteins regulate chondrogenesis.

P19 mouse embryonal carcinoma cell line is an excellent model cell system to study RA-regulated gene expression and differentiation (24). The information derived using this cell line has often been used to understand the regulation of gene ex-
pression by RA during embryonic development. For example, the mechanism of RA-dependent induction of a number of HOX proteins was studied in these cells, and this knowledge has been applied to animal studies (25–28). In addition, PBX1/23 mRNAs and proteins are induced during RA-dependent endodermal and neuronal differentiation of mouse embryonal carcinoma P19 cells in a RAR-dependent manner (29, 30).

In an effort to elucidate the role of PBX during RA-dependent differentiation of P19 cells, we prepared P19 cells that constitutively overexpress PBX1b antisense mRNA (PBX1b-AS cells). These cells display a greatly reduced RA-dependent induction of PBX1b protein levels. Although primary RA-induced events such as the elevation of RAR/2 mRNA and MEIS2 mRNA levels still occur in these PBX1b-AS cells, they do not undergo either endodermal or neuronal differentiation. In addition, PBX proteins were found to bind to the promoter of bone morphogenetic protein 4 (Bmp4) and Decorin (Dcn), two important regulators of chondrogenesis, and to induce the expression of these genes in wild type P19 cells but not in PBX1b-AS cells. Furthermore the mRNAs of two BMP receptors and BMP2 are also induced by RA treatment but in a PBX-independent manner in P19 cells. Taken together these data demonstrate that PBX proteins are required for RA-induced differentiation of P19 cells and that PBX proteins regulate the expression of BMP4 and Dcn during this differentiation process.

**EXPERIMENTAL PROCEDURES**

Cell Culture and Differentiation—Mouse embryonal carcinoma P19 cell line was purchased from American Type Culture Collection. All cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) with 10% fetal bovine serum and 2 mM L-glutamine, 100 μg/ml penicillin, and 100 units/ml streptomycin. For endodermal differentiation, 1 × 10⁵ cells/100-mm tissue culture dish were treated with 10⁻⁷ M all-trans-RA for 4 days and then 2 days without RA. For neuronal differentiation, 1 × 10⁵ cells/ml of DMEM containing 10⁻⁷ M RA were placed in 100-mm bacterial Petri dishes on day 0. The cells were collected and resuspended in fresh DMEM containing 10⁻⁷ M RA and returned to fresh 100-mm bacterial Petri dishes on day 2. Finally on day 4 the cells were collected, trypsinized, plated on tissue culture dishes, and incubated with DMEM without RA for an additional 3 days. Cells were photographed at the end of the treatment period using a Nikon Eclipse TE300 microscope with Nikon digital camera DXM1200 attachment and the ACT-1 software from Nikon. 400× magnification was used.

Preparation of PBX1b-AS P19 Cells—The mouse PBX1b full-length cDNA was amplified from 11 day postcoitus mouse liver RNA using the RT-PCR technique with the following primers: sense, 5'-AAATGAGATCATGGACGACCAGCACCGAGGCT-3'; antisense, 5'-TAGTCTAGAGTTGATTCCTGGTAACCGAATG-3'; antisense, 5'-ATCTCGAGAATTTGATATCTTCTCTCTG-3'; antisaense primer matching the transcribed vector-specific sequence (5'-ATCTCGAGCAGATTTGCACACTTCTCGG-3'). The PCR product was cloned into pCR4-TOPO vector, and the PBX1b DNA was confirmed by sequencing to be 100% homologous to the previously reported PBX1b cDNA sequence (GenBank™ accession number AF020197). This PBX1b cDNA was then subcloned into pcDNA4/TTO vector (Invitrogen) in the antisense orientation with the cytomegalovirus promoter upstream. This construct was stably transduced into P19 cells using the calcium phosphate method (31) followed by selection with 200 μg/ml Zeocin. Isolated stable clones were screened by RT-PCR analysis testing the expression of exogenous antisense mRNA with the sense PBX1b sequence (5'-TTCCATATGCTGACACATTGGCTG-3') and the antisense primer matching the transcribed vector-specific sequence (5'-ATTCTCGAGAATTTGATATCTTCTCTCTG-3'). The clones were then treated with 10⁻⁷ M RA for 24 h and then incubated with 10⁻⁷ M RA for an additional 3 days. Coverslips were photographed at the end of the treatment period using a Nikon Eclipse TE300 microscope with Nikon digital camera DXM1200 attachment and the ACT-1 software from Nikon. 400× magnification was used.

