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Potentiation of GATA-2 Activity through Interactions with the Promyelocytic Leukemia Protein (PML) and the t(15;17)-Generated PML-Retinoic Acid Receptor α Oncoprotein

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The hematopoietically expressed GATA family of transcription factors function as key regulators of blood cell fate. Among these, GATA-2 is implicated in the survival and growth of multipotential progenitors. Here we report that the promyelocytic leukemia protein (PML) can complex with GATA-2 and potentiate its transactivation capacity. The binding is mediated through interaction of the zinc finger region of GATA-2 and the B-box domain of PML. The B-box region of PML is retained in the PML-RARα (retinoic acid receptor alpha) fusion protein generated by the t(15;17) translocation characteristic of acute promyelocytic leukemia (APL). Consistent with this, we provide evidence that GATA-2 can physically associate with PML-RARα. Functional experiments further demonstrated that this interaction has the capacity to render GATA-dependent transcription inducible by retinoic acid, raising the possibility that GATA target genes may be involved in the molecular pathogenesis of APL.

The GATA factors comprise a family of transcriptional regulatory proteins characterized by the ability to bind a common conserved DNA sequence (W*GATA*R) by virtue of evolutionarily conserved C_4 zinc finger domains (48, 56). Within the hematopoietic system, three members of the GATA family, GATA-1, -2, and -3, are expressed. The phenotypes of knockout mice, deficient in GATA-1, -2, or -3, suggest that these factors play critical but distinct roles in hematopoiesis. Thus, GATA-1 is implicated in the maturation and terminal differentiation of erythroid and megakaryocytic cells (20, 54, 57, 68), whereas GATA-2 appears critically involved in the survival and growth of multipotential progenitors (61). Mice deficient in GATA-3 display abnormalities in T-cell development and differentiation (26, 58). Much attention has focused on how these different factors, which seemingly bind to similar (or identical) *cis* elements, carry out their distinct biological functions. Part of the answer may be attributed to their different expression profiles: GATA-1 is expressed at a high level in erythroid cells, mast cells, megakaryocytes, and eosinophils and at a low level in multipotential progenitors (18, 40, 62, 81), whereas GATA-2 is more broadly expressed among hematopoietic cells, with particularly prominent expression in early progenitors, as well as megakaryocytes and mast cells (45, 47, 48). GATA-3 expression within hematopoiesis is confined to T lymphocytes (33, 48).

Functional experiments in which the deficiency caused by loss of function of a given GATA family member is rescued by enforced expression of a different family member in part support the notion that GATA factors are interchangeable (4, 60). However, the rescue is yet to be completely effective, thereby implicating the existence of intrinsic differences in the functional potentials of the different GATA factors. That there are such intrinsic differences in the biological properties of different GATA factors has also been argued on the basis of ectopic expression experiments conducted in both erythroid cells (6) and multipotent progenitor cells (27, 53). These intrinsic differences may relate to subtle differences in binding site affinities or preferences. Some evidence for this has been obtained in vitro (32, 42, 52), but how these findings relate to the in vivo situation will require a fuller understanding of different bona fide GATA target genes.

Modifications of GATA proteins, through both phosphorylation (8, 51, 59) and acetylation (5, 28), provide additional control points for the regulation of GATA factor functions, as does the interaction of GATA proteins with other regulatory protein factors. In this context, several molecules that bind GATA factors and possibly regulate their transcription activity have been identified. These efforts concentrated primarily on GATA-1, although many of the proteins identified by virtue of their interaction with GATA-1 were subsequently shown to interact with other GATA family members. GATA-1 can form homotypic interactions with itself, as well as heterotypic interactions with the other hematopoietically expressed GATA family members GATA-2 and GATA-3 (7). GATA-1 has also been shown to bind to other zinc finger-containing transcription factors such as Sp1, EKLF (43), and most recently a multiple-zinc-finger protein, FOG (friend of GATA-1) (65). FOG binds to the N-terminal finger (N-finger) of GATA-1 and has been implicated in GATA-1 function in erythroid and megakaryocytic cells (64). It has also been reported that GATA-1 can associate with the LIM domain protein Lmo2 (Rbtn2), which acts as a bridging molecule assembling Tal-1/SCL, Ldb, and GATA-1 on a split GATA-E box motif (50, 69). Furthermore, GATA-1 has been reported to bind to the transcriptional integrator CBP (CREB-binding protein) (3). The majority of factors so far identified as binding to GATA factors have been implicated in red cell or megakaryocyte function (3, 43, 64), reflecting the tissue distribution of GATA-1 expression and its primary use in screening.

All of the interactions described so far are mediated through the zinc finger regions of GATA-1. Given the high degree of

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conservation of the zinc finger domain between different GATA family members (80), it is perhaps not entirely surprising that many of the factors identified as potential GATA-1 partners are also able to bind GATA-2. Some of these, such as Lmo2, share overlapping domains of expression and roles in hematopoiesis with GATA-2 and thus have the potential to be of physiological significance with regard to GATA-2 function (13, 74). In this study, we examined whether GATA partner proteins other than those identified in erythroid and megakaryocytic cell screens might exist in the stem/progenitor cell compartment of hematopoiesis. Since GATA-2 is the predominant GATA factor expressed within this compartment (34, 45, 49), we have used GATA-2 as a bait in a yeast two-hybrid screen of a hematopoietic progenitor cell library.

These experiments have identified the promyelocytic leukemia protein (PML) as a GATA-2 binding activity. PML is a RING finger protein initially isolated as a fusion counterpart of the retinoic acid receptor alpha (RARα) in t(15;17)-associated acute promyelocytic leukemia (APL) (11, 30). The functions of the wild-type PML protein are not completely understood, although it has been implicated in retinoic acid (RA) pathways, growth suppression, and apoptosis (46, 55, 70, 71, 79), and also as a context-dependent modulator of transcription (15, 25, 46, 67, 78). Consistent with the latter role as an enhancer of transcription, we show that PML potentiates the transactivation capacity of GATA-2. Finally, we provide evidence for altered functional interactions between GATA-2 and the PML-RARα fusion protein, raising the possibility that subversion of GATA-2 function may be a component of PML-RARα-mediated leukemogenesis.

MATERIALS AND METHODS

Two-hybrid screens and two-hybrid assays in yeast. Cloning vectors and yeasts for two-hybrid screening were obtained from Clontech (Palo Alto, Calif.) and used according to the manufacturer's instructions. The bait plasmid was constructed by inserting the *Nco*I restriction fragment of human GATA-2 (hGATA-2) cDNA (14) (a gift from S. H. Orkin, Harvard Medical School), which encodes the complete hGATA-2 sequence but lacks the last amino acid residue, into the *Nco*I site of pAS2-1 (pAS2-1/hGATA-2). *Saccharomyces cerevisiae* reporter strain Y190 (containing *GAL4-lacZ* and *GAL4-HIS3* reporters) was transfected sequentially with pAS2-1/hGATA-2 and then the library plasmids, which were derived from murine immature hematopoietic cell H7 and constructed in pGAD10 vector (a gift from T. Ito, University of Texas) (76). Cells were plated on Trp-deficient (Trp−), Leu−, His− agarose plates containing 3-amino-1,2,4-triazole (3-AT; 25 mM). $LacZ^+$ clones were identified by a standard β-galactosidase filter assay. A well-isolated $LacZ^+$ colony was cultured in SD Trp−, Leu−, His−, 3-AT+ medium, and then the library plasmids were isolated and transformed into the yeast containing the bait plasmid to confirm the reproducibility of the results of the β-galactosidase filter assay. The library plasmid was then partially sequenced, and the sequence obtained was subjected to a homology search using the BLAST program.

