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Retinoic Acid Regulates the Expression of PBX1, PBX2, and PBX3 in P19 Cells Both Transcriptionally and Post-Translationally

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Abstract Pre-B cell leukemia transcription factors (PBXs) are important co-factors for the transcriptional regulation mediated by a number of Hox proteins during embryonic development. It was previously shown that the expression of several *Pbx* genes is elevated in mouse embryo limb buds and embryonal carcinoma P19 cells upon retinoic acid (RA) treatment although the mechanism of this induction is not well understood. In this report, we demonstrate that PBX1a, PBX1b, PBX2, and PBX3 mRNAs and PBX1/2/3 proteins are induced during endodermal and neuronal differentiation of P19 cells in a RAR-dependent subtype-unspecific manner following RA treatment. The increases in both PBX1 mRNA and PBX3 mRNA levels are secondary responses to RA treatment requiring new proteins synthesis while the increase in PBX2 mRNA is a primary response. The RA-dependent increases in PBX1 mRNA, PBX2 mRNA, and PBX3 mRNA levels are likely to be transcriptionally regulated since the stability of these mRNAs does not change. In addition, the half-lives of PBX1/2/3 proteins are significantly extended by RA treatment. Two possible mechanisms could contribute to the stabilization of PBX proteins: PBX proteins associate with RA-dependent increased levels of MEIS proteins, and RA may decrease the proteasome dependent degradation of PBX proteins. J. Cell. Biochem. 92: 147–163, 2004. © 2004 Wiley-Liss, Inc.

Key words: PBX; retinoic acid; RAR; P19 cells

Retinoic acid (RA), the most active form of vitamin A, and its derivatives are important nutrients for growth, immune function, embryonic development, and differentiation. The actions of RA are mediated by two classes of nuclear receptors, RA receptors (RARs) and retinoid X receptors (RXRs) (for review, see [Chambon, 1996]). RARs and RXRs, which belong to the multigene family of the steroid and thyroid hormone receptor superfamily, are RA-dependent transcriptional regulatory factors. The RARs and RXRs are each composed of three receptor subtypes termed α, β, and γ, encoded by unique genes. Upon RA binding, RARs and RXRs form heterodimers (RAR/RXR) or homodimers (RXR/RXR) and transduce the RA signal at the level of transcription via RA response elements (RAREs) and retinoid X response elements (RXREs) located in the regulatory region of target genes. In vitro binding studies have demonstrated that the natural metabolites, all-trans-RA and 9-cis-RA, are high affinity ligands for RARs, whereas only 9-cis-RA has been shown to bind to RXRs [Heyman et al., 1992; Levin et al., 1992].

Pre-B cell leukemia transcription factor 1 (PBX1) was discovered in 1990 as a chromosome 1:19 translocation in pre-B cell leukemia [Kamps et al., 1990; Nourse et al., 1990]. This chromosomal translocation results in an E2A-PBX1 fusion protein (homeodomain of PBX1 and the transcriptional activation domain of E2A) that alters the normal transcriptional

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regulation by PBX1. Expression of E2A-PBX1 causes the transformation of NIH 3T3 fibroblast cells and induces myeloid leukemia in mice [Kamps and Baltimore, 1993; LeBrun and Cleary, 1994; Lu et al., 1994; Monica et al., 1994]. Two additional subtypes, PBX2 and PBX3, have been identified and found to have 77 and 84% amino acid sequence homology to PBX1, respectively [Monica et al., 1991]. Furthermore, there are multiple isoforms of PBX1 and PBX3 that are generated by alternative splicing.

PBX proteins are members of the TALE (three-amino-acid-loop extension) family of homeodomain proteins. This family of proteins is unique in that it has three conserved amino acid residues between helix 2 and helix 3 of its homeodomain [Burglin, 1997]. PBX proteins interact with a number of Hox proteins through the YPWM motif N terminal to the homeodomain of Hox proteins [Chang et al., 1995; Neuteboom et al., 1995; Shen et al., 1996; Shanmugam et al., 1997]. The formation of the PBX–Hox complex has been shown to enhance both Hox DNA-binding specificity and affinity [Lufkin, 1996; Mann and Chan, 1996; Sanchez et al., 1997; Shen et al., 1997]. Additional TALE proteins, including MEIS and PREP, can also associate with the PBX–Hox dimer, and the resulting trimeric complex can further regulate transcription due to its higher DNA binding specificity [Jacobs et al., 1999; Shanmugam et al., 1999; Ferretti et al., 2000; Penkov et al., 2000]. Interestingly, MEIS and PREP are able to associate with PBX in the cytoplasm and induce a conformational change in PBX. This interaction exposes the PBX nuclear localization signal and causes the translocation of the dimeric protein to the nucleus [Saleh et al., 2000]. In the developing mouse limb buds, MEIS expression is restricted to the proximal region causing PBX to localize to the nucleus in this region. Treatment with RA leads to a distal expansion of MEIS expression which is believed to cause the nuclear localization of PBX in the distal region of the limb buds [Mercader et al., 1999]. This could be one of the factors that contribute to RA-dependent proximalization of limb buds.

It is generally believed that RA functions during embryonic development and differentiation through the regulation of expression of genes such as *Hox* genes. Previously, we found that a single teratogenic dose of RA at gestation day 11 quickly elevates PBX mRNA and protein levels in mouse embryo limb buds [Qin et al., 2002]. In addition, Knoepfler and Kamps [1997] have demonstrated that PBX expression is induced during the neuronal differentiation of mouse embryonal carcinoma P19 cells after 1–7 days of RA treatment. To further understand the role of PBX during development, we have examined the mechanism of regulation of PBX expression by RA in P19 cells. Consistent with prior reports [Knoepfler and Kamps, 1997; Ferretti et al., 2000; Qin et al., 2002], PBX1/2/3 protein and PBX1a, PBX1b, PBX2, and PBX3 mRNA levels are elevated in P19 cells induced to differentiate to either endoderm-like cells or neuron-like cells following RA treatment. This requires the activation of RARα, RARγ, or RARβ/γ. The increases in both PBX1 and PBX3 mRNA levels are secondary responses to RA treatment requiring new protein synthesis although the induction in PBX2 mRNA level is a primary response. Furthermore, the RA-dependent increase in PBX expression is complex involving both transcriptional regulation and stabilization of PBX protein. This increase in the stability of PBX proteins upon RA treatment is likely to be related to a decrease in proteasome-mediated degradation of PBX and an increase in the association between PBX and MEIS proteins.

MATERIALS AND METHODS

Cell Culture and Differentiation

Mouse embryonal carcinoma P19 cell line was purchased from American Type Culture Collection and the RAC65 cell line was a generous gift from Dr. Michael W. McBurney (University of Ottawa, Canada). All cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen Corporation, Carlsbad, CA) with 10% fetal bovine serum and 2 mM L-glutamine, 100 μg/ml penicillin, and 100 U/ml streptomycin. For endodermal differentiation, 2×10^5 cells were plated on 100 mm tissue culture dishes with 10^{-7} M all-trans-RA, dissolved in ethanol. For neuronal differentiation, 7×10^5 cells in 7 ml DMEM containing 10^{-7} M all-trans-RA were plated on bacterial dishes.

RT-PCR Analysis

Total cellular RNA was purified using RNA-zol B reagent (Tel-test, Inc., Friendswood, TX). RNA concentration was obtained by measuring

the absorbance at 260 nm, and the purity of the RNA was determined by measuring the absorbance at 260 and 280 nm. The cDNA was prepared from total RNA with oligo(dT) priming and MMLV Reverse Transcriptase using the Advantage™ RT-for-PCR Kit (BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer's protocol. Primer sequences for PCR analysis were described previously [Mendelsohn et al., 1994; Qin et al., 2002]. For each [α-^{35}S]-dATP labeled PCR reaction, 1 μl (20 μM) of the appropriate sense primer and antisense primer along with 5 μl of cDNA were heated to 94°C for 2 min, and then quickly quenched on ice. Then 93 μl of Master Mix [10 μl reaction buffer, 1 μl dATP (10 mM), 1 μl dTTP (10 mM), 1 μl dCTP (10 mM), 1 μl dGTP (10 mM), 6 μl $MgCl_2$ (25 mM), 1 μl Taq DNA polymerase in storage buffer A (Promega Corp., Madison, WI), 1 μl of [α-^{35}S]-dATP (10 mCi/ml, 500 Ci/mmol, Perkin Elmer Life and Analytical Sciences, Inc., Boston, MA) and 71 μl of H_2O] was added to each PCR reaction. PCR thermal cycling program for the PBXs and GAPDH was 94°C for 30 s, 68°C for 2 min for variable number of cycles, final extension at 68°C for 5 min, and the PCR program for RARβ2 was 94°C 30 s, 55°C 1 min, 72°C 2 min for a variable number of cycles, final extension at 72°C for 7 min. The PCR products from the different cycle numbers were separated on a 10% polyacrylamide gel. The gel was dried and exposed to a phosphorimager screen (Packard Instrument Co., Meriden, CT) and analyzed with a phosphorimager (Packard Cyclone) and OptiQuant software (Packard). The mRNA level of each sample was normalized on the basis of the intensity of band representing GAPDH mRNA.