Preparation of siRNAs— siRNAs were designed using RNAiZol B reagent (Tel-test Inc., Friendswood, TX). The sequence for PBX1 siRNA was selected using the siRNA Target Finder program on Ambion’s Website. A region in the PBC-A small interfering RNA against PBX1 (PBX1 siRNA) was synthesized using the Silencer™ siRNA construction kit from Ambion Inc. (Austin, TX). The sequence for PBX1 siRNA was selected using the siRNA Target Finder program on Ambion’s Website. A region in the PBC-A domain of PBX1 (5'-AACGCTGCTTGTGTGGAAGAT-3') was chosen as the target for PBX1 siRNA. The scrambled GAPDH siRNA was purchased from Ambion Inc. (Austin, TX). 2 × 10⁵ P19 cells were plated on 100-mm tissue culture dishes on day 0, and transfections were performed on day 2 using the siPORT™ Lipid transfection agent from Ambion Inc. For transfection, we first added 9 μl of siPORT Lipid to 45 μl of DMEM, and then mixed this mixture was incubated at room temperature for 10–30 min. 50 pmol of each siRNA sample was then added into DMEM to a final concentration of 50 pmol/100 μl, mixed with the diluted siPORT Lipid, and incubated at room temperature for 15–20 min. The cells were washed with 5 ml of DMEM, and then 2.4 ml of fresh DMEM were added to each plate. All of the siPORT Lipid with siRNA (600 μl) was then added dropwise onto the plates and mixed by
rocking back and forth without swirling. The plates were then incubated in a 37 °C CO2 incubator for 4 h, and then 9 ml of fresh DMEM were added to each plate to maximize cell growth and reduce cytotoxicity. The next day, these transfected P19 cells were trypsinized, replated on 100-mm dishes, and treated with ethanol or 10−7 M RA. Nuclear protein extracts were harvested 24 h following RA treatment to examine PBX1/2/3 protein levels using Western blot. Total RNA samples were harvested 72 h following RA treatment for analysis of DCN and BMP4 mRNA levels using RT-PCR. Transfection of scrambled GAPDH siRNA (Ambion Inc.) was performed as a control. The scrambled GAPDH siRNA should not affect the expression of either PBX or GAPDH.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assays were used to examine the association between PBX proteins and the promoter of the Bmp4 and Dcn genes. Wild type P19 cells or PBX1b-AS cells were plated on 100-mm cell culture dishes in the presence or absence of 10−7 M RA for 3 days. Chromatin immunoprecipitation assay was performed according to the manufacture’s protocol for the ChIP assay kit (Upstate Biotechnology Inc., Lake Placid, NY).

Briefly 37% formaldehyde was added to the cells on tissue culture dishes to a final concentration of 1% in the medium for 10 min at room temperature to cross-link DNA with associated proteins. The medium was removed, and the plates were washed with PBS twice. 1 × 106 cells in 200 μl of SDS lysis buffer (supplied with the kit) were subjected to sonication to break chromatin into small pieces containing 500–2000 bp of DNA. The sonication was performed at 30 s/cycle (0.2 s on and 0.2 s off, 50% amplitude using a Branson digital sonifier) for 12 cycles with samples submerged in an ice-water bath. The sample was subjected to centrifugation at 20,800 × g for 30 min at 4 °C. The pellet was discarded, and the supernatant (~200 μl) was diluted 10 times with ChIP dilution buffer (supplied with the kit) to a total volume of 2 ml (Sample I).

A fraction (20 μl of Sample I) was saved as total input, and the remainder of Sample I was mixed with or without 1 μg of anti-PBX1/2/3 antibody on a rotating platform at 4 °C overnight. The DNA-protein antibody complex was collected using protein A-agarose beads, washed with buffers supplied with the kit, and finally released from the beads. The cross-link between DNA and associated proteins in immunoprecipitated samples and total input samples was reversed by incubating the samples at 65 °C with 0.2 M NaCl for 4 h. The samples were further purified according to the instructions of the kit, finally dissolved in 30 μl of water, and subjected to PCR.