The yeast two-hybrid assay was performed essentially as described elsewhere (39). pAS2-1/hGATA-2 (amino acids [aa] 1 to 389), pAS2-1/hGATA-2 (aa 390 to 475), and pAS2-1/hGATA-2 (aa 195 to 389) were constructed by using convenient restriction sites; pAS2-1/hGATA-2 zinc fingers (aa 272 to 389) was constructed by PCR. *S. cerevisiae* Y190 was transformed with the indicated combinations of plasmids simultaneously and grown on Trp−, Leu− agarose plates for 4 days. Colonies appearing on the plates were subjected to the β-galactosidase filter assay.

Expression plasmids. The hGATA-2/pMT2 expression plasmid was generously provided by S. H. Orkin. cDNAs for hPML and hPML-RARα (30) were provided by A. Kakizuka (Kyoto University, Kyoto, Japan). hPML/pMT2, hPML-RARα/pMT2, Flag-hPML/pCMV, Flag-hPML-RARα/pCMV, Flag-hGATA-2/pCMV, myc-hPML/pCMV, and myc-GATA-2/pCMV were constructed by using appropriate restriction sites in either pMT2, pFlag-CMV2 (Eastman-Kodak, New Haven, Conn.), or pcDNA3.1-myc tag (a gift from H. Osada, Aichi Cancer Center, Aichi, Japan) expression vector.

Cells. COS and 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). Ba/F3 cells engineered to express hPML (Ba/F3-hPML cells; a gift from T. Naoe, Nagoya University, Nagoya, Japan) were maintained in RPMI 1640 medium supplemented with 10% FCS and 5 ng of murine interleukin-3 (a gift from Kirin Brewery Co., Ltd., Tokyo, Japan) per ml. Human leukemia NB4 cells (35) were maintained in RPMI 1640 medium supplemented with 10% FCS.

Antibodies. The rat monoclonal anti-GATA-2 antibody RC 1.1, which recognizes both mouse and human GATA-2, was a generous gift from M. Yamamoto (Tsukuba University, Tsukuba, Japan). Anti-hPML antiserum (77) was kindly received from T. Naoe, and anti-GATA-2 antiserum was a kind gift from S. H. Orkin. Anti-Flag antibody M2, biotinylated M2, and avidin-agarose beads were purchased from Sigma (St. Louis, Mo.). Anti-Flag (D8), anti-Myc (A14), and anti-SMRT polyclonal antibodies and blocking peptides corresponding to the immunogen for anti-SMRT serum were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). A commercially available (Santa Cruz Biotechnology) agarose-conjugated anti-GATA-2 monoclonal antibody was used to immunoprecipitate GATA-2; agarose-conjugated normal mouse immunoglobulin (Santa Cruz Biotechnology) served as a control in these experiments.

Protein interaction assays in cells. COS cells (5×10^5) grown in 10-cm-diameter plates were transfected with the indicated expression plasmids using a standard DEAE-dextran method. The total amount of plasmids was equalized by the addition of the corresponding empty vectors. Forty-eight hours later, nuclear extracts were prepared as described elsewhere (12) and immunoprecipitated with anti-GATA-2 antibody conjugated to agarose or biotinylated anti-Flag antibody M2 in combination with avidin-agarose beads in the binding buffer (20 mM HEPES-KCl [pH 7.9], 140 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5 mg of bovine serum albumin per ml, 5% protease inhibitor cocktail [Sigma], 0.01% NP-40). After four washes with the binding buffer, immune complexes were analyzed by Western blotting using the indicated antibodies as described previously (59).

Immunoprecipitations using hematopoietic cells were also performed. Nuclear extracts from 10^8 cells were immunoprecipitated with anti-GATA-2 conjugated to agarose in the binding buffer described above. Immunocomplexes were analyzed by Western blotting.

Protein interaction assays in solution. Fragments of cDNA encoding hGATA-2 and hPML were produced using convenient restriction enzymes and PCR methods and then cloned into the glutathione *S*-transferase (GST) fusion vector pGEX 5x-1 (Pharmacia, Uppsala, Sweden). The GST constructs were transformed into *Escherichia coli* strain DH5α or XL1-Blue, and the GST fusion proteins were obtained according to the manufacturer's instructions.

Nuclear extracts of COS cells transfected with an expression plasmid for Flag-hPML or Flag-hGATA-2 were incubated with the indicated GST fusion proteins bound to the resin in the binding buffer (50 mM Tris-HCl [pH 7.5], 140 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5 mg of bovine serum albumin per ml, 5% protease inhibitor cocktail [Sigma], 0.05 or 0.01% NP-40). After gentle rocking at 4°C overnight, the resin was washed four times with the binding buffer, and the bound protein was analyzed by Western blotting with anti-Flag antibody M2.

DNA binding assay. For electrophoretic mobility shift assays (EMSAs), nuclear extract from Ba/F3-hPML cells was incubated in 10 μl of binding buffer [10 mM Tris-HCl (pH 7.5), 75 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 4% Ficoll, 0.5 μg of poly(dI-dC)] (75) and a ^{32}P-labeled double-strand oligonucleotide probe (CACT**TGATAA**CAGAAAG**TGATAA**CTCT; Santa Cruz Biotechnology). After 20 min, the protein-DNA complexes were resolved on a 4% native polyacrylamide gel and visualized by autoradiography. A 200-fold molar excess of unlabeled oligonucleotide probe or unlabeled oligonucleotide mutated in a core recognition sequence from GATA to CTTA (Santa Cruz Biotechnology) was used for competition experiments, and 1 μl of each antibody was used for supershift.

Transactivation assays. A luciferase reporter plasmid in which a murine GATA-1 promoter (positions −798 to −574) containing a double GATA site (63) was arrayed upstream of the β-globin minimal promoter (29) (designated GATA-1/Luc.) was a gift from M. Yamamoto. A luciferase reporter plasmid in which two copies of back-to-back double GATA sites in the mouse CD34 promoter were placed upstream of the β-globin minimal promoter driving the luciferase gene (designated CD34x2/Luc.) was constructed by inserting *Eag*I fragments of a double-strand oligonucleotide (AAAAA*CGGCCG*TATTTT**TAT CTGATA**GGAAGT*CGGCCG*TTTTT) into *Eag*I sites (CGGCCG) of GATA-1/Luc. from which the murine GATA-1 promoter was excised by *Eag*I digestion. The mutant reporters in which core recognition sites were mutated from GATA to TTTA (mutant GATA-1/Luc. and mutant CD34x2/Luc.) were constructed by PCR-mediated site-directed mutagenesis. Luciferase reporters GATA-1-TK/Luc. and mutant GATA-1-TK/Luc. were constructed by replacing the β-globin minimal promoter with the thymidine kinase promoter in GATA-1/Luc. and mutant GATA-1/Luc. plasmids and used for assays in hematopoietic cells.

