Protein Kinase A-dependent Transactivation by the E2A-Pbx1 Fusion Protein*

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The chimeric gene E2A-PBX1 is formed by the t(1;19) chromosomal translocation exclusively associated with pediatric pre-B cell acute lymphoblastic leukemia (pre-B ALL). The resultant fusion protein from this chimeric gene contains the DNA-binding homeodomain of Pbx1. The first and only functional Pbx1 binding site has been localized in bovine CYP17 to a sequence (CRS1) that participates in cAMP-dependent transcription of this gene encoding the steroid hydroxylase, 17α-hydroxylase cytochrome P450. Because Pbx1 is not expressed in pre-B cells, it may be possible that the E2A-Pbx1 fusion protein expressed in pre-B cells having this translocation will activate, in response to cAMP, transcription of genes not normally expressed in these cells leading to arrest of differentiation at the pre-B cell stage. We have now shown that reporter genes comprising CRS1 are activated transcriptionally by protein kinase A (PKA) in the pre-B cell line 697, which endogenously expresses the fusion protein, and that overexpression of E2A-Pbx1 in additional cell lines enhances transcription of reporter genes in a PKA-dependent fashion. Thus, it seems plausible that arrest in the pre-B stage leading to pre-B ALL includes cAMP-dependent activation of E2A-Pbx1.

Acute lymphoblastic leukemia (ALL) is predominantly a disease of children and young adults. Approximately 1800 new cases are diagnosed each year in the United States in individuals under 15 years of age. A chromosomal translocation t(1;19)(q23;p13.3) is observed in 25% of the cases of pediatric pre-B cell ALL (1, 2). This translocation producing the E2A-PBX1 chimeric gene results in synthesis of transcripts which encode the N-terminal half of the E2A gene product and the C-terminal 75% of Pbx1 (3, 4). The E2A gene product belongs to the helix-loop-helix family of transcription factors and dimerizes with itself or with other family members in order to bind DNA (5, 6). Dimerization and DNA binding functions of E2A are closely linked in a domain located in the C-terminal half of the protein. The N-terminal half of E2A contains two transactivation domains that can confer a strong transactivation function upon heterologous DNA-binding domains (7). In the E2A-Pbx1 fusion protein, the dimerization and DNA-binding domains of E2A have been replaced by the majority of the Pbx1 sequence including the DNA-binding homeodomain.

PBX is a homeobox gene family consisting of at least three members, Pbx1, Pbx2, and Pbx3 (8). Alternative splicing of the PBX1 transcript generates two different Pbx1 proteins, Pbx1a and Pbx1b, which vary in their C-terminal sequence. In the same way as Pbx1, alternative splicing of E2A-Pbx1 transcripts leads to the production of two different E2A-Pbx1 fusion proteins, E2A-Pbx1a and E2A-Pbx1b (3, 4). These fusion proteins containing the transactivation domain of E2A and the DNA-binding homeodomain of Pbx1 have been investigated intensively and shown to be capable of transformation in fibroblast (9), myeloid (10), and T lineage cells (11). Intriguingly, no lymphoid tumors have been observed in transgenic mice expressing E2A-Pbx1 (11). E2A-Pbx1 as well as Pbx family members have been reported to bind the Pbx1 recognition sequence, designated PRS, which was identified by the polymerase chain reaction-mediated random DNA binding site selection (12, 13). Independently, we discovered the Pbx1 binding site in a cAMP-regulatory sequence, designated CRS1, which enhances the expression of 17α-hydroxylase cytochrome P450 (P450c17) encoded by the CYP17 gene (14, 15). Both Pbx1a and Pbx1b are involved in the cAMP-dependent regulation of CYP17 (15). Although E2A-Pbx1 is reported to enhance reporter gene transcription through PRS (12, 13, 16), its role as a transcription factor in leukemogenesis remains unknown.

Because E2A gene products are required for B cell formation (17, 18), and E2A-Pbx1 is under the control of the E2A promoter, E2A-Pbx1 is expected to be expressed and activate target genes at a certain stage of B cell differentiation leading to pre-B ALL. Since E2A-Pbx1 retains a putative protein kinase A (PKA) recognition site derived from Pbx1, and Pbx1 is involved in cAMP-dependent transcription of CYP17, the possibility arose that E2A-Pbx1 is activated by a cAMP signaling pathway. In the B cell developmental process, an increase of intracellular cAMP level plays an important role in both differentiation and apoptotic cell death (19, 20), since induced expression of interleukin-2 receptor was observed in 70Z/3 pre-B cells in the presence of both cAMP and interleukin-1. Therefore, if E2A-Pbx1 responds to cAMP to activate transcription of target genes, this event may be involved in pre-B cell leukemogenesis.