2 μl of Sample I (total input) or 3 μl of the immunoprecipitated sample (equivalent to ~200 μl of Sample I) were used as template for PCR. PCR primers for BMP4 ChIP assays were 5′-CTCCAAATCCAT-TAAAGCCAAAGCTGCACC-3′ and 5′-TTGGCTGTGTTAGAAGCTGT-3′, which flank the putative PBX-HOX binding site in the promoter region of Bmp4 (see Fig. 7A). PCR primers for the Dcn ChIP assay were 5′-GGGTACGTTT-3′ and 5′-ACAAGGCAGCTCCAGAGATTCTAATTCTG-3′, which flank two putative PBX-HOX binding sites in the promoter region of Dcn (see Fig. 7B). The PCR parameters were 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 2 min for 38 cycles with 94 °C for 5 min for initial denaturation and 72 °C for 10 min for final extension.

RESULTS

Preparation of PBX1b-AS P19 Cells—The levels of PBX1, PBX2, and PBX3 are elevated in a RAR-dependent manner in mouse P19 embryonal carcinoma cells during RA-dependent endodermal and neuronal differentiation (29, 30). To elucidate the role of PBX in the RA-mediated differentiation of P19 cells, PBX1b-AS P19 cells that constitutively overexpress full-length PBX1b antisense mRNA were prepared and studied following treatment with RA.

Using RT-PCR with primers that map to PBX1b cDNA and plasmid vector we specifically detected the expression of the exogenous antisense PBX1b mRNA but not the endogenous PBX1b mRNA. Fig. 1A shows that this PBX1b antisense mRNA is only expressed in PBX1b-AS cells but not in wild type P19 cells or empty vector cells. Overexpression of this antisense PBX1b mRNA reduced the levels of PBX1/2/3 proteins before RA addition (Fig. 1B, compare the ethanol-treated control lanes). Moreover the RA-dependent elevation in PBX1/2/3 protein levels is also greatly reduced in these PBX1b-AS cells when compared with that of wild type P19 cells and empty vector cells grown in both monolayer and as aggregates (Fig. 1B). We obtained four stable PBX1b-AS P19 clones (clones 2, 8, 9, and 12) that demonstrated greatly reduced RA-dependent induction of PBX1/2/3 proteins. All four clones reacted similarly to RA treatment, and the results from one representative clone (clone 2) are shown in Figs. 1–4.

PBX1b-AS Cells Do Not Differentiate upon RA Treatment—Undifferentiated wild type P19 cells grow densely packed and display a characteristic cuboidal morphology that is evident using a phase-contrast microscope. For endodermal differentiation, wild type P19, empty vector cells, and PBX1b-AS cells were plated in monolayer for 4 days with 10−7 M RA followed by an additional 3 days without RA. After this RA treatment procedure, wild type P19 cells and empty vector cells differentiated into much larger endoderm-like cells that grew slower than undifferentiated P19 cells (Fig. 2, A and B). Immunohistochemical analysis demonstrated that over 95% of these endodermal cells lost the expression of SSEA-1, a marker for undifferentiated P19 embryonal carcinoma cells (Fig. 2A). Furthermore greater than 95% of these endodermal cells gained the expression of TROMA-I, a marker for endoderm-like cells (Fig. 2B) (36). However, after RA treatment, essentially all of the PBX1b-AS cells remained morphologically undifferentiated, retained the expression of SSEA-1, and did not express TROMA-I (Fig. 2, A and B). Western blot analysis also demon-
strated that the level of TROMA-I was strongly elevated in wild type P19 cells and empty vector cells but not in PBX1b-AS cells after this RA treatment procedure (Fig. 3). Thus, the induction of PBX proteins appears to be required for the RA-induced endodermal differentiation of P19 cells.

