Modulation of CREB binding protein function by the promyelocytic (PML) oncoprotein suggests a role for nuclear bodies in hormone signaling

(coactivator/nuclear receptors/transcription/acute promyelocytic leukemia)

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ABSTRACT Disaggregation of the spherical nuclear bodies termed promyelocytic (PML) oncogenic domains (PODs) is a characteristic of acute promyelocytic leukemia. Here, we demonstrate that the cAMP enhancer binding protein (CREB)-binding protein (CBP) associates with PML *in vitro* and is recruited to the PODs *in vivo*. Through its association with CBP, wild-type PML dramatically stimulates nuclear receptor transcriptional activity. These results demonstrate that a fraction of CBP is compartmentalized to the POD through its association with PML and thus suggest that PML and other POD-associated proteins may play an unexpectedly broad role in aspects of transcriptional regulation and human disease.

Research on nuclear compartments has uncovered evidence for transcription-related proteins in nuclear substructures and suggests potential relevance to transcriptional regulation (1, 2). The cell nucleus contains a variety of morphologically distinct substructures called nuclear bodies, which include the sphere organelles, coiled bodies (3–5), and the promyelocytic (PML) oncogenic domains (PODs) (for review, see ref. 2). The PODs (also known as nuclear domain 10 or Kr bodies) are macromolecular multiprotein complexes that are present in all cultured cell lines and are also present *in vivo*. A major component of the POD is the PML protein, which originally was identified as the fusion partner of the retinoic acid receptor α (RARα) in the chromosomal translocation t(15;17), resulting in the PML-RARα fusion product (6–11). PML and PML-RARα proteins have been shown to modulate the activity of a set of downstream target genes, although it is not clear whether this is a direct or indirect effect on transcription (1, 12, 13). In leukemic cells from patients with acute promyelocytic leukemia who carry the translocation t(15;17), the expression of the PML-RARα fusion protein disrupts the integrity of the POD. The POD structure is reformed after treatment with all-trans retinoic acid (14–16). The integrity of the compartment also is altered during adenovirus infection, when it appears that POD-associated proteins are released to viral replication domains (17). More recently, the POD has been shown to be a target of herpes, papillomavirus, and other viral proteins (for review, see refs. 2 and 18). Finally, the spinocerebellar ataxia 1 neurodegenerative disorder-associated protein (SCA1) also has been shown to colocalize with PML and to alter POD morphology (19, 20).

The cAMP enhancer binding protein (CREB)-binding protein (CBP) (21) functions as a transcriptional coactivator for a variety of transcription factors, including jun, fos, nuclear receptors (NRs), NF-kB, and the STAT proteins (22–26). The N-terminal region of CBP includes domains for association with the glucocorticoid receptor (GR) and the retinoid X receptor (RXR) (CBP amino acids 1–170), with CREB, cJun, Myb, and Sap-1a and with the HTLV-1 Tax viral protein (CBP amino acids 451–662). In addition, CBP interacts with the tumor suppressor p53 as a coregulatory factor (27–29). CBP contains an intrinsic histone acetyltransferase activity (30, 31) and, in addition, associates with other coactivators, such as P/CAF, SRC-1, TIFII (SRC-2), and ACTR (SRC-3) (24, 32–34). The plethora of cellular and viral proteins that interact with CBP suggests that it may serve as a transcriptional integrator of multiple signaling pathways involved in cell growth, differentiation, and viral pathogenesis. As part of our study of PML and CBP, we made the interesting observation that these two proteins can physically associate and can be colocalized to the POD. In exploring the consequence of this association, PML was shown to promote increased localization of CBP to the POD and also to function as a potent NR coactivator.

MATERIALS AND METHODS

Plasmids. Apol4-tk-Luc contains four synthetic oligonucleotides linked to the minimal thymidine kinase promoter upstream of the coding sequence for the luciferase gene. The oligonucleotides correspond to the A site of the apolipoprotein AI promoter (35). MMTV-Luc, Gal4-tk-Luc, CMXβgal, CMX-RXRα, CMXCBP (mouse), CMXPML, and CMXPML-RAR have been described (1, 17, 25). The pcDNA3CBP(1-1100) and pGEXCBP expression vectors contain the indicated CBP amino acid domains in-frame with polyHis (Invitrogen) and glutathione *S*-transferase (GST) protein, respectively. pSVPMLΔ216-331 and pSVPMLΔ271-331 have been described (1). The expression vectors CMXGal-4CBP and CMX-Gal-4CBP(1-1100), (1-551), (311-521), (1-311), (1077-1461), (1431-2414) contain the full-length mouse CBP coding sequence or the indicated CBP amino acid domains in-frame with the Gal-4DNA binding domain downstream of the cytomegalovirus promoter. The CMX Gal-4RXRαF contains the full-length mouse RXRα coding sequence in-frame with the Gal-4 DNA binding domain downstream the cytomegalovirus promoter.