Western Blot

P19 cells were washed with cold phosphate buffered saline twice. Cells were homogenized in Lysis buffer I (10 mM HEPES, pH 7.9, 1 mM EDTA, 60 mM KCl, 0.5% NP40, 1 mM DTT, 1 mM PMSF, 0.5 μg/ml Leupeptin, 0.5 μg/ml Pepstatin A, and 0.5 μg/ml Aprotinin). A small aliquot was removed, stained with 0.4% Trypan Blue, and examined microscopically to ensure intact nuclei. The homogenates were incubated on ice for 10 min and then the nuclei were pelleted by centrifugation at 1,500*g* for 20 min. The nuclear pellet was resuspended in Lysis Buffer II (same as Lysis buffer I except that 0.5% NP40 was omitted) and centrifuged at 1,500*g* for 20 min to remove any remaining cytosolic protein. The pellet, representing intact nuclei, was resuspended in Nuclear Resuspension Buffer (250 mM Tris, pH 7.8, 60 mM KCl, 1 mM DTT, 1 mM PMSF, 0.5 μg/ml Leupeptin, 0.5 μg/ml Pepstatin, and 0.5 μg/ml Aprotinin) and vortexed briefly. For lysis, nuclei were then subjected to 5 freeze (dry-ice)/thaw (37°C) cycles of approximately 20 min each, vortexing vigorously between cycles for 1 min. The nuclear suspension was centrifuged at 10,600*g* for 10 min and a small aliquot of the supernatant was used for the determination of the protein concentration using the Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA) and the remainder was quickly frozen at −70°C as the nuclear fraction. All procedures were performed on ice or at 4°C except where indicated.

Twenty five micrograms of nuclear protein extracts were fractionated by discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis using a 5% stacking gel and a 10% resolving gel. Proteins were electroblotted to PVDF membranes (Millipore Immobilon-P, Billerica, MA). Blots were blocked overnight at 4°C in Tris-buffered saline (20 mM Tris HCl, pH 7.4, 0.15 M NaCl) containing 7% nonfat dry milk and 0.1% Tween-20. The membranes were incubated with primary antibody for 1 h at room temperature. PBX1/2/3, RARβ, and MEIS1 antibodies were purchased from Santa Cruz, Inc., Santa Cruz, CA. The secondary antibody (anti-rabbit HPR-conjugated) was incubated with the membrane for 1 h at room temperature. The "Enhanced Chemiluminescence Plus" (ECL-Plus) kit (Amersham Biosciences, Piscataway, NJ) was used for detection.

Cycloheximide and DRB Treatment

To determine if new protein synthesis is required for the RA-dependent increase in PBX mRNA levels, P19 cells were pretreated with 10 μg/ml cycloheximide (Sigma, St. Louis, MI) for 15 min followed by the addition of 10^{-7} M RA. Twenty-four hours later total RNA was isolated and PBX mRNA levels were determined by RT-[α-^{35}S]-dATP labeled PCR.

In order to test the stability of PBX mRNAs after RA treatment, P19 cells were treated with 10^{-7} M RA or ethanol for 24 h and then treated with 40 μg/ml DRB (5,6-Dichlorobenzimidazole 1-b-D-ribofuranoside, Sigma). Total RNA was harvested from cells 0, 3, 6, 12 h following DRB treatment. RT-[α-^{35}S]-dATP labeled PCR was

performed to examine PBX mRNA levels in each sample.

Co-Immunoprecipitation Assay

P19 cells were treated with either ethanol or 10^{-7} M RA for 24 h. Whole cell protein lysates were prepared by incubating the cells for 20 min on ice in 1× TNE buffer (0.05 M Tris pH 8.0, 0.15 M NaCl, 1% NP-40, and 2 mM EDTA pH 8.0) followed by centrifugation at 18,000*g* for 10 min. The supernatant was saved as whole cell protein lysates. The protein concentration was measured using Bio-Rad Protein Assay reagent. Nuclear protein extracts were prepared as described in the Western blot section. Three hundred micrograms of whole cell protein or 100 μg of nuclear protein were mixed with 2 μg of anti-MEIS1 antibody (Santa Cruz, Inc) and incubated at 4°C on a rotating platform overnight. Fifty microliters of protein-A agarose beads were added to the sample and incubated for an additional 2 h at 4°C on a rotating platform. The beads were then washed five times with 1 ml of 1× TNE buffer. The antibody and protein were released from the beads by boiling the beads in 50 μl of 2× sample buffer (120 mM Tris, 20% glycerol, 2% 2-mercaptoethanol, 0.01% bromophenol blue, pH 6.8). The sample was then centrifuged at 18,000*g* for 2 min. Twenty five microliters of the supernatant was loaded on SDS–PAGE gel and the level of PBX1/2/3 proteins was determined by Western blot analysis.

Pulse Labeling

On day 1, 3×10^5 P19 cells were plated on 100 mm dish. They were treated with 10^{-7} M RA on day 3 to induce the expression of PBX proteins. Twenty-four hours following RA treatment (day 4) DMEM was removed and the cells were washed with methionine-free/cysteine-free DMEM (MCFM) twice. The cells were then incubated with 5 ml MCFM at 37°C for 15 min to deplete the intracellular pool of methionine and cysteine. The MCFM was then replaced with MCFM containing EXPRE^{35}S^{35}S Protein Labeling Mix (>1,000 Ci/mmol, Perkin Elmer Life and Analytical Sciences) at a final concentration of 0.1 mCi/ml. The cells were incubated with this pulse-labeling medium for 30 min at 37°C. The labeling medium was removed and the cells were washed with PBS twice. DMEM with ethanol or 10^{-7} M RA was added to the labeled cells. The cells were allowed to grow for an additional 0, 3, 6, 9, and 21 h and then nuclear protein was harvested as described above. PBX proteins contained in 60 μg of nuclear protein from the ethanol treated cells or 10 μg of nuclear protein from RA treated cells were immunoprecipitated using 5 μg of anti-PBX1/2/antibodies and 50 μl Protein A-Agarose beads (Santa Cruz Biotech., CA) per sample as described above. The immunoprecipitated samples were fractionated on SDS–PAGE and the amount of ^{35}S-labeled PBX1/2/3 protein was determined by phosphorimage analysis as described above.

Proteasome Inhibitor Treatment

Clasto-lactacystin β-lactone (Calbiochem, Inc., San Diego, CA) is a highly specific, cell-permeable, irreversible inhibitor of the 20S proteasome. 5×10^5 P19 cells were plated on 100 mm dish for 2 days. After 48 h the cells were treated with either DMSO or 2×10^{-6} M clasto-lactacystin β-lactone in DMSO for 6 h. Nuclear protein extracts were harvested following clasto-lactacystin β-lactone treatment and Western blot was performed to determine the amount of PBX1/2/3 protein.

RESULTS

PBX Is Strongly Induced by RA in P19 Cells

Previously our laboratory reported that the expression of PBX1a, PBX1b, PBX2, and PBX3 is elevated in gestation day 11 mouse limb buds following a teratogenic dose of RA using microarray and RT-PCR analysis [Qin et al., 2002]. In this study, we have used P19 embryonal carcinoma cells to further elucidate the mechanism of RA-mediated elevation of PBX expression. The P19 cell line is an excellent model to study the mechanism of RA mediated gene regulation and differentiation [McBurney, 1993]. Upon RA exposure, P19 cells differentiate into endoderm-like cells when grown in monolayer or neuron-like cells when grown as aggregates. Figure 1A shows that treatment of P19 cells grown in monolayer for 24 h with 10^{-7} M RA elevates the mRNA levels of PBX1a, PBX1b, PBX2, and PBX3 (5.5-, 5.4-, 3.4-, and 1.8-fold, respectively). Additional studies demonstrate that the mRNA levels of these *Pbx* genes are also elevated to a similar extent in P19 aggregates after 24 h of RA exposure (data not shown). Furthermore, PBX1/2/3 protein levels are elevated greater than 12-fold in P19 cells treated for 24 h with RA