For neuronal differentiation, P19 cells were allowed to form aggregates in bacterial Petri dishes in the presence of 10^{-7} M RA for 4 days. These aggregates were collected, trypsinized, and then plated on tissue culture dishes for an additional 3 days without RA. After this treatment, wild type P19 cells and empty vector cells differentiated into neuron-like cells that are characterized by axon-like structures extending between cells (Fig. 2C). Greater than 95% of these neuronal cells lacked the expression of SSEA-1 (Fig. 2D) and gained the expression of neurofilament-H, a marker of neuronal cells (Fig. 2E). However, this treatment procedure did not differentiate PBX1b-AS cells into neuronal cells as characterized by their morphology, their continued expression of SSEA-1, and their lack of expression of neurofilament-H (Fig. 2, C, D, and E). Therefore, PBX proteins appear to be required for both RA-induced endodermal differentiation and neuronal differentiation of P19 cells.

RA Increases the mRNA Levels of the Primary Response Genes in PBX1b-AS Cells—To rule out the possibility that the
PBX1b-AS cells are totally unresponsive to RA, the expression of RARβ2 and MEIS2 (primary response genes normally induced within 3 h of RA treatment in wild type P19 cells (37, 38)) was examined in wild type P19 cells, empty vector cells, and PBX1b-AS cells. The mRNA levels of RARβ2 and MEIS2 are elevated following RA treatment in these PBX1b-AS cells to a level similar to that observed in wild type P19 cells and empty vector cells (Fig. 4). These data demonstrate that the PBX1b-AS cells are able to respond to RA treatment by inducing the mRNA levels of at least two primary response genes. However, these cells did not differentiate to either endoderm-like cells or neuron-like cells indicating that these differentiation processes are blocked at the PBX induction step. Thus, the RA-dependent induction in PBX protein levels is required for RA-dependent differentiation of P19 cells.

**PBX Proteins Regulate the Transcription of BMP4 but Not BMP2 during Endodermal Differentiation**—During the RA-mediated differentiation of P19 cells, MEIS, PREP1, and a number of HOX proteins, including HOXA1 and HOXB1, are induced (20, 25–28, 38). These proteins can form dimeric or trimeric complexes with PBX proteins to regulate the target genes involved in differentiation processes. Since the PBX1b-AS cells do not differentiate upon RA treatment, they are useful for identifying genes that are under the regulation of PBX proteins during RA-induced differentiation of these cells.

PBX1 knock-out mice display widespread patterning defects of the skeleton suggesting a role for PBX1 in chondrogenesis (21). Interestingly, during sequence analysis of the promoter of Bmp2 and Bmp4, two important regulators of chondrogenesis, we identified putative PBX-HOX binding sites that could be important for the transcriptional regulation of these Bmps by PBX proteins. RT-PCR was used to study the expression of BMP2 and BMP4 in P19 cells treated with 10−7 M RA in monolayer (Fig. 5). Clearly the mRNA level of BMP4 is elevated after 2 days of RA treatment and continues to rise at least until 4 days of RA treatment in wild type P19 cells but not in PBX1b-AS cells (clones 2 and 9, data for clone 2 is shown). On the other hand, the mRNA level of Bmp2 is induced by 24 h of RA treatment in both wild type P19 cells and PBX1b-AS cells. Thus, it appears that an increase in PBX protein levels is required for the induction of BMP4 mRNA but not for the induction of BMP2 mRNA in P19 cell following RA treatment.

To further confirm the role of PBX proteins in the induction of BMP4 mRNA level, we used the siRNA technique to reduce the elevation of Bmp1/2/3 proteins during the RA induced differentiation of wild type P19 cells (Fig. 6). The PBX1 siRNA was first transiently transfected into wild type P19 cells. These cells were then treated with 10−7 M RA for 1 and 3 days. This PBX1 siRNA successfully reduced the RA-dependent elevation in Bmp1/2/3 protein levels to about 56% after 1 day of RA treatment. Similarly the RA-dependent increase in BMP4 mRNA level after 3 days of RA treatment was also reduced to about 60%, proportional to the reduction in Bmp1/2/3 protein levels (Fig. 6). These data further confirm that PBX proteins are required for the induction of BMP4 during the RA-mediated differentiation of P19 cells.