Cells. CV1 and Hep-2 cells were maintained as monolayers in DMEM supplemented with 100 units/ml penicillin–streptomycin and 10% resin–charcoal-stripped (36) bovine calf serum or 10% FCS, respectively (GIBCO). Cultures were maintained at 37°C and in 7% CO_2. For immunofluorescence,

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Abbreviations: PML, promyelocytic; POD, PML oncogenic domain; RARα, retinoic acid receptor α; CBP, CREB-binding protein; NR, nuclear receptor; GR, glucocorticoid receptor; RXR, retinoid X receptor; GST, glutathione *S*-transferase; CREB, cAMP enhancer binding protein.

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cells were grown on round coverslips (Corning) in 6-well plates.

Antibodies. Affinity-purified polyclonal rabbit antiserum against human PML, Ab PML 5311, the mAb 5E10, and the PML mouse Ab (PG-M3) (Santa Cruz Biotechnology) have been described (17). The CBP A22 and CBP C20 antibodies were purchased from Santa Cruz Biotechnology. The CBP N-5729 and CBP Kix 5214 were kindly provided by M. Montminy and have been described (37, 38). Secondary antibodies labeled either with FITC or Texas red were purchased from The Jackson Laboratory.

***In Vitro* Binding Assays.** GST-CBP and His-Tag CBP were prepared as indicated (Amersham Pharmacia and Qiagen, Chatsworth, CA, respectively). Radiolabeled PML wild-type and mutant protein, PML-RAR, and Tax proteins were prepared by coupled *in vitro* transcription–translation (Promega) by using the corresponding expression vector as template DNA

for each case. The PML proteins are functional *in vitro* as described (ref. 1; data not shown). For the *in vitro* binding assays, 30–50 μl of glutathione Sepharose or Ni–nitrilotriacetic acid agarose associated with the corresponding recombinant proteins were incubated with 3–5 μl of S^{35}-radiolabeled proteins for 30 min at 4°C. Complexes then were centrifuged, were washed five times in appropriate buffers, and were separated by SDS/PAGE, and gels were exposed to x-ray films for 4–8 h.

Transfection. For transient transfections, CV-1 and Hep2 cells were grown in 6- or 48-well plates to 50–80% confluence in the corresponding medium. Twelve hours later, the cells were transiently transfected with the indicated expression vectors by lipofection by using *N*-[2-(2, 3)-dioleoyloxy)propyl-*N,N,N*-trimethyl ammonium methyl sulfate] (Dotap) according to the instructions of the manufacturer (Boehringer Mannheim). For a 6-well plate, 1–4 μg of the indicated expression vector driven by simian virus 40 or cytomegalovirus promoter
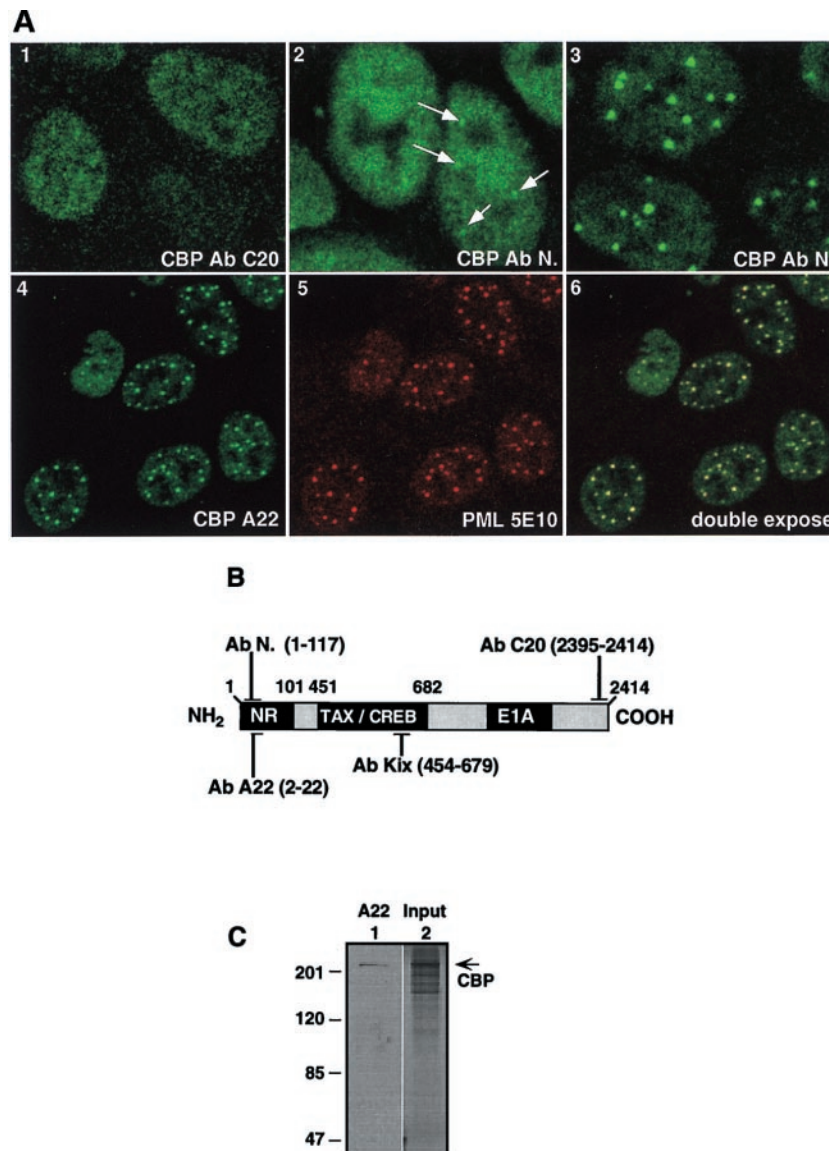