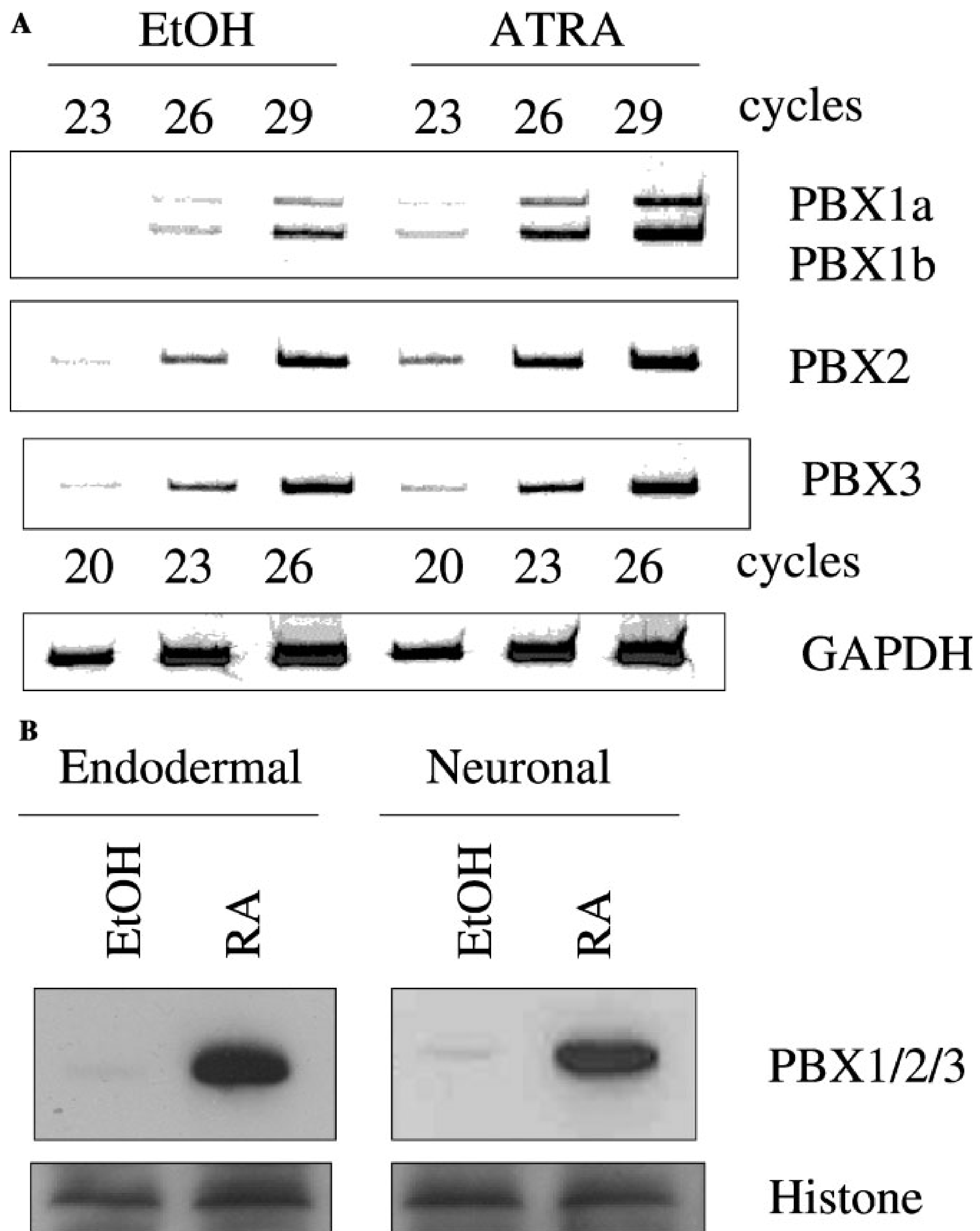


Fig. 1. RA induces PBX mRNA and protein levels in P19 cells. **A**: P19 embryonal carcinoma cells were plated in monolayer and treated with 10^{-7} M RA for 24 h. Total cellular RNA was isolated and [α-^{35}S]-dATP labeled RT-PCR was performed to measure the mRNA levels of PBX1a, PBX1b, PBX2, and PBX3. GAPDH mRNA was used for normalization of RNA levels. Samples were removed at different PCR cycles to ensure that the reactions were not saturated. **B**: P19 cells were plated in monolayer (endodermal) or as aggregates (neuronal) and treated with 10^{-7} M RA for 24 h. Nuclear protein extracts were harvested and examined for the expression of PBX1/2/3 proteins by Western blot. Blots were reprobed for histone protein levels as a control for protein loading.

during both endodermal and neuronal differentiation (Fig. 1B). Finally, dose response studies show that treatment with as little as 10^{-8} M RA increases the PBX1a, PBX1b, PBX2, and PBX3 mRNA levels as well as protein levels of PBX1/2/3 in P19 cells (Fig. 2).

Increases in PBX1 mRNA and PBX3 mRNA Levels Are Secondary Responses to RA Treatment Whereas the Elevation in PBX2 mRNA Level Is a Primary Response

Time course studies demonstrate that both PBX1 mRNA and PBX3 mRNA levels (Fig. 3A) are not elevated until 12 h following RA treatment. However, the mRNA level of PBX2 is increased as early as 3 h following RA treatment. PBX1/2/3 protein levels are elevated between 6 and 12 h following RA treatment and continue to gradually rise until 72 h following RA treatment (Figs. 3B and 4B and data not shown). Interestingly, the protein and mRNA levels of *RARβ2*, a primary response gene to RA treatment in P19 cells [Shen et al., 1991], are elevated as early as 3 h after RA exposure (Fig. 3A,B). This suggests that the elevations in both PBX1 and PBX3 mRNA levels are secondary responses to RA treatment whereas the elevation in PBX2 mRNA is a primary response.

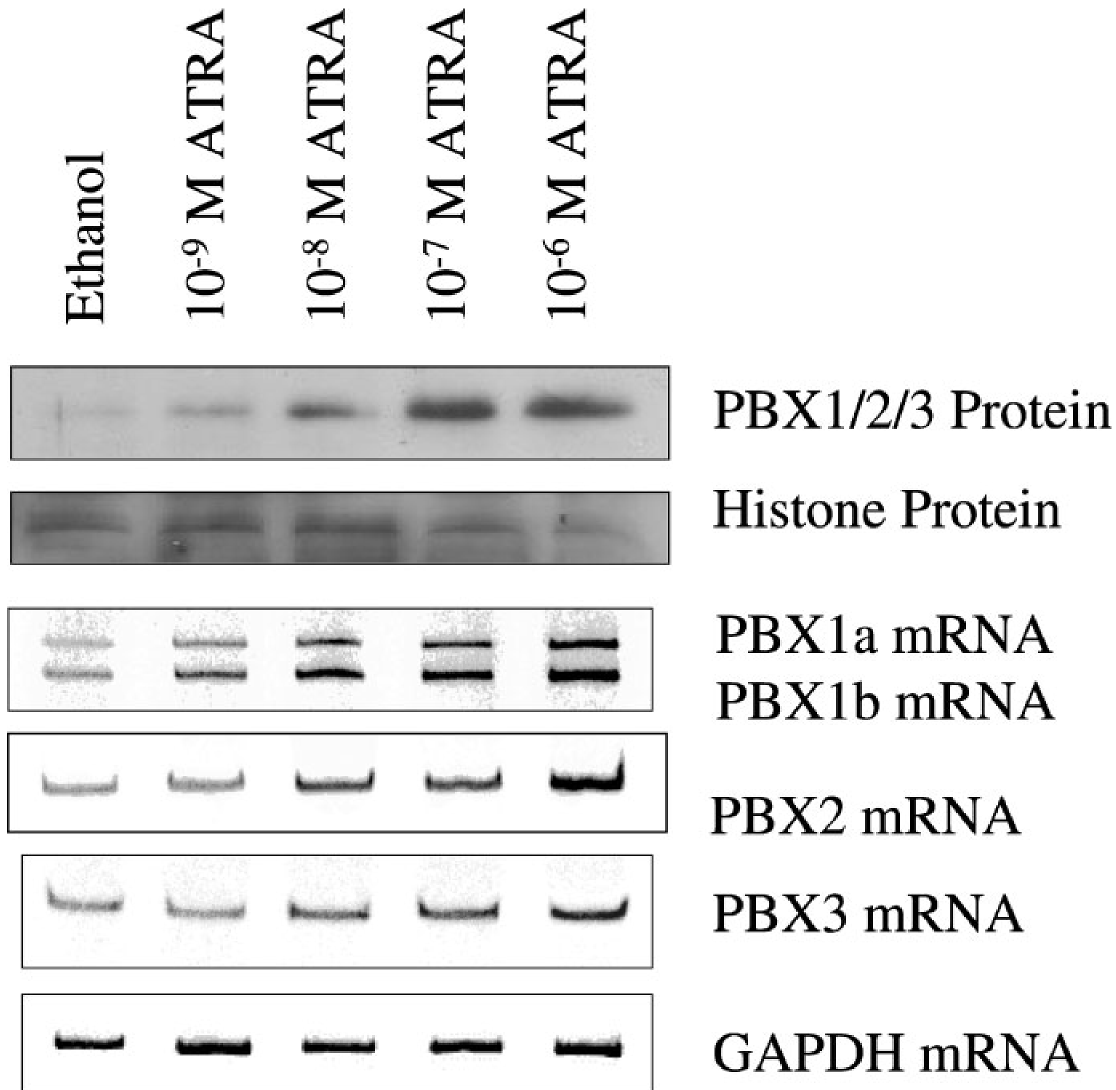


Fig. 2. Dose response of PBX mRNA and protein to RA. P19 cells were plated in monolayer and treated with RA at the indicated concentration for 24 h. PBX mRNA and proteins levels were determined by [α-^{35}S]-dATP labeled RT-PCR and Western blot, respectively. GAPDH mRNA and histone protein are shown for normalization.