ChIP assays were used to determine whether PBX1/2/3 proteins bind in vivo to the Bmp4 promoter following RA treatment. Wild type P19 cells and PBX1b-AS cells were treated with 10−7 M RA for 3 days in monolayer. After chromatin immunoprecipitation with anti-PBX1/2/3 antibody and PCR amplification using the primers shown in Fig. 7A that flank the putative HOX-PBX binding site, we detected a PCR product only in the RA-treated wild type P19 cell samples but not in any other samples including the RA-treated PBX1b-AS cells (Fig. 7C, clone 2). This demonstrates that PBX1/2/3 proteins bind to the Bmp4 promoter, most likely to the putative HOX-PBX binding site, upon RA treatment only in wild type P19 cells but not in PBX1b-AS cells.

**RA Is Likely to Regulate the Expression of DCN and BMP Signaling during Endodermal Differentiation**—Encouraged by our finding on the transcriptional regulation of Bmp4 by PBX proteins, we performed a mouse osteogenesis microarray using RNA isolated from wild type P19 cells and PBX1b-AS cells treated with 10−7 M RA for 3 days to identify additional genes that might be regulated by PBX proteins (data not shown). This array contains cDNAs of about 100 mouse genes whose functions are related to chondrogenesis. Since P19 cells are not specialized chondroblasts, we only observed expression of a small fraction of genes including Den, Madh2 (mouse MAD homolog 2 (Drosophila)), Madh3, Madh4, Vegfa (vascular endothelial growth factor a), Bmpr1a, and Bmpr2. The mRNA of DCN was found to be elevated in the RA-treated wild type P19 cells but not in the RA-treated PBX1b-AS cells (data not shown). However, the expression of most of the other genes was weak, and it was difficult to compare their -fold inductions by
RA treatment. Thus, $\alpha$-$^35$S-dATP RT-PCR analysis was used to further examine the mRNA levels of these genes.

The mRNA levels of MADH2, MADH3, MADH4, and VEGFa were not altered by RA treatment in either the wild type P19 cells or PBX1b-AS cells (data not shown). However, DCN mRNA levels were induced about 8-fold in wild type P19 cells but not induced in PBX1b-AS cells following RA treatment (Fig. 8). This suggests that PBX proteins also regulate the expression of Dcn in P19 cells. Again using PBX1 siRNA we reduced the RA-dependent elevation of DCN mRNA to $\sim$43%, proportionate to the reduction in the RA-dependent elevation in PBX1/2/3 protein levels in wild type P19 cells (Fig. 6). Using the ChIP assay, we found that PBX proteins bind to a region of the Dcn promoter where there are two putative PBX-HOX binding sites in the RA-treated wild type P19 cells but not in the PBX1b-AS cells (Fig. 7, B and C). These data further confirm that PBX proteins bind to the promoter of Dcn and induce its transcription during the RA-dependent differentiation of P19 cells.

Interestingly the mRNA levels of BMPR1A and BMPR2 were induced similarly in both wild type P19 cells and PBX1b-AS cells following RA treatment (Fig. 8). This suggests that these BMP receptors along with BMP2 are induced by RA treatment in P19 cells in a PBX-independent manner. Furthermore, since BMP2, BMP4, BMPR1A, and BMPR2 are all induced by RA treatment, it is likely that BMP signaling pathways are activated during the RA-dependent differentiation of P19 cells.

**DISCUSSION**

Previously we demonstrated that PBX proteins are induced by RA treatment in mouse limb buds and mouse embryonal carcinoma P19 cells (23, 29). In this report we prepared PBX1b-AS P19 cells in an effort to elucidate the role of PBX proteins in the RA-induced differentiation of P19 cells. Due to the high homology in cDNA sequences among PBX subtypes, overexpression of full-length PBX1b antisense mRNA in these PBX1b-AS cells greatly reduced the RA-dependent induction of PBX1/2/3 protein levels. Although at least two primary responses (induction of RAR/2 and MEIS2 mRNA levels) in these PBX1b-AS cells greatly reduced the RA-dependent induction of PBX1/2/3 protein levels. Although at least two primary responses (induction of RAR/2 and MEIS2 mRNA levels) in these PBX1b-AS cells greatly reduced the RA-dependent induction of PBX1/2/3 protein levels. Although at least two primary responses (induction of RAR/2 and MEIS2 mRNA levels) in these PBX1b-AS cells greatly reduced the RA-dependent induction of PBX1/2/3 protein levels. Although at least two primary responses (induction of RAR/2 and MEIS2 mRNA levels) in these PBX1b-AS cells greatly reduced the RA-dependent induction of PBX1/2/3 protein levels.
and Dcn and are required for the induction of the expression of these two genes during the RA-dependent endodermal differentiation of P19 cells. Since BMP receptors and BMP2 are also induced by RA treatment, it is likely that the BMP signaling pathways and DCN play important roles in the RA-dependent differentiation of P19 cells.