FIG. 1.   Differential localization of CBP in the nucleus. (*A*) Immunohistochemistry of CV1 cells, fixed at 80–90% confluence and analyzed in confocal microscopy. Green corresponds to the CBP staining revealed with the FITC-conjugated secondary Ab, red corresponds to the PML staining revealed with the Texas red-conjugated secondary Ab, and the yellow color in the double-exposure image indicates the sites where PML and CBP colocalize. Primary Abs are used as indicated. (*2*) The arrow shows the CBP speckled-like structures that colocalize with PML protein. *2* and *3* show two independent, randomly selected, fields of asynchronous cells populations. *1-5* are single-exposure photographs, and *6* is a double exposure. (*B*) Schematic representation of CBP primary structure. The location of the epitopes for the corresponding CBP Abs is indicated. (*C*) immunoblotting of total reticulocyte extracts expressing the full-length CBP protein. Proteins were analyzed in a 7.5% SDS gel and were probed with the A22 CBP Ab. The arrow shows the 270-kDa CBP.

were used in the transfection. Cells were analyzed as for the expression levels of different proteins and the localization of the overexpressed and/or the endogenous proteins in the nuclei.

Reporter Assays. Luciferase and β-galactosidase were assayed as described (36). Extracts were prepared 24–30 h after transfection. Equal quantities of extract protein were assayed in each point. Results are given as a relative activity, based on the positive control activity (arbitrarily set at 1) observed in each described experiment, in the absence of coactivators.

Immunohistochemistry. CV-1 and Hep-2 cells were fixed as described (17). In summary, cells were grown in coverslips fixed either at room temperature in 3.7% formaldehyde and 0.2% Triton X-100 (vol/vol) or at −20°C for 5 min with fresh and cold methanol, acetone, respectively. Antigen localization was determined after incubation of permeabilized cells with rabbit antiserum or mAb diluted in PBS for 1 h at room temperature. Secondary mAbs conjugated to fluorescein or Texas red were applied for 1 h at room temperature in a humidified chamber. For double immunofluorescence, permeabilized cells were incubated with the two Abs under the same conditions. Cells then were stained for DNA with 0.5 µg/ml bis benzimide (Hoechst 33258; Sigma) in PBS and were mounted with gelvatol or Fluoromount G (Fischer Scientific). The two fixation methods gave comparable results. Fluorescence images were analyzed in confocal microscopy. Confocal images represent single optical sections. For the *in vitro* immunodetection assay, crude cell extracts were analyzed in a 12% SDS/PAGE gel, were transferred to a nitrocellulose membrane, and were probed with the corresponding Abs as described (39).