To confirm this hypothesis, P19 cells were pretreated with cycloheximide, a drug that blocks protein translation, and then 10^{-7} M RA was added for 24 h. As expected for a primary response gene, RARβ2 mRNA level is elevated in cells treated with both RA alone and RA plus cycloheximide (Fig. 3C). The mRNA level of PBX2 is also induced by RA in the presence of cycloheximide suggesting that it is a primary response gene to RA treatment. However, RA is not able to elevate the mRNA levels of PBX1a, PBX1b, and PBX3 in the presence of cycloheximide (Fig. 3C). This demonstrates that the RA-dependent elevations in PBX1 mRNA and PBX3 mRNA levels in P19 cells are secondary responses to RA treatment and their induction by RA requires new protein synthesis. On the other hand, the RA-dependent induction of PBX2 mRNA is likely to be a primary response and does not require new protein synthesis.

Involvement of RARs in the RA-Dependent Induction of PBX

To determine the role of RARs in the RA-dependent induction of PBX levels, we studied the ability of RA to induce PBX1/2/3 expression in RAC65 cells. RAC65 cells are derivatives of P19 cells that carry a mutant RARα with a truncated ligand-binding domain. This mutation is dominant negative to all the RARs in these cells which makes RAC65 cells resistant

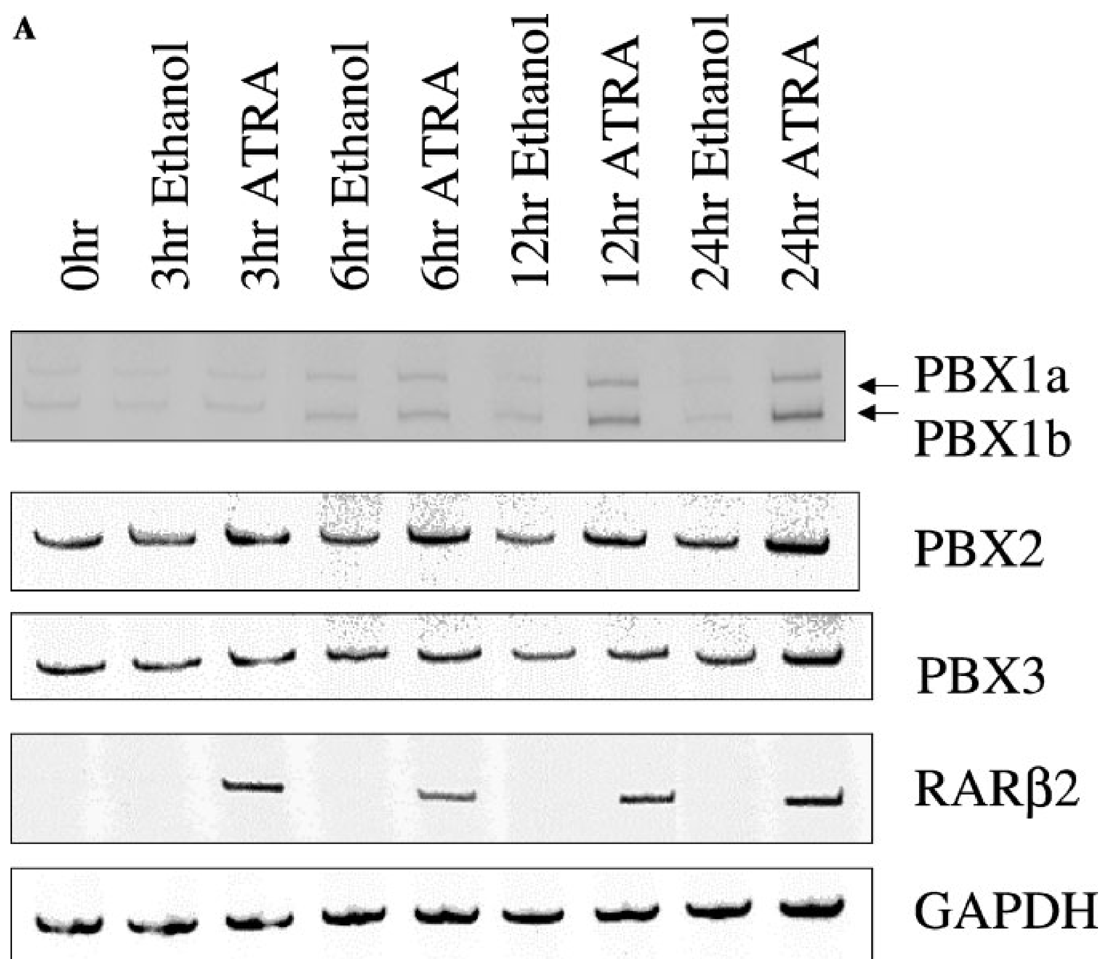
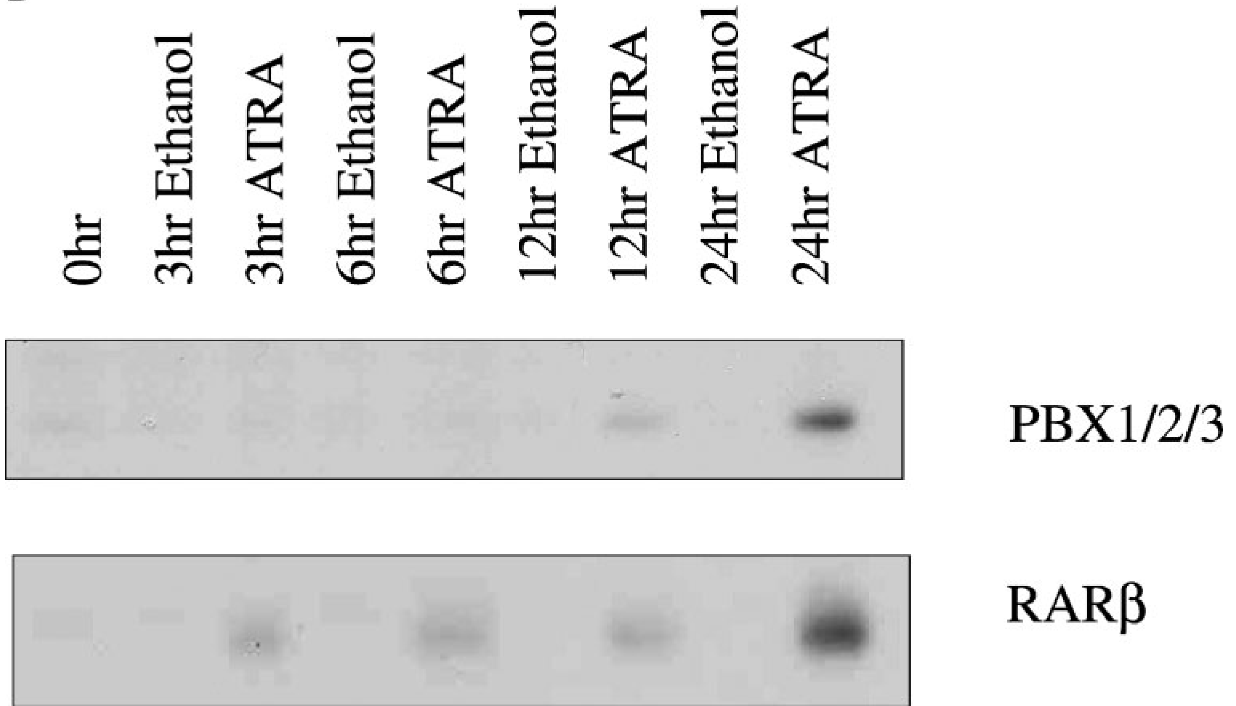


Fig. 3. The increases in PBX1 mRNA and PBX3 mRNA levels in P19 cells are secondary responses to RA treatment whereas the elevation in PBX2 mRNA is a primary response. **A** and **B**: P19 cells were plated in monolayer and treated with 10^{-7} M RA for the indicated times. The mRNA levels of PBX1a, PBX1b, PBX2, PBX3, and RARβ2 (A), and protein levels of PBX1/2/3 and RARβ (B) were determined by [α-^{35}S]-dATP labeled RT-PCR and Western blot, respectively. **C**: P19 cells plated in monolayer were pretreated with 10 μg/ml cycloheximide (CHX) for 15 min to block protein synthesis. RA was added to the cells at a final concentration of 10^{-7} M and incubated for an additional 24 h. Total cellular RNA sample was harvested and [α-^{35}S]-dATP labeled RT-PCR was performed to determine the levels of PBX1a, PBX1b, PBX2, PBX3, and RARβ2 mRNAs.