In this study, we have investigated whether the fusion protein E2A-Pbx1a transactivates transcription in a cAMP-dependent manner. Utilizing cotransfection of expression constructs of E2A-Pbx1a and the PKA catalytic subunit (PKA-C) with a luciferase reporter construct containing the CRS1 sequence in different cell lines, we demonstrate that the transactivation of the fusion protein is PKA-dependent but not tissue-specific.
The expression of Pbx1-related proteins in Y1 (mouse E13.5) cells was examined by Western blot analysis of nuclear extracts—

- Pbx1a and Pbx1b
- E2A-Pbx1a
- 43-kDa CREB

were expressed only in Y1 cells. Instead of Pbx1 proteins, E2A-Pbx1a was detected as the major E2A-Pbx1 fusion protein in 697 cells by both anti-Pbx1 (Fig. 1A) and anti-E47 on the same membrane (Fig. 1B). It is not clear whether the lower band of the doublet in 697 cells detected by anti-E47 is due to E47 and/or E2A-Pbx1b proteins whose mobilities on SDS-polyacrylamide gel electrophoresis are near one another. Neither Pbx1 proteins nor E2A-Pbx1 fusion proteins were detected in S194 cells. Lymphocytes are known not to contain Pbx1 proteins, although they contain PBX2 and PBX3 gene products (16). These are either not detectable with anti-Pbx1 or are too low abundance to be observed in S194 cells.

**Gel Shift Analysis of Nuclear Extracts from S194 and 697 Cells**—The pattern observed with S194 nuclear extract is indistinguishable from the two-banded pattern observed with Y1 cells which is attributed to Pbx1-containing complexes (15), except that 2–3 times as much S194 extract was necessary to observe a pattern similar to that from Y1 cells. As demonstrated previously (25), the transcriptional activity of the RSV promoter is only weakly induced by forskolin. Thus, the probability is low that Pbx1 enhances transcription of the RSV promoter in E2A-Pbx1 RSVpBK.

Luciferase assays were performed using a kit (Promega) following the manufacturer's instructions. Light production from luciferase reaction mixtures was measured by a liquid scintillation counter (Wallac 1409, Pharmacia Biotech Inc.).

**RESULTS**

**Immunodetection of Pbx1-related Proteins in Nuclear Extracts**—The expression of Pbx1-related proteins in Y1 (mouse adrenal tumor cell), S194 (mouse myeloma B cell), and pre-B ALL 697 cells was examined by Western blot analysis of nuclear extracts (Fig. 1). While anti-CREB showed approximately the same signal intensity in these cell lines, Pbx1a and Pbx1b

**MATERIALS AND METHODS**

Plasmid Constructs—The CREB (cAMP-responsive element binding protein) expression plasmid has been described previously (15). The cDNA clone encoding human E2A-Pbx1a (3) was inserted downstream of the RSV promoter in RSVpBK (Stratagene). DNA fragments containing four tandem repeated copies of CRS1 (4 × CRS1) (14), of PRS (4 × PRS) (12, 13), and of CRE (4 × CRE) (14) were placed upstream of an SV40 minimal promoter fused to the luciferase gene in the pECo vector (Promega) to yield 4 × CRS1-Luc, 4 × PRS-Luc, and 4 × CRE-Luc, respectively. The expression vector (PKAp) for the PKA catalytic subunit (PKA-C) (21) was a generous gift from Dr. G. S. McKnight (University of Washington).

Cell Culture, Co-transfection, and Luciferase Assay—Y1 and HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.) supplemented with 10% bovine calf serum (22). Jg3 cells were cultured in DMEM with 5% horse serum and 5% fetal calf serum. S194 cells were cultured in DMEM with 10% horse serum. The pre-B 697 cells (23) were cultured in RPMI media (Life Technologies, Inc.) with 10% fetal calf serum and 2 mM glutamine. The plasmid 4 × CRS1-Luc or 4 × PRS-Luc was co-transfected into Y1, HepG2, and Jg3 cells with the indicated expression vectors by the calcium phosphate precipitation method as described (15). S194 cells were co-transfected by the DEAE-dextran method (13), while pre-B 697 cells were co-transfected by electroporation (Bio-Rad, Gene Pulser) under optimized conditions (960 microfarads and 450 V in culture media supplemented with serum) (24). After transfection, cells were incubated for 24 h, treated with or without forskolin for an additional 48 h, and harvested for luciferase assay. When PKAp was co-transfected, cells were harvested 48 h later. All transfections were performed at least three times, and typical data are shown. As demonstrated previously (25), the transcriptional activity of the RSV promoter is only weakly induced by forskolin. Thus, the probability is low that Pbx1 enhances transcription of the RSV promoter in E2A-Pbx1 RSVpBK.