RESULTS

Differential Distribution of Endogenous CBP in the Nucleus. Initial studies with CBP antibodies in CV1 and Hep-2 cells suggested a uniform or diffuse nuclear distribution similar to that seen in Fig. 1*A* using a C-terminal specific CBP Ab (C20) (Fig. 1*A 1*; data not shown). Unexpectedly, two N-terminal Abs, 5729 and A22, revealed a different pattern that is clearly reminiscent of that of the PODs (Fig. 1*A 2-4*; data not shown). C20 Ab recognizes an epitope between amino acids 2,395 and 2,414 whereas the A22 and N-5729 Abs recognize nonoverlapping but adjacent N-terminal epitopes (Fig. 1*B*). The immunoreactivity of A22 peptide Ab, which is able to reveal punctate CBP, was further confirmed in an *in vitro* immunodetection assay (Fig. 1*C*). Although each of these Abs is specific for CBP, as described (ref. 37 and *Materials and Methods*), we speculate that the C20 epitope is masked in certain microenvironments. Indeed, the intensity of the visualization of CBP in speckled-like structures even with the A22 and N-5729 Abs can vary in asynchronous cell populations, suggesting that CBP may exist in several transitional states (Fig. 1*A*, compare *2* and *3*, labeled with CBP N-5729 Ab; data not shown). After overexpression of CBP, an Ab to the Kix domain (Kix 5614 Ab) (38) also detects compartmentalized CBP, and, in addition, a transiently expressed N-terminal (but not a C-terminal) tagged CBP adopts the same pattern (see Fig. 3; data not shown). Taken together, the data suggest that CBP may be found in at least two different physical states: homogeneously diffused and compartmentalized to a speckled-like domain.

CBP Colocalizes with PML in the POD. To investigate whether PODs coincide with the speckled CBP domain, a double immunofluorescence experiment was undertaken in asynchronous CV1 cells by using the mAb 5E10 (PML-specific) and the CBP polyclonal A22 Ab. As shown, the PML localization to the POD structure was virtually identical with the CBP speckled pattern (Fig. 1*A 4-6*). This finding suggests that the nuclear structure identified by CBP Abs A22 and N-5729 corresponds to the PML nuclear compartment (Fig.
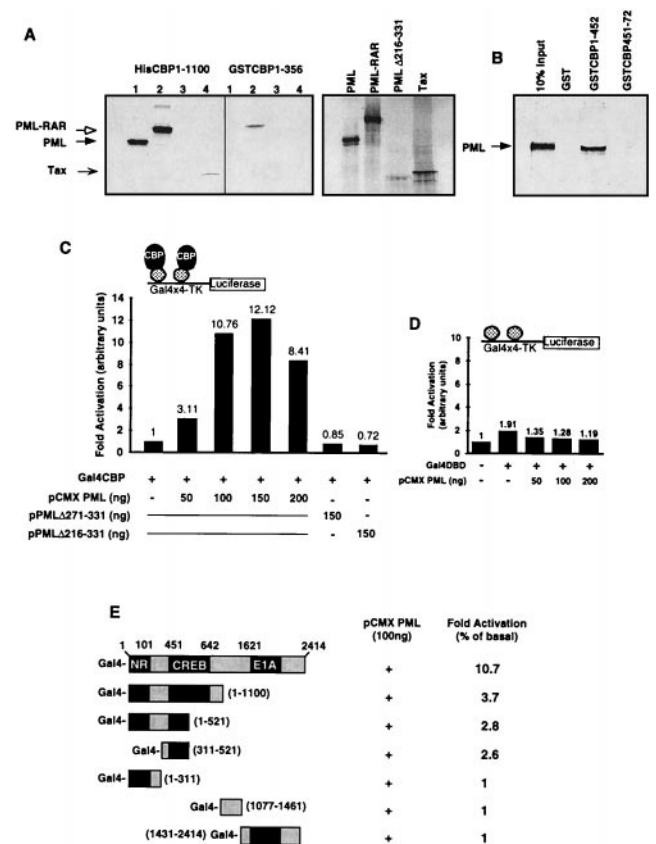

Fig. 2. PML and CBP proteins interact *in vivo* and *in vitro*. (*A*) *In vitro* association of bacterial expressed CBP and S35 radiolabeled PML wild-type and mutant PMLΔ216-331, PML-RAR, and Tax proteins. In the pull-down experiment, the loaded proteins were used at 5:1 ratio compared with the input proteins represented on the right part of the figure. All samples were analyzed in a 4–20% SDS gradient gel, as indicated. (*B*) *In vitro* mapping of the PML-CBP interaction domain. GST fusion proteins containing the indicated residues of CBP were tested for binding to radiolabeled PML. CBP451-722 contains the Tax/CREB interaction domain (i.e., amino acids 451–661). (*C*) PML-CBP interaction in a one-hybrid analysis. CV1 cells (48-well plates) were transiently cotransfected with 100 ng of Gal4-tk-Luc reporter construct, 90 ng of CMXβgal, 60 ng of Gal4CBP, and the expression vectors for PML wild type and mutant, as indicated, per transfection point. All of the transfection points were equalized for the total amount of expression vectors, i.e., CMX empty vector, at a 200-ng final concentration. All points were performed in triplicate and varied by <10%. The presented values correspond to a representative experiment of at least four independent assays. (*D*) Transfections using the Gal4 DNA-binding domain as a control. (*E*) Mapping the PML-CBP interaction domain *in vivo*. CV1 cells were transfected and analyzed as in *C*. Expressions vectors at 100 ng were used as indicated.