This is the first report to demonstrate that an elevation in PBX protein levels is required for the RA-induced differentiation of P19 cells to both endodermal and neuronal cells. The RA-dependent differentiation of P19 cells is a very complex process that involves many proteins. Perturbation of the expression of critical proteins can block the differentiation process or push the differentiation of these cells to a specific pathway. For example, overexpression of a dominant negative RARα totally blocks both endodermal and neuronal differentiation (39, 40), ectopic expression of Axin blocks RA-dependent neuronal differentiation (41), and overexpression of N-cadherin differentiates P19 cells into neurofilament-expressing neurons in the absence of RA (42). On the other hand, gene knock-out studies in mice demonstrate that inactivation of Pbx1 is lethal but not until embryonic day 15 (21). Taken together, these data suggest that PBX2 and PBX3 can substitute for the role of PBX1 only in the early stages of embryonic development or that PBX2 and/or PBX3 have an important unique function(s) during early embryonic development.

Using both the PBX1b-AS cells and PBX1 siRNA, we demonstrate that an elevation in PBX1/2/3 protein levels is required for the RA-dependent induction of Bmp4 mRNA and Dcn mRNA. In these studies, the RA-dependent induction of PBX1/2/3 protein levels blocked the differentiation of P19 cells to both neuronal cells and endodermal cells suggesting that PBX proteins are critical during the early stages of embryonic development. On the other hand, gene knock-out studies in mice demonstrate that inactivation of Pbx1 is lethal but not until embryonic day 15 (21). Taken together, these data suggest that PBX2 and PBX3 can substitute for the role of PBX1 only in the early stages of embryonic development or that PBX2 and/or PBX3 have an important unique function(s) during early embryonic development.
Values are mean ± S.E. of three independent experiments. Wt, wild type; C, ethanol-treated control.

homeodomains of these proteins. On the other hand, the PBX1 siRNA is only 21 base pairs in length. This PBX1 siRNA is 100% homologous to PBX1 mRNA (both PBX1a and PBX1b), 81% homologous to PBX2 mRNA, and 57% homologous to PBX3 mRNA. It is likely that this PBX1 siRNA only efficiently inhibits the expression of PBX1 but not PBX2 and PBX3 and thus reduces the RA-dependent induction of PBX1/2/3 protein levels only to a moderate extent. Furthermore PBX1 siRNA was introduced into cells by the transient transfection technique that does not always deliver siRNA into 100% of the cells, whereas PBX1b-AS cells are a homogenous population in which every cell expresses the PBX1b antisense mRNA. Nonetheless the percentage of reduction in PBX1/2/3 protein levels by both methods is proportional to the reduction in BMP4 and DCN mRNA levels demonstrating the involvement of PBX proteins in the RA-dependent induction of BMP4 and DCN.

Besides the PBX response element, binding sites for other transcription factors such as upstream stimulating factor, RAR, chicken ovalbumin upstream promoter-transcription factor, and CBFA1 have been described in the promoter of the Bmp4 gene using in vitro gel shift assays (44–47). Therefore, the regulation of Bmp4 is rather complex potentially involving multiple transcription factors. In this report, ChIP assays demonstrate that PBX proteins associate with Bmp4 promoter in a RA-dependent fashion in vivo. In addition, PBX1b-AS cells and siRNA confirm that PBX proteins are required for the RA-dependent induction of BMP4 mRNA. On the other hand, BMP4 expression has been reported to be repressed by RA in F9 cells (48, 49). We did not observe any RA-dependent induction in PBX levels in F9 cells following RA treatment. This could explain why the level of BMP4 mRNA is not increased upon RA treatment of F9 cells.