293T cells (2×10^5/35-mm-diameter plate) were transfected with the indicated expression plasmids by a standard calcium phosphate coprecipitation method. Cell lysates were prepared 36 to 48 h after transfection and assayed for luciferase activity using a luciferase assay system (Promega) according to the manufacturer's instructions. Total amounts of plasmids used for transfection were equalized by the addition of the corresponding empty vectors. Transfection efficiency was normalized on the basis of β-galactosidase activity expressed from cotransfected pCMV/β-gal plasmids (a gift from N. Emi, Nagoya University) or *Renilla* luciferase activity from cotransfected pRL-TK-*Renilla* luciferase plasmids (Promega), with essentially the same results. For assays with PML-RARα expression vectors, the indicated concentrations of all-*trans* RA (Sigma) were added to the culture medium 24 h after transfection, and luciferase activity was measured after a further 24 h. The relative luciferase activities presented reflect duplicate or

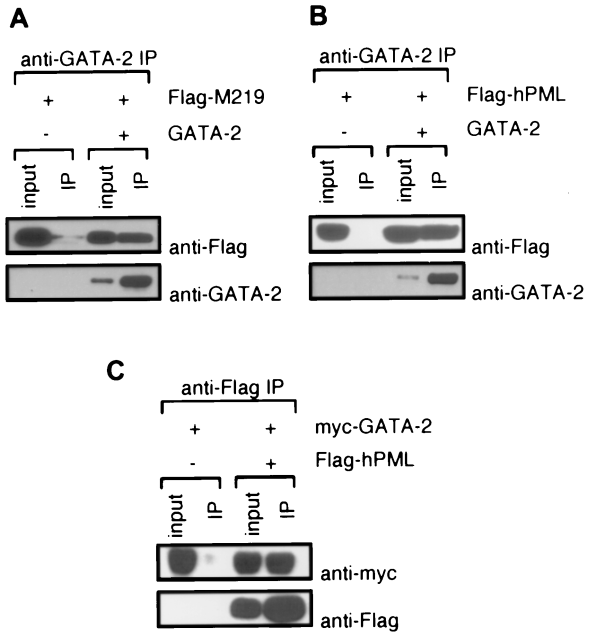

FIG. 1. Interaction of GATA-2 with PML in mammalian cells. Nuclear extracts of COS cells transfected with expression plasmids encoding the indicated proteins were immunoprecipitated (IP) with anti-GATA-2 (A and B) or anti-Flag (C) antibody and analyzed by immunoblotting with anti-Flag and anti-GATA-2 (RC1.1) antibodies (A and B), or anti-Myc and anti-Flag antibodies (C). Nonimmunoprecipitated material (10% input) was analyzed as a control for appropriate expression of proteins programmed by transfected plasmids. M219 is the mouse PML clone identified as interacting with GATA-2 in a yeast two-hybrid screen. Note that both mouse (A) and human (B) PML coprecipitate with hGATA-2. In reciprocal immunoprecipitation experiments, GATA-2 coprecipitated with hPML (C).

triplicate values from a representation of no less than three independent experiments.

For assays using hematopoietic cells, cells were transfected with the indicated reporter plasmids, together with pRL-TK-*Renilla* luciferase plasmid as an internal control, by electroporation using a Gene Pulser (Bio-Rad, Richmond, Calif.) with a capacitance setting of 960 μF and 250 V. Data are presented as relative luciferase activity.

RESULTS

Isolation of PML as a binding partner of GATA-2. We have used a yeast two-hybrid screen to search for potential GATA partner proteins in hematopoietic progenitor cells. Using full-length hGATA-2 cDNA fused to the GAL4 DNA binding domain as bait, we screened a total of 10^7 primary transformants of a murine immature hematopoietic cell (H7) (11) cDNA expression library and obtained three positive clones. Sequencing revealed that two of the three clones (M218 and M219) encoded the murine homologue (21) of hPML (data not shown).

To confirm that GATA-2 could interact with PML in a mammalian cell context, COS cells were cotransfected with expression vectors harboring hGATA-2 and an epitope-tagged (Flag) version of one of the mouse PML cDNAs (clone M219) isolated by yeast two-hybrid screening. Two days following transfection, cell lysates were prepared and subjected to immunoprecipitation using a monoclonal anti-GATA-2 antibody. The immunoprecipitated material was fractionated by polyacrylamide gel electrophoresis and subsequently analyzed by Western blotting using an anti-Flag or anti-GATA-2 antibody (Fig. 1A). The results show that mouse PML coimmunoprecipitated with GATA-2 in this system, suggesting that GATA-2 and PML can complex in mammalian cells. Results similar to those obtained with the Flag-tagged mouse PML clone were obtained using a Flag-tagged hPML cDNA (Fig. 1B). Reciprocal immunoprecipitation was also conducted. COS cells were cotransfected with expression plasmids for Myc-tagged hGATA-2 and Flag-tagged hPML, and then cell lysates were immunoprecipitated with anti-Flag (i.e., anti-PML) antibody (Fig. 1C). The results show that GATA-2 coimmunoprecipitated with hPML, confirming the specificity of the association of the two proteins.

Mapping the sites of interaction between GATA-2 and PML. We wished to determine the region in GATA-2 that was required for its interaction with PML. Our initial mapping was conducted using the yeast two-hybrid system. Plasmids encoding various parts of GATA-2 fused to the GAL4 DNA binding domain were assessed for the ability to interact with the mouse PML (clone M219)-GAL4 activation domain fusion protein in yeast (Fig. 2A). The results show that aa 272 to 389 of GATA-2, which encompass both N- and C-zinc fingers, are sufficient for binding to PML, although neither zinc finger alone showed binding activity to PML in this assay system (data not shown). The most carboxyl-terminal portion of GATA-2 (aa 390 to 475) is not involved in the interaction of GATA-2 and PML in the yeast system. The role of the N-terminal region (aa 1 to 193) of GATA-2 in binding to PML could not be satisfactorily assessed by the yeast two-hybrid assay because this region, when fused to the GAL4 DNA binding domain, strongly activated LacZ production from the reporter gene in the absence of the PML-GAL4 activation construct.

We next conducted pull-down assays to more precisely determine the binding sites. Various parts of GATA-2 fused to GST were produced in bacteria and tested for binding to Flag-tagged hPML expressed in COS cells (Fig. 2B). The zinc fingers were again found to bind PML, with either the N- or C-finger alone being sufficient to retain PML. The reason why a single finger (N- or C-finger alone), which showed binding activity to PML in pull-down assays, failed to show such activity in yeast two-hybrid assays is not clear, although such discrepancies have been previously reported (19). In pull-down assays, the amino-terminal portion (aa 1 to 193) failed to show affinity for PML. Unfortunately, preparations of the most carboxyl portion of GATA-2 (aa 390 to 475) fused to GST were consistently highly degraded and thus not suitable for analysis in this assay (data not shown). Taken together, the yeast two-hybrid and pull-down assays suggest that the primary site within the GATA-2 molecule responsible for interaction with PML lies within the zinc finger region.

We next performed similar pull-down experiments to determine the region within PML involved in binding to GATA-2 (Fig. 2C). These experiments showed that the RING+B-box region, or B-box region alone, bound to GATA-2, whereas the RING or coiled-coil region did not. On the basis of these experiments, we conclude that the interaction between GATA-2 and PML involves the zinc finger regions of GATA-2 and the B-box domain of PML.