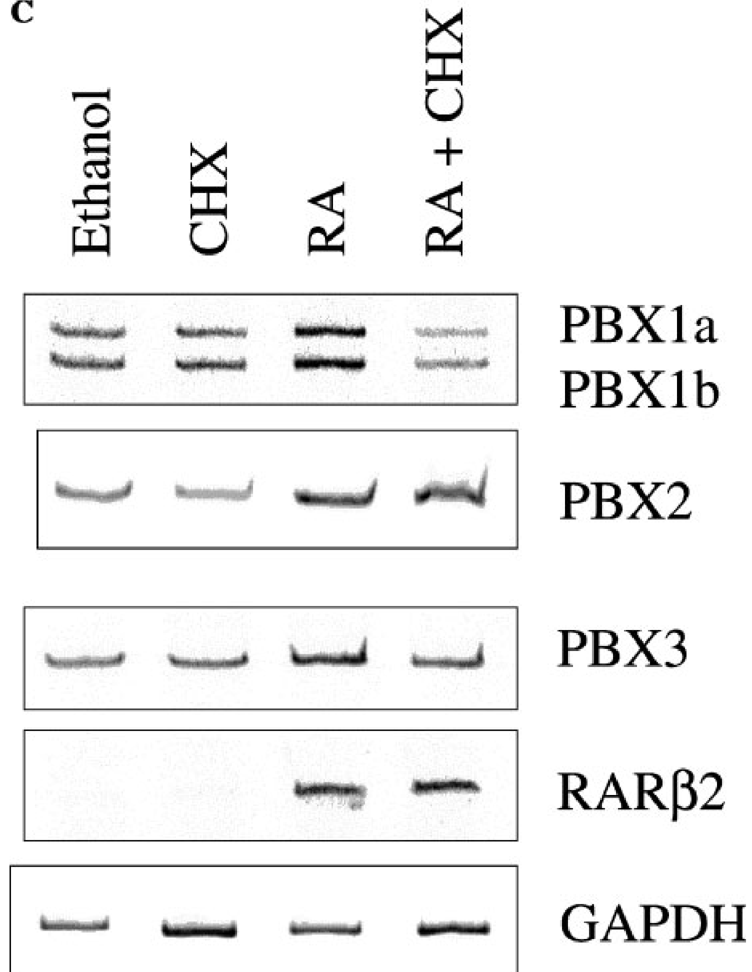


Fig. 3. (*Continued*)

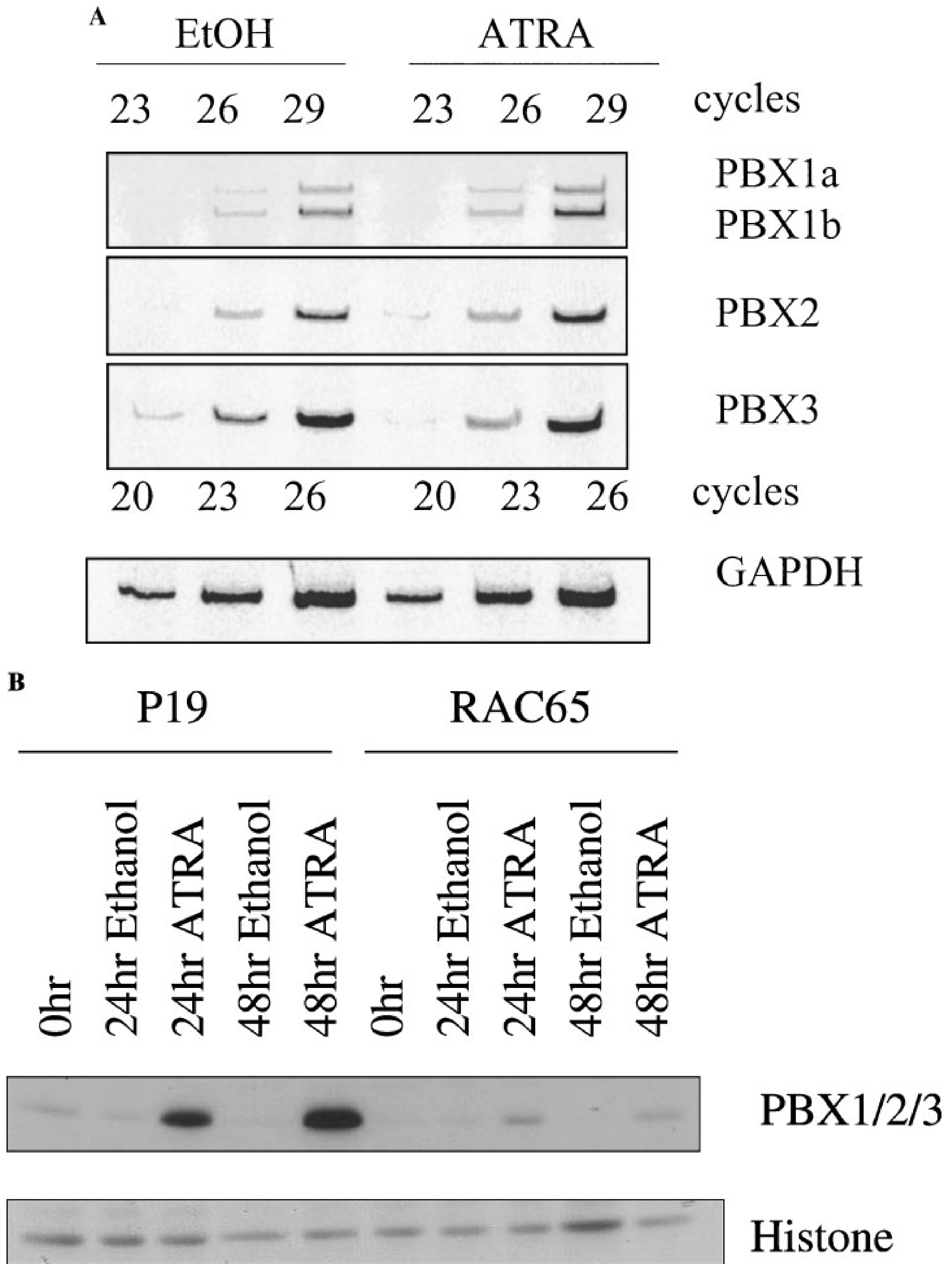


Fig. 4. RARs are involved and required for the RA-dependent elevation in P19 cells. **A**: The levels of PBX1a, PBX1b, PBX2, and PBX3 mRNAs were determined by [α-^{35}S]-dATP labeled RT-PCR using total RNA isolated from RAC65 cells following treatment with 10^{-7} M RA for 24 h. **B**: PBX1/2/3 protein levels were determined by Western blot in nuclear protein extracts of P19 and RAC65 cells following treatment with 10^{-7} M RA for 24 and 48 h. **C**: P19 cells were plated in monolayer and treated with different RAR-selective agonist at the indicated concentrations for 24 h. Nuclear protein extracts were harvested and PBX1/2/3 protein levels were determined by Western blot. These agonists were dissolved in either ethanol or DMSO.