Luciferase assays were performed using a kit (Promega) following the manufacturer's instructions. Light production from luciferase reaction mixtures was measured by a liquid scintillation counter (Wallac 1409, Pharmacia Biotech Inc.).

**Gel Shift Analysis**—The antiserum against Pbx1 (13) and preparation of nuclear extracts and gel shift analyses (22) have been described previously. The CRS1 DNA fragment was end-labeled by T4 DNA kinase with [γ-32P]ATP, isolated from an 8% polyacrylamide gel, and used for gel shift analysis. The labeled consensus GC-box sequence which binds Sp1 (sp1) is attributed to Pbx1-containing complexes (15), except that 2–3 times as much S194 extract was necessary to observe a pattern similar to that from Y1 cells (a GC-box control indicates Sp1 concentrations are similar in Y1 and S194 extracts). This signal can be both depleted and supershifted with anti-Pbx1 (Fig. 2). The lower band essentially disappears, and the upper band is depleted by this antibody. Presumably, this gel shift pattern arises from Pbx2 and/or Pbx3, and, therefore, anti-Pbx1 can recognize one or both. Using the pre-B ALL cell line, a single gel shift band is observed migrating to a point between the upper and lower bands produced by S194 extracts. This band is both diminished and supershifted by anti-Pbx1. The mobility of the supershifted band was again slightly different from that observed with the S194 extract. Since no upper or lower band is observed with the 697 extract, it is concluded that, if expressed, Pbx2 and/or Pbx3 are at very low levels in this cell line. Although anti-E47 recognized the E2A-Pbx1 protein on immunoblot analysis (Fig. 1B), it did not show supershifted signals with the 697 extract on gel shift analysis (data not shown). Thus, the question of whether the gel shift pattern observed with the 697 extract is due to E2A-Pbx1 remains unclear.

Transactivation by E2A-Pbx1a in Response to PKA Mediated through CRS1—Both Pbx1a and Pbx1b are nuclear protein factors involved in the cAMP-dependent transcription promoted by CRS1 (15). Therefore, we have investigated the activation of endogenous E2A-Pbx1a in 697 cells by overexpression of PKA catalytic subunit (PKA-C), which is capable of phosphorylating substrate proteins directly, using a luciferase reporter plasmid 4 × CRS1-Luc. As shown in Fig. 3A, the luciferase
activity was greatly enhanced by overexpression of PKA-C, suggesting that PKA-C activates endogenous E2A-Pbx1a resulting in transactivation of the luciferase gene through CRS1.

The S194 B cells show very low CRS1-mediated PKA-dependent transactivation (Fig. 3B). However, when S194 cells were co-transfected with PKA-C and E2A-Pbx1a expression plasmids (PKAp and E2A-PBX RSV, respectively) and 4 × CRS1-Luc (Fig. 3B), a low basal level of luciferase activity without the expression of PKA-C but a strong activity with the expression of PKA-C (approximately 70-fold enhancement) was observed. This enhancement by the fusion protein was 2-2.5-fold greater than that by Pbx1a (Pbx RSV) in response to PKA-C. As control experiments, co-transfection with PKAp and CREB RSV (a CREB expression plasmid) and 4 × CRE-Luc was performed. Because of the presence of endogenous CREB in S194 cells (Fig. 1), PKAp weakly enhanced the luciferase activity through 4 × CRE without co-transfection of CREB RSV. When CREB RSV was transfected, the co-transfection of PKAp greatly enhanced the luciferase activity. The level of the enhancement by PKA-C with CREB was approximately the same as that with E2A-Pbx1a, suggesting that the transactivation by E2A-Pbx1a in response to PKA is as strong as that observed with CREB.

To examine whether the transactivation of E2A-Pbx1a is cell-specific, similar co-transfection experiments have been performed utilizing Y1 (mouse adrenal tumor), Jeg3 (human choriocarcinoma), and HepG2 cells (human hepatocarcinoma). Because Y1 cells express Pbx1b and Pbx1a (Fig. 1A), luciferase activities were enhanced without co-transfection of E2A-PBX RSV by either forskolin treatment, a stimulator of intracellular cAMP, or cotransfection of PKAp. Y1 cells transfected with E2A-PBX RSV showed a basal activity of the reporter gene slightly higher than that of control plasmid-transfected cells (Fig. 4A). Either forskolin treatment or PKAp co-transfection greatly enhanced the activity. Activation levels in different cell lines vary, perhaps due to experimental variation, however, the transactivation by E2A-Pbx1a was greatly enhanced by the co-expression of PKA-C in both Jeg3 and HepG2 (Fig. 4, B and C). These results indicate that the cAMP-dependent transactivation of E2A-Pbx1a is not tissue-dependent. Thus, E2A-Pbx1 appears to require no complex formation with tissue-specific factors to transactivate its target genes.