PML potentiates GATA-2 activity. We next asked whether the association of PML with GATA-2 had any functional consequences for GATA-2 activity. Since PML is capable of modulating transcription depending on context (15, 25, 46, 67, 78), we examined the effect of PML on GATA-2's ability to potentiate the transcriptional activity of a luciferase reporter gene linked in *cis* to a GATA recognition motif. Because there are as yet no known GATA-2 target genes in hematopoietic cells, we surveyed a range of potential GATA-dependent reporters

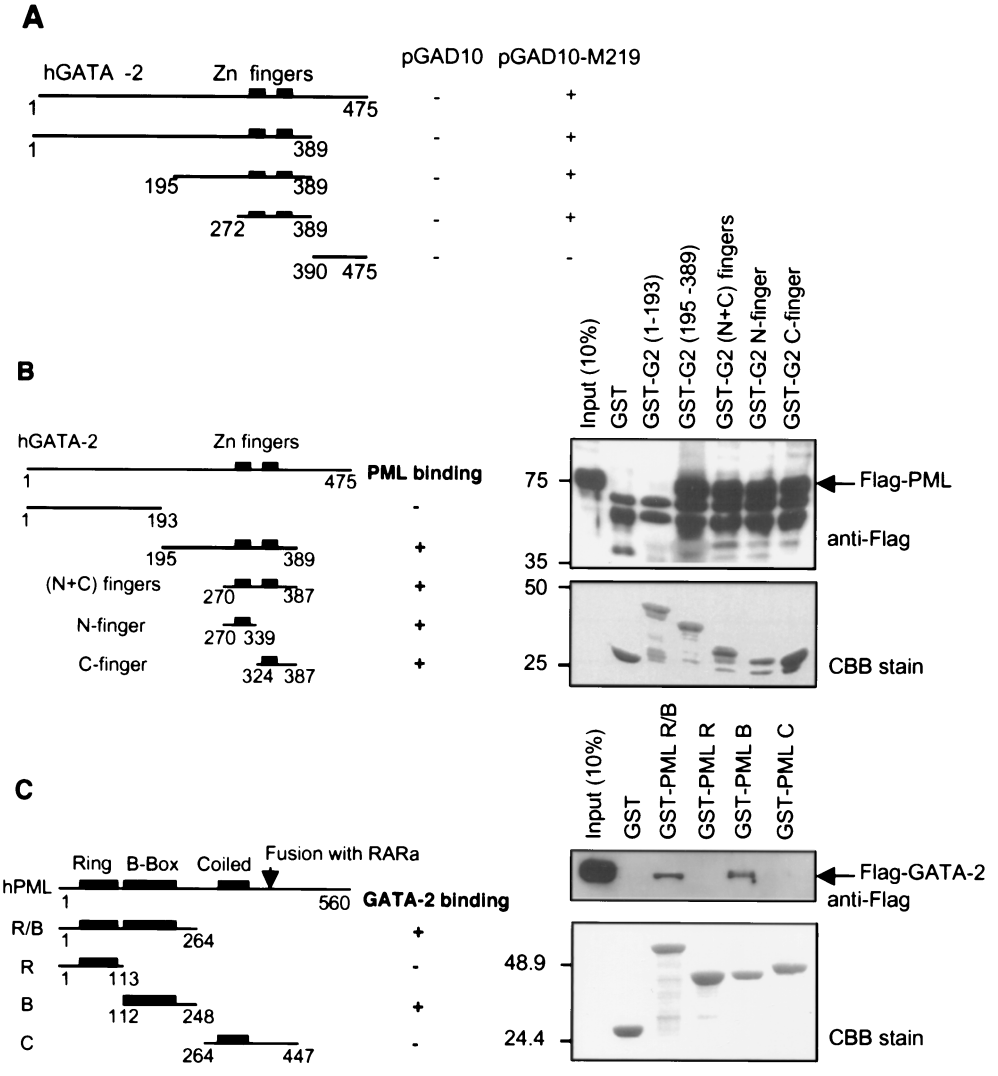

FIG. 2. Mapping regions of GATA-2 and PML involved in interaction as determined by yeast two-hybrid (A) and pull-down (B and C) assays. (A) The indicated regions of GATA-2 were fused to the GAL4 DNA binding domain and assayed for the ability to interact with a mouse PML (M219)-GAL4 activation domain fusion protein after transfection into the reporter yeast strain Y190. The pGAD10 vector-only construct served as a control for interaction-dependent reporter activity. Schematic drawings of the parts of GATA-2 tested (with the first and last amino acids present in the constructs indicated) and summary of the interaction, where + and − denote $LacZ^+$ and $LacZ^-$, respectively (determined by β-galactosidase filter assays), are presented. (B) GST fusion proteins containing the indicated portions of GATA-2 were tested for the ability to bind Flag-tagged hPML contained in COS cell nuclear extract programmed with Flag-tagged hPML expression vectors. The first and last amino acids of the GATA-2 region present in the various GST fusions are indicated, and the abilities of the proteins to bind PML are summarized. Western blot analysis of the pull-down material using an anti-Flag antibody is shown on the right (upper panel), and Coomassie brilliant blue (CBB) staining is presented in the lower panel to allow assessment of the quality and quantity of the various GST–GATA-2 proteins used. Numbers on the left indicate positions of molecular mass markers in kilodaltons. (C) Reciprocal pull-down analysis in which various GST-PML fusion proteins were assayed for the ability to bind to Flag-tagged GATA-2.

for utility in our experiments. Reporters based on GATA elements from SCL, erythropoietin receptor, EKLF, mast cell carboxypeptidase A, and α1-globin (48, 56) showed no GATA-2-dependent transactivation (data not shown). However, GATA-2 modestly (<2-fold) but reproducibly transactivated GATA-1/Luc., a reporter containing a double GATA element derived from the GATA-1 gene (63); mutation of the GATA binding site in the reporter abolished GATA-2-dependent transactivation (mutant GATA-1/Luc.) (Fig. 3A). Using this reporter system in 293T cells, PML was found to enhance the transactivation potential of GATA-2 (Fig. 3A) and to do so in a dose-dependent manner (Fig. 3B). Although in some experiments PML slightly increased reporter activity in the absence of GATA-2 expression (Fig. 3A and B and data not shown), the reporter activity achieved by coexpression of GATA-2 and PML was significantly higher than the simple sum of the activities achieved by either GATA-2 or PML alone. To confirm the potentiating effects of PML on GATA-2 activity, we constructed canonical luciferase reporters. Since a back-to-back double GATA site in the mouse CD34 promoter has been shown to have high affinity for GATA-2 (G. May and T. Enver, unpublished observations), we placed two copies of the double GATA sites upstream of β-globin minimal promoter driving luciferase. This reporter (CD34x2/Luc.) displayed GATA-2-dependent activity, and the activity was again enhanced by PML (Fig. 3C). The GATA-2-dependent activity and its enhancement by PML were abrogated when the GATA sites in the reporter were disrupted (mutant CD34x2/Luc.), confirming the strict dependence of the observed effects on the presence of the GATA motifs. PML did not alter the expression level of