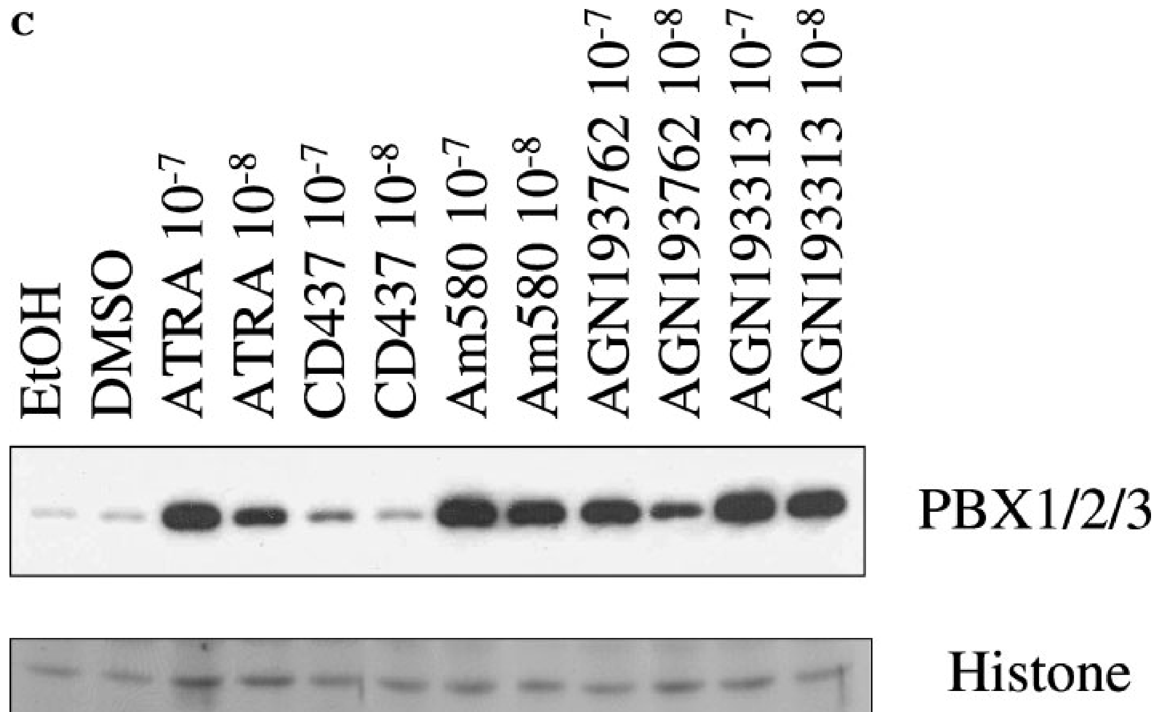


Fig. 4. (*Continued*)

to RA-dependent differentiation [Song and Siu, 1989; Pratt et al., 1990; Kruyt et al., 1992]. PBX1a, PBX1b, PBX2, and PBX3 mRNA levels are not induced in RAC65 cells after 24 h of RA treatment although they are increased between 1.8- and 5.5-fold in wild type P19 cells (compare Fig. 1A with Fig. 4A). The basal level of PBX1/2/3 protein is lower in RAC65 cells compared to P19 cells (compare 0 h samples in Fig. 4B). PBX1/2/3 protein levels are only slightly increased in RAC65 cells even after 48 h of RA treatment, significantly less than that of the wild type P19 cells (Fig. 4B). These data strongly suggest that activation of RARs is required for this elevation in PBX1/2/3 expression.

RARs are widely expressed during embryogenesis although each of the three subtypes display their own individual expression pattern suggesting that there are functional differences among RAR subtypes (reviewed in [Ross et al., 2000]). To study which RAR subtype is responsible for the induction of PBX1/2/3 protein in P19 cells, we treated P19 cells with different RAR-selective agonists for 24 h and quantitated PBX1/2/3 levels by Western blot. Activation of RARα alone (AM580, K_d for RARα = 8 nM, K_d for RARβ = 131 nM, and K_d for RARγ = 450 nM [Bernard et al., 1992]), or RARβ/γ together (AGN 193313 and AGN 193762, K_d for RARβ and RARγ = 2–6 nM and, K_d for RARα > 1,000 nM [Gambone et al., 2002]) strongly elevates PBX1/2/3 protein levels to a similar extent as RA (Fig. 4C). The RARγ selective agonist CD437 (K_d for RARγ = 75 nM, K_d for RARα = 6,500 nM, and K_d for RARβ = 2,480 nM [Bernard et al., 1992]) also elevates PBX1/2/3 protein levels however to a lesser extent (Fig. 4C). This is most likely due to the low binding affinity between CD437 and RARγ. These data suggest that the RA-dependent induction of PBX1/2/3 is mediated by RARs and does not appear to be receptor subtype specific.

PBX Levels Are Regulated by RA Both Transcriptionally and Post-translationally

To study the mechanism of the RA-dependent increase in PBX expression, we first examined the stability of PBX mRNAs following RA treatment. P19 cells were treated with ethanol or RA for 24 h and then DRB was added to block RNA transcription in these cells. The residual PBX1 mRNA in the cells was measured by [α-^{35}S]-dATP labeled RT-PCR at various times after DRB treatment (Fig. 5A). It is clear that the degradation curves of PBX1a and PBX1b mRNAs are very similar in both the RA and ethanol treated P19 cells with a half-life of about 8 h (Fig. 5B). In addition, the half-lives of PBX2

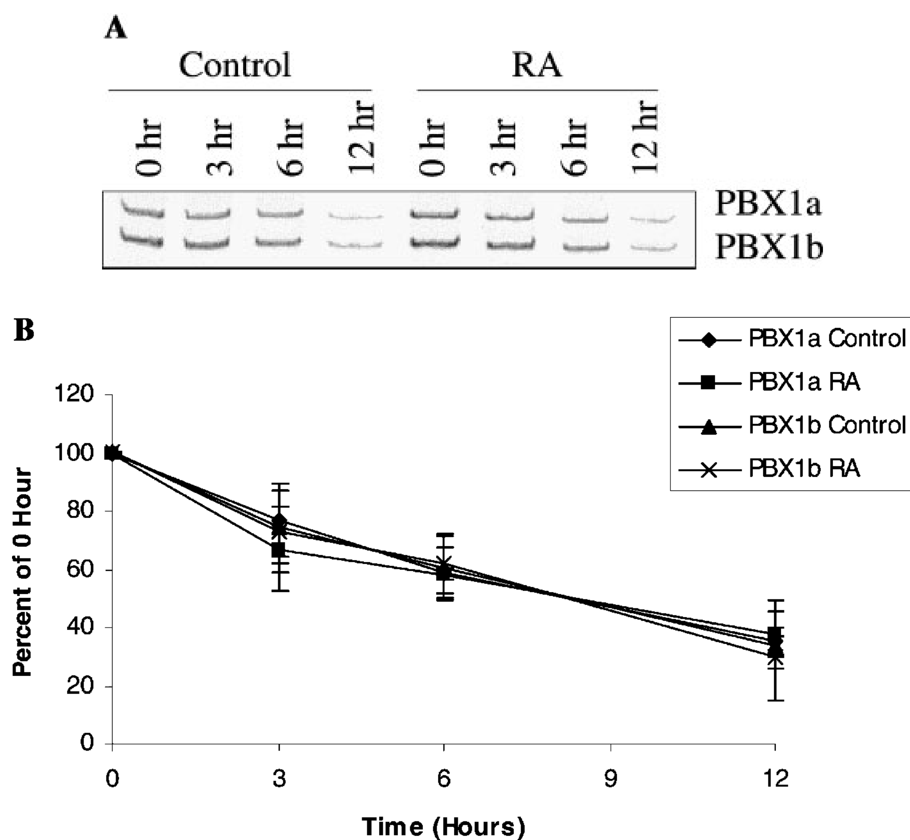


Fig. 5. Stability of PBX mRNA following RA treatment in P19 cells. P19 cells were plated in monolayer and treated with ethanol (control) or 10^{-7} M RA for 24 h. Cells were then treated with 40 μg/ml DRB and the residual PBX1 mRNA level in the cells was measured by using [α-^{35}S]-dATP labeled RT-PCR at various time after DRB treatment. **A**: Representative gel from four independent experiments is shown. **B**: Plot of residual PBX mRNA levels. Data points are mean ± SEM.

mRNA and PBX3 mRNA are similar to that of PBX1 mRNA and are not altered by RA treatment (data not shown). These data demonstrate that the stability of PBX mRNAs remains the same following RA exposure and suggest that an increase in transcription of these genes is likely to be responsible for the elevation in PBX mRNAs.

Since we observed a much larger increase in PBX1/2/3 protein levels than the increase in their mRNA levels during endodermal differentiation of P19 cells (Fig. 1A,B), the turnover rate of PBX1/2/3 proteins was examined by pulse labeling studies (Fig. 6A,B). P19 cells were first treated with ethanol or 10^{-7} M RA for 24 h. The cells were then pulse labeled with ^{35}S-methionine and ^{35}S-cysteine for 30 min. Following the labeling period, the ^{35}S-labeling medium was replaced with regular growth medium. Nuclear protein extracts were harvested at different time points following the removal of the labeling media. PBX1/2/3 was immunoprecipitated from these protein extracts with anti-PBX1/2/3 antibody and the immunoprecipitate subjected to SDS–PAGE. The amount of radiolabeled PBX1/2/3 proteins was quantified by phosphorimage analysis. The half-lives of PBX1/2/3 proteins in RA treated cells (about 12 h) are approximately two times greater than the ethanol treated cells (about 6 h) indicating that these proteins are stabilized by RA treatment. This increased protein stability is very likely to contribute to the RA-dependent increase in PBX1/2/3 expression.