In the regulatory region of the CYP17 gene, the first example of a native target gene for Pbx1, the Pbx-binding site of CRS1 (TTGATGGAC) is placed in the opposite orientation to PRS (ATCAAATCAA) (12, 13, 16). To compare activation by both the difference in orientation and DNA sequence, we constructed the 4 × PRS-Luc plasmid containing the consensus PRS sequence in the same orientation as used by other groups (12, 13, 16) in the luciferase reporter plasmid. In S194 cells, 4 × PRS-Luc also showed little enhancement of basal transcription upon overexpression of E2A-Pbx1a but showed a dramatic enhancement with the co-expression of E2A-Pbx1a and PKA-C as did 4 × CRS1-Luc (Fig. 5). This indicates that the orientation and sequence variation between CRS1 and PRS are not critical for the cis-activation of reporter genes. We also examined the promoter dependence of the reporter gene activation by replacing the SV40 minimal promoter in 4 × CRS-Luc with β-globin and CYP17 minimal promoters. Although the absolute level of luciferase activities varied depending on minimal promoters, the pattern of activation by PKA-C showed no difference (data not shown). These results indicate that E2A-Pbx1a is able to transactivate its target genes upon stimulation by cAMP via PKA and also suggest that the activation of E2A-Pbx1a by PKA in the B cell lineage may trigger gene transcription leading to the development of pre-B ALL.

**DISCUSSION**

In this study we have shown that 697 pre-B ALL cells, which express E2A-Pbx1, have a gel shift pattern clearly distinct from cells which express members of the Pbx gene family, yet they enhance transcription mediated by the Pbx binding site in a cAMP-dependent fashion. Not only does endogenous E2A-Pbx1 have this effect in pre-B ALL cells, but co-expression of E2A-Pbx1 shows this effect in several different cell types.

Extradenticle, a Drosophila homologue of Pbx proteins, interacts with homeotic selector (HOM) proteins to modulate the developmental specificity (28–30). In addition, E2A-Pbx1 and Pbx1a have also been shown to interact in vitro with Hox proteins, mammalian counterparts of Drosophila HOM proteins (31). Lu et al. (32) have reported that E2A-Pbx1 and Pbx1 form complexes with Hox proteins leading to repression of transactivation by co-transfection analysis using the PRS sequence. Intriguingly, they have also reported that Pbx1 does...
not form complexes with Hox proteins when the CRS1 sequence is used as a Pbx binding site (32). In fact, the gel shift pattern observed with 697 cells in this study showed no slower migrating complexes that have been reported previously for E2A-Pbx1. The reporter gene vectors containing 4 × CRS1 (4xCRS1-Luc) and 4 × Pbx1 (4xPbx1-Luc) as cis-activating sequence were co-transfected with E2A-PBX RSV and PKAp into S194 cells as described in Fig. 3.

Fig. 5. Comparison of 4 × CRS1 and 4 × Pbx1 as cis-activating sequence. The reporter gene vectors containing 4 × CRS1 (4xCRS1-Luc) and 4 × Pbx1 (4xPbx1-Luc) as cis-activating sequence were co-transfected with E2A-PBX RSV and PKAp into S194 cells as described in Fig. 3.

basal transcription with previous reports (12, 13, 16) was not resolved in this study. Because the transactivation level of E2A-Pbx1a induced by PKA is in a comparable range with the SV40 enhancer and the CRE/CREB system-positive controls in all cell types examined, we conclude that this PKA-dependent transactivation of E2A-Pbx1a is biologically significant.

Although the detailed cellular mechanisms are not known, the cAMP signaling pathway is important for differentiation and apoptosis of lymphocytes (18, 19, 35, 36). The expression of the E2A-Pbx1 fusion protein is exclusively associated with a subgroup of childhood leukemia that exhibit arrest at the stage of pre-B cell differentiation (1, 2). The present study therefore suggests that a cAMP signaling pathway required for proper differentiation and/or apoptotic cell death may activate the target genes of E2A-Pbx1 resulting in triggering the malignant transformation and proliferation of pre-B cells containing the t(1;19) translocation.

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