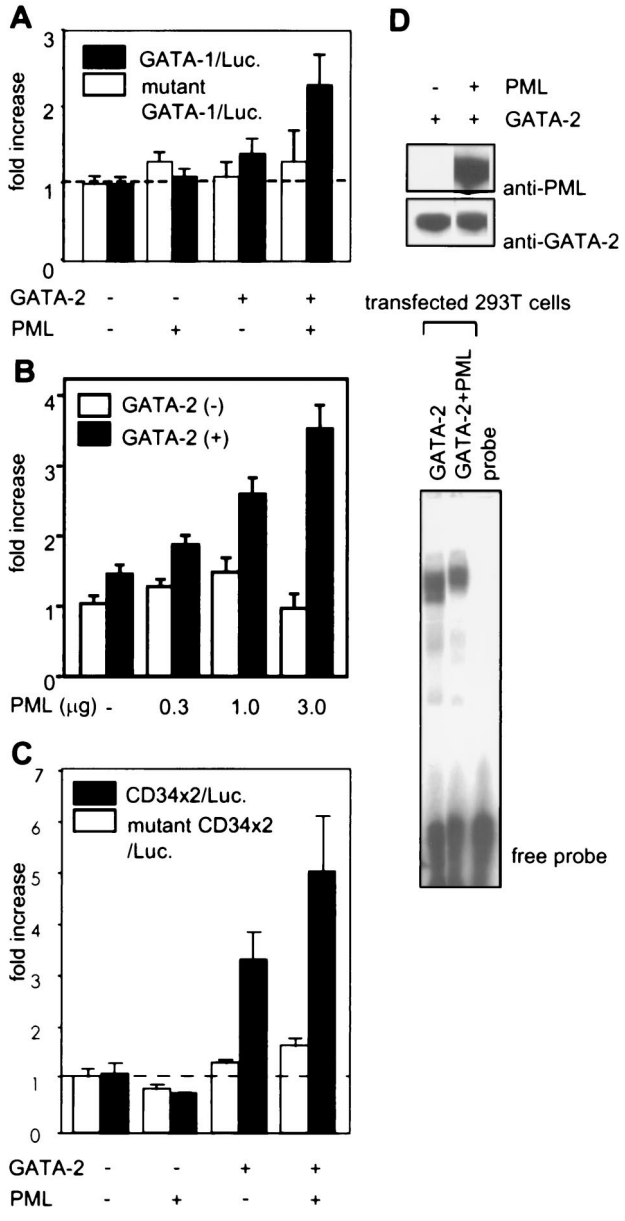

FIG. 3. PML potentiates GATA-2-dependent reporter gene activity in transient transfection assays. (A) 293T cells were transfected with a luciferase reporter plasmid containing double GATA sites from the promoter of mouse GATA-1 (GATA-1/Luc., 0.5 μg; solid bars) or its mutant in which GATA sites were mutated from GATA to TTTA (mutant GATA-1/Luc., 0.5 μg; open bars), together with expression plasmids for GATA-2 (pMT2/GATA-2; +, 30 ng) and PML (pMT2/PML; +, 1 μg), as indicated. (B) Effects of increasing amounts of PML on GATA-2 activity were examined in 293T cells using the GATA-1/Luc. reporter. GATA-1/Luc. (0.5 μg), pMT2/GATA-2 (+, 30 ng), and the indicated amounts of pMT2/PML were used. (C) Experiments similar to those represented in panel A were conducted using a luciferase reporter plasmid containing two copies of back-to-back double GATA sites from the mouse CD34 promoter (CD34x2/Luc., 0.5 μg; solid bar) or its mutant in which GATA sites were disrupted (mutant CD34x2/Luc., 0.5 μg; open bar), together with pMT2/GATA-2 (+, 30 ng) and pMT2/PML (+, 1 μg). Luciferase activity is standardized against *Renilla* luciferase (A and C) or β-galactosidase (B) activity from cotransfected control reporters (pRL-TK-*Renilla* luciferase or pCMV/β-gal) and expressed as fold increase of the activity of reporter (GATA-1/Luc. or CD34x2/Luc.) alone. Amounts of plasmids used were equalized by the addition of corresponding empty vectors. (D) 293T cells were transfected with pMT2/GATA-2 (+, 3 μg) in combination with pMT2/PML (+, 30 μg) or pMT2 (−, 30 μg). The resultant nuclear extracts were used for Western analysis with anti-PML and anti-GATA-2 antisera (top panel) and EMSA (lower panel). Note that PML expression affected neither the expression level of GATA-2 nor GATA-2 binding to DNA.

GATA-2, as judged by Western blotting (Fig. 3D, top panel), nor did it significantly affect GATA-2 binding to DNA in EMSAs (Fig. 3D, lower panel), suggesting that PML acts as a coactivator for transactivation by GATA-2. Thus, we conclude that PML is capable of potentiating GATA-2-mediated transactivation.

Binding of a GATA-2–PML complex to a GATA motif in DNA. We next asked whether GATA-2 could recruit PML to a GATA recognition site in DNA in hematopoietic cells. These experiments were performed with cell lysates from the murine hematopoietic progenitor cell line Ba/F3. Ba/F3 cells are factor dependent for growth and survival and, importantly, express endogenous GATA-2 (59). Previous studies in our laboratories have made experimental use of the Ba/F3 cell system for the analysis of GATA-2 binding to DNA, GATA-2 regulation by receptor-mediated cell signaling (59), as well as GATA-2 function using genetic manipulation of its activity within cells (27). Since antisera to murine PML were not readily available, we used a Ba/F3 cell clone (Ba/F3-hPML) engineered to express hPML. EMSAs using an oligonucleotide containing GATA-recognition sites and Ba/F3-hPML nuclear extracts revealed a protein-DNA complex (Fig. 4A, lanes 2 and 9), which was competed by wild-type oligonucleotide (lane 3) but not by an oligonucleotide in which the GATA site is mutated (lane 4). Supershift assays using antibodies to GATA-2 (lane 5 and 10) or PML (lane 12) indicated the presence of both these proteins in the complex. Simultaneous incubation with antibodies to both proteins further supershifted the DNA-protein complex (lane 7 and 13).

In similar EMSAs conducted with hGATA-2-expressing hematopoietic cell lines, evidence for a GATA-2–PML complex bound to a GATA site in DNA was not found. Furthermore, attempts to coimmunoprecipitate endogenous PML and GATA-2 from these human cell lines were not successful. The immunolocalization of GATA proteins in hematopoietic cells has previously been addressed (17). Our attempts to immunolocalize GATA-2 and PML within cells have been technically difficult. However, the limited preliminary data that we have obtained suggest that the bulk of GATA-2 and PML localize differently within the nucleus (data not shown). These data raise the possibility that the interaction of GATA-2 and PML seen in COS cell transfectants and Ba/F3-hPML cells may arise a consequence of overexpression. We therefore compared the level of expression of hPML in Ba/F3-hPML cells with that of endogenous hPML in human HEL and KG1 cells by Western blotting. The results shown in Fig. 4B are consistent with a relatively mild (two- to threefold) increased level of PML in Ba/F3-hPML relative to HEL and KG1.

These experimentally induced conditions of mild overexpression of PML may be met in particular situations in vivo, since PML levels are known to fluctuate considerably within cells in response to physiological cues (such as interferon) (36). The potential for GATA-2–PML interaction could also be enhanced through alterations in the intracellular localization of the proteins. PML, unlike GATA-2, is known to reside in discrete nuclear bodies. However, the localization of PML is significantly altered in t(15;17) APL.

Interaction of GATA-2 and PML-RARα. PML was originally cloned as a fusion partner of RARα in a PML-RARα chimeric protein generated by the t(15;17) translocation, which is typically associated with APL (11, 30). PML-RARα is known to complex with PML in APL cells and display an intracellular localization distinct from PML in other hematopoietic cells (16, 31, 72). The B-box region of PML is retained in the PML-RARα chimera (Fig. 2C), raising the possibility that the