Possible Mechanisms of the RA-Dependent Increase in PBX Protein Stability

It was recently suggested that members of the MEINOX family of proteins can interact with PBX proteins to stabilize them in mouse teratocarcinoma F9 cells and zebrafish hind-

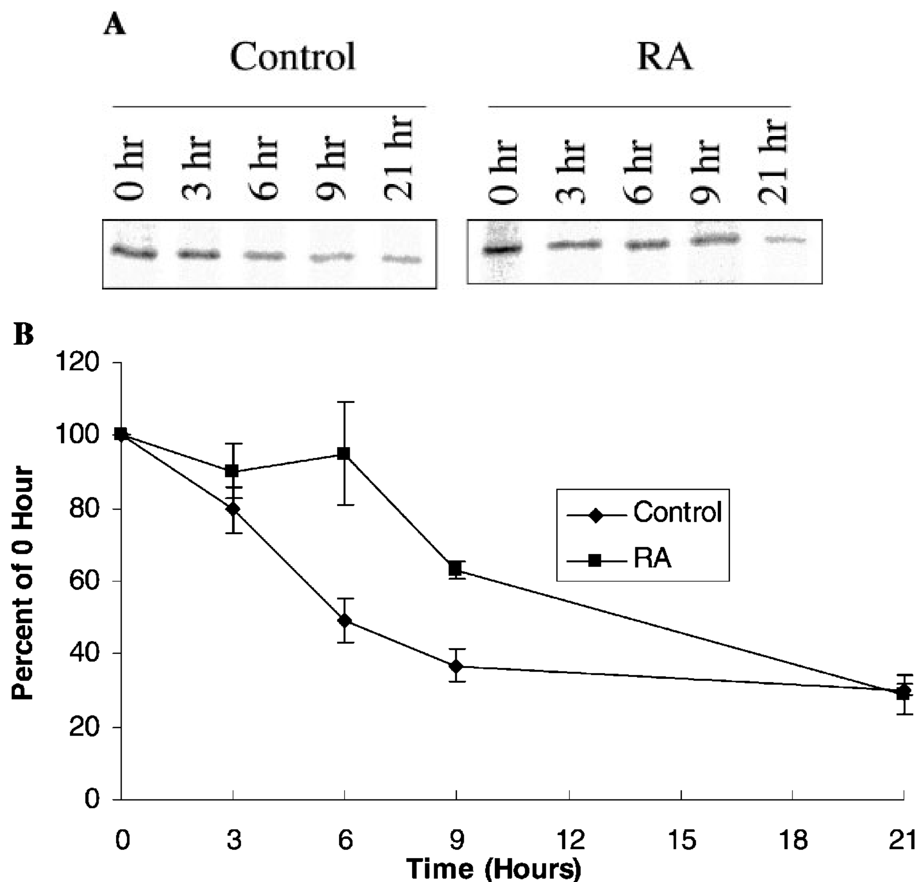


Fig. 6. Stability of PBX proteins following RA treatment in P19 cells. **A**: P19 cells were plated in monolayer and treated with ethanol (control) or 10^{-7} M RA for 24 h. The cells were then incubated with MCFM containing ^{35}S-methionine and ^{35}S-cysteine for 30 min to pulse-label proteins that were being synthesized in the cells. The labeling medium was then replaced with DMEM containing either ethanol or 10^{-7} M RA. Nuclear proteins were extracted from these cells at the indicated time. Sixty micrograms of nuclear proteins from control samples and 10 μg of nuclear proteins from RA treated samples were subjected to immunoprecipitation using anti-PBX1/2/3 antibody and the immunoprecipitates resolved on SDS–PAGE. The dried gel was exposed to phosphorimager screen. **Panel A** is a representative gel of four independent experiments. The level of residual PBX1/2/3 protein was quantitated by Optiquant software and plotted in **panel B**. Data points are mean ± SEM.

brain [Waskiewicz et al., 2001; Longobardi and Blasi, 2003]. PREP1 protein and other members of the MEINOX family of proteins including MEIS1 and MEIS2 have been demonstrated to be upregulated by RA in P19 cells [Oulad-Abdelghani et al., 1997; Ferretti et al., 2000]. Using co-immunoprecipitation assays we found that the amount of PBX1/2/3 proteins associated with MEIS1 (Fig. 7) and MEIS2 (data not shown) is greatly increased upon RA treatment in P19 cells. This interaction may play an important role in stabilizing PBX1/2/3 proteins in RA-treated P19 cells.

Besides stabilization of PBX by interaction with MEINOX proteins, PBX proteins have also been suggested to be degraded through the ubiquitin-proteasome pathway [Longobardi and Blasi, 2003]. To determine the role of proteasome degradation in the increase in PBX1/2/3 protein levels in P19 cells, we treated these cells with clasto-lactacystin β-lactone, a highly specific 20S proteasome inhibitor for 6 h and determined the levels of PBX1/2/3 protein. Blocking the proteasome activity in these cells results in an elevation in the levels of PBX1/2/3 proteins (Fig. 8). Since it has been demonstrated that RA regulates the proteasome mediated degradation of a number of transcription factors such as RB2/p130 and RARγ, RA is likely to reduce the proteasome mediated degradation of PBX proteins in P19 cells which can contribute to the elevation in PBX1/2/3 protein levels [Kopf et al., 2000; Gianni et al., 2002; Vuocolo et al., 2003].

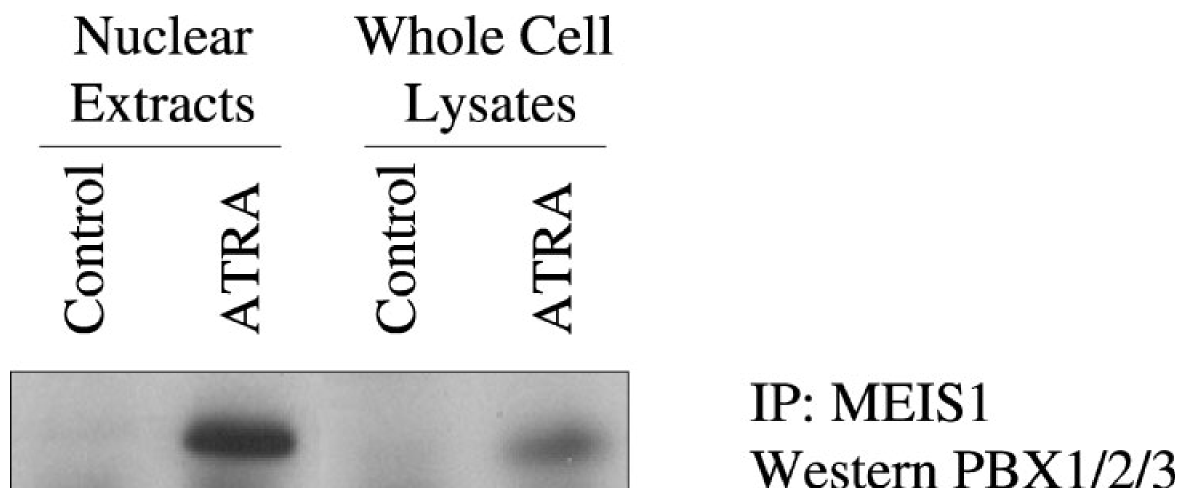


Fig. 7. The association between PBX and MEIS proteins. P19 cells were treated with ethanol (control) or 10^{-7} M RA for 24 h. Whole cell protein lysates and nuclear protein extracts were obtained. The samples were immunoprecipitated with anti-MEIS1 antibody and resolved on SDS–PAGE followed by Western blot using anti-PBX1/2/3 antibody.

DISCUSSION

In this study, we focused on the early events following RA treatment of P19 cells to elucidate the mechanism of RA-dependent regulation of PBX expression. PBX1a, PBX1b, PBX2, and PBX3 mRNA and PBX1/2/3 protein levels are elevated in P19 cells during differentiation to either endodermal or neuronal cells following RA treatment in a RAR-dependent subtype unspecific manner. Furthermore, PBX1 and PBX3 mRNA levels are not elevated until after 6 h of RA treatment and their induction is a secondary response requiring new protein synthesis. On the other hand, the level of PBX2 mRNA is increased as early as 3 h following RA treatment and its induction is a primary response. Furthermore, the RA-dependent induction of PBX expression is complex involving both an increase in the transcription of *Pbx* genes and an increase in the half-life of PBX1/2/3 proteins. This stabilization of PBX proteins is likely to be related to an increase in the association between PBX and MEIS, and a decrease in proteasome-mediated degradation of PBX.