Similarly the murine Dcn promoter also contains binding sites for many transcription factors including glucocorticoid receptor, AP-1, AP-2, serum response factor, and Oct-1 that could be important for the regulation of its expression (50). We demonstrate here that RA induces the expression of Dcn in P19 cells in a PBX-dependent manner and that PBX binds in vivo to the promoter of Dcn in a RA-dependent fashion. Dcn has also been shown to be induced by hepatocyte growth factor treatment of interstitial fibroblasts, repressed by glucocorticoid treatment of mesangial cells, repressed by tumor necrosis factor-α treatment of normal fibroblasts, reduced in quiescent fibroblast cells, and reduced in normal fibroblasts that overexpress c-Jun (51–54). Thus, the regulation of transcription of Dcn is also quite complex and may involve different transcription factors in different cell types.

In this study, we observed a dramatic RA-dependent increase in the mRNA levels of BMP2, BMP4, and two BMP receptors. The elevation in BMP4 mRNA is PBX-dependent, while that of BMP2 mRNA and the two BMP receptor mRNAs is PBX-independent. Previous studies have demonstrated that the growth and differentiation of F9 cells is affected by addition of BMP2 (49). Also RA and a high dose of recombinant BMP4 can induce apoptosis in P19 cells (55, 56). Thus, BMP2 and BMP4 proteins are likely to act in an autocrine fashion to activate the BMP signaling pathway and contribute to the RA-induced differentiation of P19 cells. In the PBX1b-AS cells, BMP signaling may be impaired due to a block in the induction of BMP4 levels thereby contributing to the inability of these cells to differentiate. Taken together these data suggest that BMP signaling could play an important role in the RA-dependent differentiation of P19 cells. However, additional studies including the use BMP receptor-activating or -blocking antibodies are needed to more definitively demonstrate the importance of BMP signaling in P19 cell differentiation.

In addition to cellular differentiation, BMP proteins are also important secreted growth factors regulating bone and cartilage formation (for reviews, see Refs. 57 and 58). They initiate their signaling through binding to the type I and type II BMP receptors and activate the intracellular signaling cascade involving SMAD proteins. Dcn is a member of the family of small leucine-rich proteoglycans. It binds to collagen fibrils in a highly regular pattern and is believed to be important for the assembly of collagen fibers (for reviews, see Refs. 59 and 60). Dcn has also been suggested to regulate cell matrix production by inhibiting transforming growth factor-β activity through direct binding. Since PBX1 has been suggested to regulate chondrogenesis during embryonic development (21), our findings suggest that PBX proteins are likely to achieve this at least in part by regulating the expression of BMP4 and Dcn.

REFERENCES

1. Chambon, P. (1996) FASEB J. 10, 940–954
2. Krumlauf, R. (1994) Cell 78, 191–201

Acknowledgments—We thank Dr. Tracee S. Panetii for providing the fluorescence and phase-contrast microscopes. The TROMA-I antibody developed by Philippe Brulet and Rolf Kemler and the SSEA-1 antibody developed by Davor Solter and Barbara B. Knowles were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, National Institutes of Health and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

---

Fig. 8. Expression of DCN and BMP receptors. Wild type P19 cells and PBX1b-AS cells were treated with 10−7 M RA for 3 days in monolayer. Total RNA was harvested, and α-32P-dATP RT-PCR was performed to examine the expression of BMP4, DCN, BMPR1A, and BMPR2. GAPDH PCR was performed for normalization. Fold inductions in the level of each mRNA normalized for GAPDH mRNA levels in both wild type cells and AS cells are indicated on the bottom of the each gel. Values are mean ± S.E. of three independent experiments. Wt, wild type; C, ethanol-treated control.
Pre-B Cell Leukemia Transcription Factor (PBX) Proteins Are Important Mediators for Retinoic Acid-dependent Endodermal and Neuronal Differentiation of Mouse Embryonal Carcinoma P19 Cells

Pu Qin, Juliet M. Haberbusch, Zhenping Zhang, Kenneth J. Soprano and Dianne R. Soprano

J. Biol. Chem. 2004, 279:16263-16271. doi: 10.1074/jbc.M313938200 originally published online January 23, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M313938200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 58 references, 25 of which can be accessed free at http://www.jbc.org/content/279/16/16263.full.html#ref-list-1