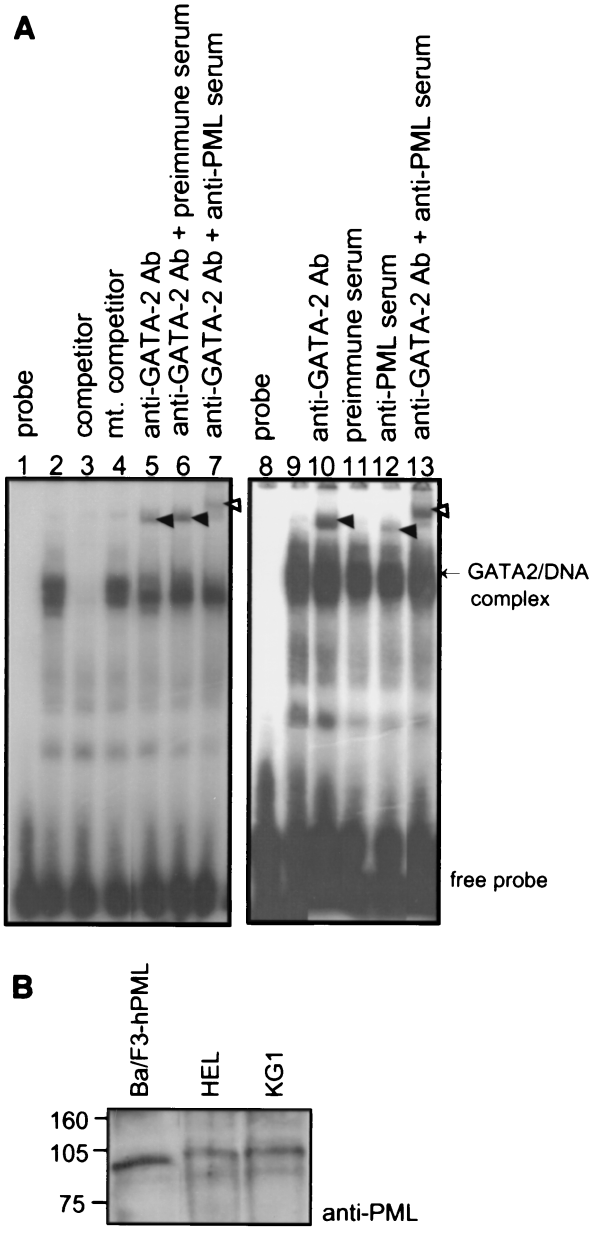

FIG. 4. A GATA-2–PML complex binds to a GATA motif in DNA. (A) Nuclear extracts from Ba/F3-hPML cells were used in EMSAs. A ^{32}P-labeled oligonucleotide containing GATA consensus recognition sites was used as a probe. The protein-DNA complex was revealed in lanes 2 and 9. Competitive experiments were performed using a 200-fold excess of either the unlabeled oligonucleotide (competitor) or a mutant (mt.) oligonucleotide in which the GATA consensus site had been disrupted (lanes 3 and 4). Supershift experiments were conducted by addition of anti-GATA-2 antibody (Ab; RC1.1) and/or anti-PML antiserum as indicated, with preimmune rabbit serum serving as a control (lanes 5 to 7 and 10 to 13). The positions of migration of free probe, specific GATA-2–DNA complexes, and the supershifted complexes (filled arrowheads) are indicated. The simultaneous addition of anti-PML and anti-GATA-2 antibodies to the reaction resulted in a further retarded complex indicated by the open arrowheads (lanes 7 and 13). (B) Nuclear extract from Ba/F3-hPML cells was analyzed for expression of hPML by Western blotting with anti-hPML antibody along with human hematopoietic HEL and KG1 cells. Equal numbers (10^6) of cells were used for the analysis. Sizes are indicated in kilodaltons.

PML-RARα oncoprotein may have the potential to associate with GATA-2, which is the predominant GATA factor expressed in early myeloid progenitor cells (44, 45, 47) representing the cellular target for transformation in APL.

We first examined this possibility using the COS cell overexpression system. Flag-tagged PML-RARα was coexpressed in COS cells together with hGATA-2, and the resultant cell lysates were immunoprecipitated using anti-GATA-2 antibodies and analyzed by Western blotting. The results show that the PML-RARα oncoprotein has the capacity to interact with GATA-2 in the context of mammalian cells (Fig. 5A). Importantly, the association of GATA-2 with PML-RARα was also revealed in NB4 cells, which were derived from an APL patient and harbor both endogenous PML-RARα and PML (35). Nuclear extracts from NB4 cells were subjected to similar immunoprecipitation experiments using anti-GATA-2 antibody. The results show that both endogenous PML-RARα and endogenous PML coimmunoprecipitated with endogenous GATA-2, demonstrating that these three proteins have capacity to associate in leukemia cells (Fig. 5B).

We next conducted reporter gene-based transactivation analysis in 293T cells to examine whether expression of the PML-RARα oncoprotein could functionally modulate GATA-2-dependent transactivation. GATA-dependent luciferase reporter constructs were transfected into 293T cells, either alone or together with PML or PML-RARα expression vectors as indicated in Fig. 5C. The experiments were conducted in the presence or absence of a GATA-2 expression vector. In both cases, one half of the experimental sample was treated with RA and the other half was treated with diluent (dimethyl sulfoxide [DMSO]) alone. The results show that PML-RARα (like PML) can stimulate GATA-2-dependent reporter gene activity without RA treatment. Strikingly, in the presence of PML-RARα, GATA-2-dependent reporter gene expression is rendered ligand inducible by RA (Fig. 5C). This effect was more striking when PML and PML-RARα were coexpressed. Results similar to those obtained using the GATA-1/Luc. reporter were obtained with the CD34x2/Luc. reporter (Fig. 5D). The effect of RA on GATA-2-dependent transactivation in the presence of PML and PML-RARα was abrogated when the GATA sites in the reporter were disrupted, confirming the strict GATA site dependence of the effects observed.

APL cells are known to be unresponsive to physiological concentrations of RA but to respond to pharmacological concentrations of RA by undergoing terminal differentiation (23). We therefore examined the RA dose dependency of this enhancement of GATA-2 activity. Physiological concentrations of RA (~1 nM) showed minimal effects on GATA-2-dependent activity, whereas pharmacological concentrations (~100 nM to ~1 μM) evidently potentiated the activity (Fig. 5E). Since PML-RARα has been known to complex with transcriptional corepressors (like SMRT, Nco-R, and Sin3A), which are released by pharmacological but not physiological concentrations of RA (22, 37), it is expected that the release of the corepressors might be responsible for the enhancement of GATA-2 activity by PML-RARα in the presence of RA. To examine this possibility, we conducted immunoprecipitation experiments using NB4 cells. Nuclear extracts from NB4 cells treated with RA (1 μM) or diluent (DMSO) for 24 h were immunoprecipitated with anti-GATA-2 antibody, and the resultant immunocomplex was analyzed by Western blotting with anti-SMRT and anti-GATA-2 antibodies. A blocking peptide to the anti-SMRT antibody was used to verify the authenticity of the SMRT signal (Fig. 5F). In the absence of RA, SMRT was detected in the immunocomplex, whereas SMRT was no longer detectable after treatment with RA. These results suggest that GATA-2 was released from the SMRT transcriptional repressor. In keeping with this, GATA-dependent reporter activity induced in NB4 cells was found to be rendered RA responsive (Fig. 5G). NB4 cells were transfected with the