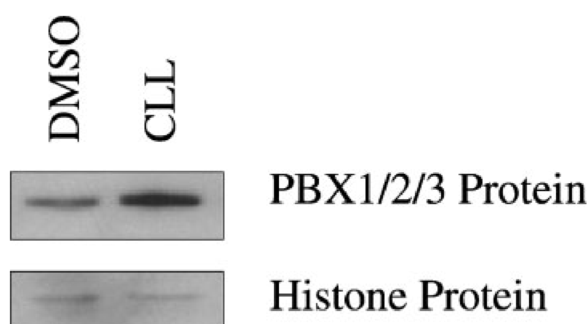


Fig. 8. Proteasome is involved in the degradation of PBX proteins. P19 cells were treated with 2×10^{-6} M clasto-lactacystin β-lactone (CLL) or DMSO for 6 h. PBX1/2/3 protein levels were determined by Western blot. Histone was used as a loading control.

Pbx1, *Pbx2*, and *Pbx3* genes are each located on distinct chromosomes and their transcription is under the control of separate promoters. However, phylogenetic trees based on the analysis of nucleotide and amino acid sequence data using Clustral W software suggest that *Pbx1* and *Pbx3* are evolutionarily closer to each other than *Pbx2* [Endo et al., 1997; Popovici et al., 2001]. Also, PBX1 and PBX3 pre-mRNAs are alternatively spliced resulting in isoforms with different carboxyl termini while a single PBX2 transcript and protein has been observed [Monica et al., 1991]. Our finding that the RA-dependent increases in PBX1 mRNA and PBX3 mRNA are secondary responses while PBX2 is a primary response in P19 cells is also consistent with *Pbx1* and *Pbx3* genes being more closely related to each other than *Pbx2*. While RARs are required for the RA-dependent induction of expression of all three *Pbx* genes in P19 cells, *Pbx1* and *Pbx3* require additional transcription factors that are different from those of *Pbx2*.

Although the RA-dependent increases in the level of PBX1 mRNA and PBX3 mRNA levels in P19 cells are secondary responses that require

new protein synthesis, we do not know the nature of these primary response protein(s). In addition to PBX2, it is known that a number of Hox proteins are induced by RA in P19 cells in a primary response fashion [Popperl and Featherstone, 1993; Pratt et al., 1993; Zwartkruis et al., 1993]. Furthermore, autoregulatory elements have been identified in the promoter of *Hoxb1*, *Hoxa4*, and *Hoxd4* which are responsible for the activation of their own transcription [Popperl and Featherstone, 1992; Di Rocco et al., 1997; Packer et al., 1998]. It is possible one or more *Hox* genes are primarily induced upon RA treatment of P19 cells and that Hox protein(s) cooperate with existing PBX proteins including the primarily induced PBX2 as a Hox/PBX heterodimer along with RARs to increase the mRNA level of PBX1 and PBX3.

It is interesting that the RA-dependent increases in PBX1 mRNA and PBX3 mRNA levels in P19 cells appear to be slower than in gestation day 11 murine limb buds (less than 3 h in the limb buds and greater than 6 h in P19 cells [Qin et al., 2002]). It is possible that the primary response proteins required for the increase in PBX1 mRNA and PBX3 mRNA levels in P19 cells may already be present in the limb buds before exposure of the mouse to a teratogenic dose of RA. Thus, the more rapid increases in PBX1 mRNA and PBX3 mRNA levels in the limb buds could be due to a primary response rather than a secondary response. It should be noted that limb buds contain a physiological concentration of RA due to the normal dietary vitamin A intake of the mice whereas P19 cells are routinely cultured without RA. This physiological level of RA could possibly maintain the basal expression of the primary responsive gene(s) required in P19 cells that contribute to the induction in PBX1 and PBX3 expression in the limb buds following exposure to a teratogenic dose of RA. Since a number of Hox proteins are normally present in the developing limb buds, it is possible that they are able to cooperate with RARs in the induction of PBX1 and PBX3 expression by teratogenic doses of RA.

The studies with RAC65 cells demonstrate that a functional RAR is required for the RA-dependent increase in the expression of all three *Pbx* genes. Since RA (pan-RAR agonist), AM580 (RARα selective agonist), CD437 (RARγ selective agonist), and the two AGN compounds (both RARβ/γ selective agonists) all induce PBX1/2/3 protein levels, this regulation in PBX expression is not RAR subtype specific in P19 cells. Since only RARα and RARγ are expressed in undifferentiated P19 cells [Kruyt et al., 1991], these are the two most likely subtypes that mediate the induction of PBX1/2/3 expression by RA. Functional redundancy among RAR subtypes has previously been observed in the regulation of some genes. For example, ALP and BMP2 mRNA levels are induced by both RARα and RARγ selective agonists in F9 cells [Gianni et al., 1993; Rogers, 1996]; and MUC2 and MUC5AC mRNAs are induced by RARα, RARβ, and RARγ selective agonists in human tracheobronchial epithelial cells [Koo et al., 1999]. On the other hand, there are genes whose expression appears to be regulated in a RAR subtype specific manner within a particular cell type. For example, RARβ2 and Hoxa1 mRNAs are strongly induced by RARγ selective agonist, but only weakly induced by RARα agonist and not induced by RARβ agonist in F9 cells; RARα agonist induced Hoxa1 and RARβ2 to a higher extent than RARγ agonist in P19 cells; and RARα is more potent in the induction of RARβ in B16 cells than RARγ [Taneja et al., 1996; Desai et al., 2000].

Since we did not observe any change in the half-lives of the three PBX mRNAs upon RA treatment, it is very likely that the increases in these PBX mRNA levels are due to an increase in the transcription of each of their respective genes. In search for the required enhancer element(s) in the promoter of *Pbx1*, we cloned a portion of the human *Pbx1* promoter extending from −5,000 bp to +550 bp (transcription start site as +1) into a luciferase reporter vector. Upon transient transfection of this construct into P19 cells, we observed strong basal promoter activity but no responsiveness to RA (data not shown). On the other hand, the endogenous PBX1 mRNA level was elevated by RA in these transfected cells. Therefore, it is likely that this piece of DNA does not contain all of the RA-dependent promoter element(s) that are required for RA action. Since a nucleotide sequence scan of this region of DNA (−5,000 bp to +550 bp) did not reveal any obvious putative RAREs, it is possible that the primary response protein enhancer element(s) and/or critical RARE is lacking. These enhancer element(s) could be either located further upstream, in an intron, or in the 3′ end of the *Pbx1* gene as in the case of *Hoxa1* and *Hoxb1* [Langston and Gudas, 1992; Marshall et al., 1994]. Identification of the

important enhancer element(s) could help in the identification of the primary response protein(s) that mediate the secondary induction of PBX1 mRNA in P19 cells following RA treatment.

Consistent with the previous findings of Knoepfler and Kamps [1997], we observed a higher fold induction in the levels of PBX proteins than the levels of PBX mRNAs following RA treatment of P19 cells. This suggests that in addition to transcriptional regulation of PBX mRNA levels there is also translation and/or post-translational regulation in the RA-dependent induction of PBX protein levels in P19 cells. In pulse labeling experiments, we demonstrate that RA treatment doubled the half-life of PBX1/2/3 proteins suggesting that protein stability is playing a role in the post-transcriptional regulation of PBX proteins. In this experiment, there are a significantly higher percentage of the radiolabeled PBX proteins remaining in the RA-treated cells after 3, 6, and 9 h than in ethanol treated cells. Although the residual percentage of PBX proteins at 21 h is comparable between the two samples, this could be due to the recycling of the [^{35}S]-labeled amino acids from the degradation of cellular proteins and their re-incorporation into newly synthesized PBX proteins resulting in a plateau in the degradation curve. In addition, the degradation of PBX proteins in ethanol-treated cells reached a plateau much earlier than in the RA-treated cells. This also indicates that PBX proteins degrade more slowly in RA-treated cells. Further studies indicate that the stabilization of PBX protein could be caused by their binding to MEIS protein and reduced proteasome-dependent degradation. On the other hand, our data do not rule out the role of translational regulation of PBX proteins that was proposed by Knoepfler and Kamps [1997]. Both mechanisms could contribute to the increased stability of PBX proteins and elevation of PBX protein levels upon treatment of P19 cells with RA.

It has been demonstrated that MEIS1 and MEIS2 proteins are quickly induced by RA treatment in P19 cells [Ferretti et al., 2000; Oulad-Abdelghani et al., 1997]. Besides interacting with PBX proteins and possibly preventing them from being degraded, these MEIS proteins can also translocate PBX proteins into the nuclei to regulate transcription. Furthermore, a number of *Hox* genes including *Hoxa1*, *Hoxb1*, and *Hoxb2* are also induced by RA in P19 cells [Langston and Gudas, 1992; Popperl and Featherstone, 1993; Zwartkruis et al., 1993]. Thus, MEIS, Hox, and PBX proteins are likely to form dimeric or trimeric complexes and regulate the transcription of their target genes following RA treatment. Expression of these target genes could contribute to the RA-dependent differentiation of P19 cells. Future experiments will focus on studying the role of PBX in the RA-induced endodermal and neuronal differentiation of P19 cells.

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