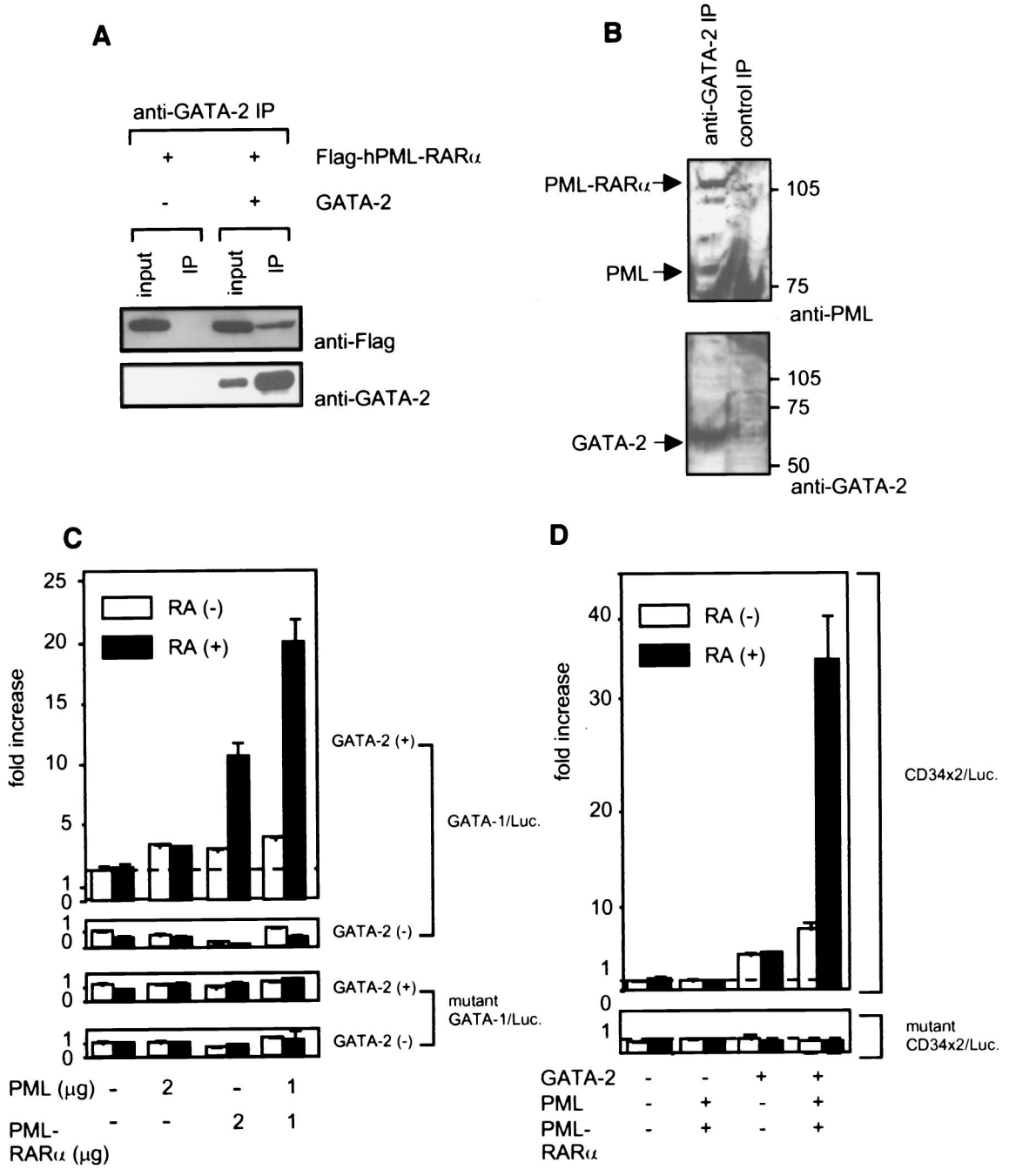

FIG. 5. Interaction of GATA-2 with PML-RARα. (A) Nuclear extracts of COS cells transfected with the indicated expression plasmids were immunoprecipitated (IP) with anti-GATA-2 antibody and analyzed by Western blotting with anti-Flag and anti-GATA-2 (RC1.1) antibodies. (B) Nuclear extracts of NB4 cells (10^8) were immunoprecipitated with anti-GATA-2 antibody or control antibody and analyzed by Western blotting with anti-PML and anti-GATA-2 antisera. Note that both PML and PML-RARα coprecipitated with GATA-2. Sizes are indicated in kilodaltons. (C) Luciferase reporter gene assays using 293T cells were conducted as described in the legend to Fig. 3. Expression plasmids for GATA-2 (pMT2/GATA-2; +, 100 ng), PML (pMT2/hPML; 1 or 2 μg), and PML-RARα (pMT2/hPML-RARα; 1 or 2 μg) were used in combination with GATA-1/Luc. or mutant GATA-1/Luc. reporter plasmids (0.5 μg), as indicated. All-*trans* RA (+, 1 μM; solid bars) was added to the culture media 24 h after transfection, and luciferase activities were measured 24 h later. Luciferase activities are normalized as described in the legend to Fig. 3 and presented as fold increase in activity from GATA-1/Luc. reporter alone in the absence of RA. (D) Experiments similar to those represented in panel C were conducted using CD34x2/Luc. (top panel) or mutant CD34x2/Luc. (lower panel). pMT2/GATA-2 (+, 100 ng), pMT2/PML (+, 1 μg) and pMT2/PML-RARα (+, 1 μg) were used as indicated. Data are presented as fold increase in activity from CD34x2/Luc. alone in the absence of RA. (E) Effects of increasing concentrations of RA on GATA-2 activity in the presence of PML and PML-RARα were examined. 293T cells were transfected with GATA-1/Luc. reporter (0.5 μg) together with expression plasmids for GATA-2 (+, 100 ng), PML (+, 1 μg), and PML-RARα (+, 1 μg), as indicated. Cells were treated with the indicated concentrations of RA and assayed for luciferase reporter activities. Data are presented as fold increase in activity from the reporter alone in the absence of RA. (F) Nuclear extracts from NB4 cells (10^8) treated with RA (1 μM, 24 h) or DMSO (solvent for RA; control) were immunoprecipitated with GATA-2 or control antibodies and analyzed by Western blotting with anti-SMRT and anti-GATA-2 antisera. Peptides corresponding to immunogen for SMRT antiserum were used (+) to confirm the authenticity of the SMRT band. (G) NB4 cells were transfected with GATA-1-TK/Luc. or mutant GATA-1-TK/Luc. as indicated; 24 h after transfections, cells were treated with RA (1 μM; solid bar) or DMSO (open bar), and luciferase activities were measured 24 h later. Luciferase activities are normalized on the basis of *Renilla* luciferase activities from cotransfected pRL-TK-*Renilla* plasmids and presented as fold increase in the activity relative to GATA-1-TK/Luc. in the absence of RA.

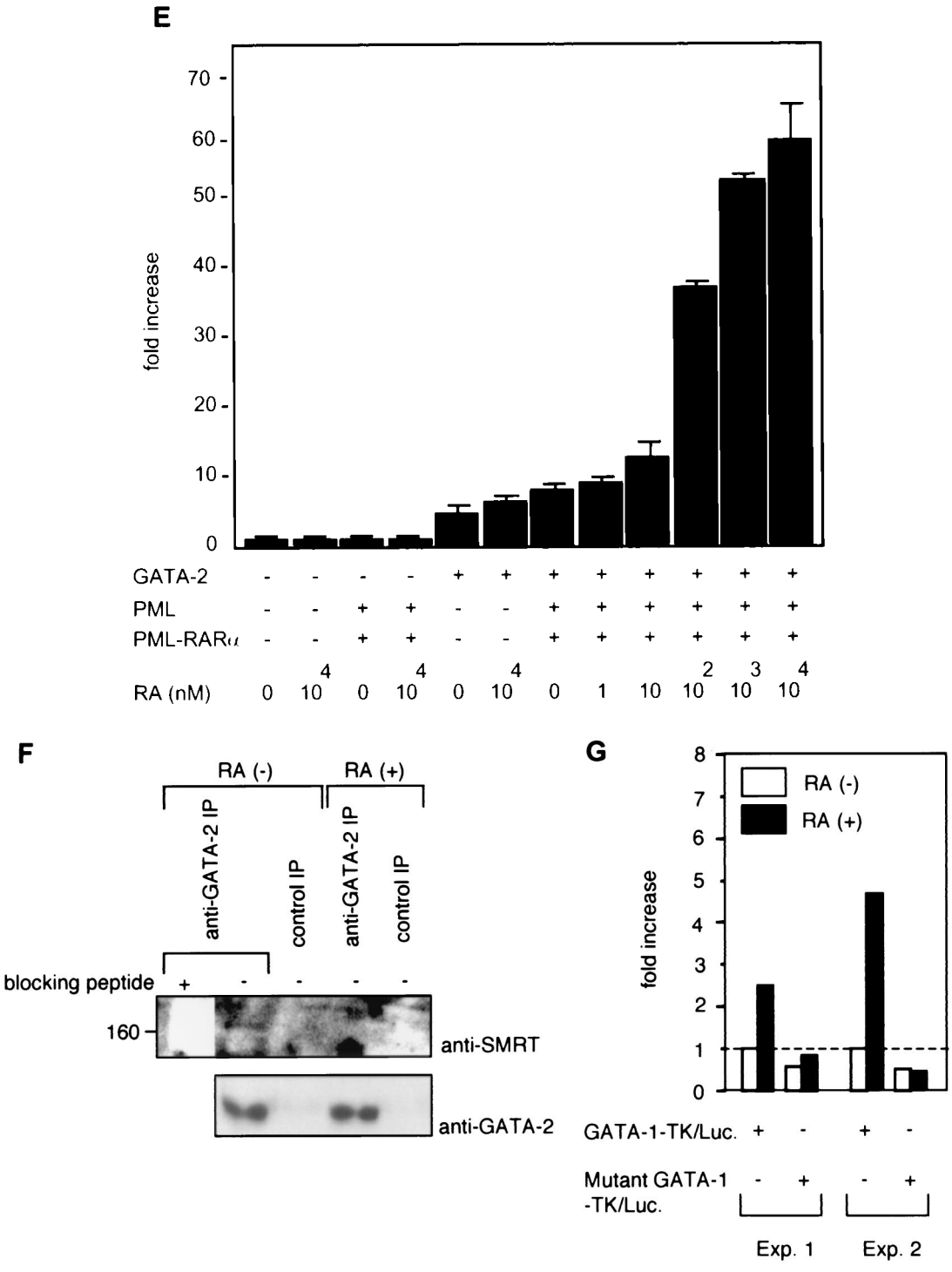

FIG. 5—*Continued.*

GATA-1-Tk/Luc. reporter and then treated with RA (1 μM) or diluent (control). The results show that RA induced higher reporter activity than the control. This effect was not observed when mutant GATA-1-Tk/Luc. was used in place of GATA-1-Tk/Luc., suggesting that the effect of RA was dependent on GATA activity. These results provide evidence for the functional relevance of a PML-RARα–GATA-2 interaction in the appropriate leukemic cells.

DISCUSSION

In this report we have presented evidence that transcription factor GATA-2 can physically associate with PML; this interaction resulted in increased functional activity of GATA-2, as judged by transactivation assays. The mechanisms whereby PML modulates GATA factor-dependent transcription are not clear from the present study. PML altered neither the expres-

sion level of GATA-2 nor GATA-2 binding to DNA in our transient transfection system, raising the possibility that PML functions directly as a transcriptional coactivator. The latter possibility is partly supported by recent data showing that PML binds to a transcriptional integrator, CBP (15). Furthermore, PML potentiates the transcriptional activity of several nuclear receptors, to which PML might bind through other proteins serving as bridging molecules (15, 79). Similarly, our results suggest that PML might be attracted to larger transcription factor complexes including GATA factors and CBP and could directly participate in transcriptional regulation.

Our data for Ba/F3-hPML cells suggest that GATA-2 and PML can interact in a hematopoietic cell context and that the GATA-2–PML complex can be recruited to a GATA recognition motif in DNA. These results thus raise the possibility that PML may modulate GATA-2 activity at GATA-2-dependent target genes in vivo. However, a GATA-2–PML interaction has not been detected in native hematopoietic cell lines. Comparison of the expression levels of PML in Ba/F3-PML and native hematopoietic cells is consistent with the possibility that mild (two- to threefold) overexpression of PML allows GATA-2 to complex with PML. PML expression is known to be induced by cytokines including interferons (36), raising the possibility that GATA-2 is complexed with, and therefore potentiated by, PML conditionally in the hematopoietic system.

Experiments directed at mapping the region of GATA-2 involved in its interaction with PML identified the zinc finger region of GATA-2 as playing a critical role. Reciprocal mapping experiments performed with PML identified the B-box region of PML as a critical determinant for its interaction with GATA-2. PML has also been shown to interact with a number of different nuclear regulatory proteins including the general transcription factor Sp1 (66), the t(11;17) APL-associated transcription factor PLZF (41), and PML itself (24). All of these factors interact with PML through its coiled-coil domain, quite unlike the interaction between GATA-2 and PML, which is mediated by the B-box zinc finger region of PML. The only other protein known to interact with PML via its B-box region is the retinoblastoma protein Rb, which binds PML via its pocket region (1). Curiously GATA-1 also binds to Rb via the pocket region (73), suggesting that PML-Rb, GATA-Rb, and PML-GATA complexes may be competitive and mutually exclusive.

The zinc finger region of the GATA factors, in addition to its role in DNA binding, has also been shown to function as a protein-protein interaction domain. GATA factors have been shown to associate with other nuclear regulatory proteins (other GATAs, Sp1, Lmo2, CBP, etc.) (3, 7, 43, 50) by virtue of the GATA C_4C_4 zinc finger region. Since the zinc finger region has been highly evolutionarily conserved throughout the GATA family (80), it is perhaps not surprising that both GATA-1 and GATA-3 also have the potential to bind to PML (data not shown), although cellular distributions of PML and GATA-1 and GATA-3 do not largely seem to overlap (9).

Within the bone marrow, PML is predominantly expressed in myeloid cells (9), and PML knockout mice show impairment in myelopoiesis both in vivo and in myeloid colony formation in vitro (70). Recently, GATA-2 has been shown to play roles in regulating proliferation of hematopoietic cells, with overexpressed GATA-2 having the ability to inhibit the cell cycle (27, 53). Since GATA-2 is a predominant GATA factor expressed in early myeloid cells (44, 47, 48), PML may have a functional link with GATA-2 in terms of proliferation and differentiation in myeloid lineages.

The interaction between GATA-2 and the PML-RARα oncoprotein is perhaps more intriguing. A number of critical hematopoietic regulators such as SCL/Tal-1, Lmo2, and AML-1 have been implicated in leukemogenic pathways (2, 38). Surprisingly, evidence linking GATA family members with leukemic transformation has been generally lacking. GATA-2 is often expressed in leukemia cells and cell lines (44, 47), but this may simply reflect the progenitor status (and hence GATA-2 positivity) of the target cell in transformation, rather than any leukemogenic effect of GATA-2 itself. Our results raise the possibility that a component of PML-RARα's leukemic potential is realized via GATA-2. By rendering a subset of GATA-2 target genes subject to regulation by the retinoid signaling pathway and its attendant corepressor and coactivator molecules (22, 37), PML-RARα may disrupt the balance between self-renewal and differentiation (27, 53), thereby exerting its leukemogenic potential. It is tempting to speculate that in APL cells GATA-2's activities are suppressed through PML-RARα-associated corepressors (like SMRT), suppression being released by RA and leading to activation of GATA-2-dependent transcription. Enhanced GATA-2 activity may partly contribute to inhibit cell cycling and allow cells to undergo differentiation, since GATA-2 has been recently shown to have such activities (27, 53).

In any event, understanding the functional significance of interactions between GATA-2 and wild-type or chimeric PML proteins will require a better understanding of GATA-2 target genes in both normal and leukemic cells.

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