1A). In addition, the weaker speckled pattern detected with Ab N-5729 and indicated by the arrows in Fig. 1*A 2* also overlaps in a single exposure and double immunofluorescence in confocal microscopy with the POD (data not shown). The nonuniform appearance of PODs in different cells in Fig. 1*A 4–6* suggests that CBP localization may be sensitive to the cell cycle, which is consistent with previous data that showed an increase in diffuse PML in the nucleus during S phase (2). These results indicate that a portion of CBP is present in nuclear PODs. This result confirms recent results from studies using electron microscopy (40).

PML Associates with the N-Terminal Domain of CBP (Amino Acids 311–521) and Potentiates CBP-Mediated Activation of Transcription. Given that CBP and PML proteins in part colocalize in the nucleus, we investigated whether PML and CBP could interact directly. We initially carried out a

series of pull-down experiments with *in vitro*-translated PML and PML-RARα and bacterially expressed HisTag-CBP(1-1100) or GST CBP(1-356) fusion proteins. As shown (Fig. 2*A*), ≈30% of input wild-type PML or fusion protein PML-RARα was bound to immobilized HisTag-CBP(1-1100) after a 30-min incubation. In contrast, only the PML-RARα protein was pulled down with the GST CBP(1-356) fusion. In addition, a PML mutant protein carrying a deletion of the coiled-coil domain (pPMLΔ216-331) did not bind either to HisTag-CBP(1-1100) or GST CBP(1-356) fusion proteins (Fig. 2*A*, respectively lane 3). Finally, as a control, a known CBP associating protein, *in vitro*-translated Tax, was shown to bind with the CBP(1-1100) but not with the CBP(1-356) fusion (Fig. 2*A*, respectively lane 4), in agreement with previous studies (41). This analysis reveals that the amino acid 216–331 domain of PML is essential for association with the N-terminal domain of CBP.

To delineate further the PML binding domain in CBP, we tested additional GST fusion proteins [CBP(1-452) and CBP(451-722)] (Fig. 2*B*). PML bound specifically to GST-CBP(1-452) and not to GST-CBP(451-722), which contains the CREB interaction domain (that is, amino acids 451–642) (41). Because CBP(1-356) does not bind PML (Fig. 2*A*), we conclude that residues 357–452 are critical for binding.

Given the results of the *in vitro* binding studies, it was important to determine whether PML and CBP functionally interact *in vivo*. We first asked whether cotransfection of a PML expression vector increased transactivation of a Gal4-

CBP fusion protein. Indeed, expression of PML in a dose-dependent manner activated Gal4-CBP transcription, resulting in a 12-fold increase with 150 ng of PML expression vector (Fig. 2*C*). Deletions of the leucine zipper and coiled-coil domains of PML (pPMLΔ271-331, pPMLΔ216-331) not only abolished transactivation of Gal4-CBP but actually showed a slight repressive effect on its basal level of activity (Fig. 2*C*; data not shown). These data indicate that tethering CBP and PML to the DNA results in enhanced transcriptional activation. Because PML does not bind to DNA, the above suggest that PML may be a novel CBP-associated cofactor. It is important to note that, under these experimental conditions, PML does not significantly affect the basal level of activity of the Gal4-tk-Luc reporter gene (Fig. 2*D*). The PML interaction site was localized by using a series of CBP deletion mutants in the Gal-"one-hybrid" detection system. In agreement with the *in vitro* studies, this analysis revealed a minimal domain (amino acids 311–521) that was sufficient for the functional interaction (Fig. 2*E*; data not shown). In contrast, Gal4-CBP (1–311), which previously was shown to interact with nuclear receptors (24, 25), showed no functional interaction with PML (Fig. 2*E*).

Reciprocal Recruitment of CBP and PML to the POD Requires the PML Association Domain. Immunohistochemical analysis was used to investigate further the functional and physical interaction between PML and CBP. We sought to address the relationship between diffuse and compartmentalized CBP by asking whether increased PML expression would alter this distribution. As previously shown, overexpression of
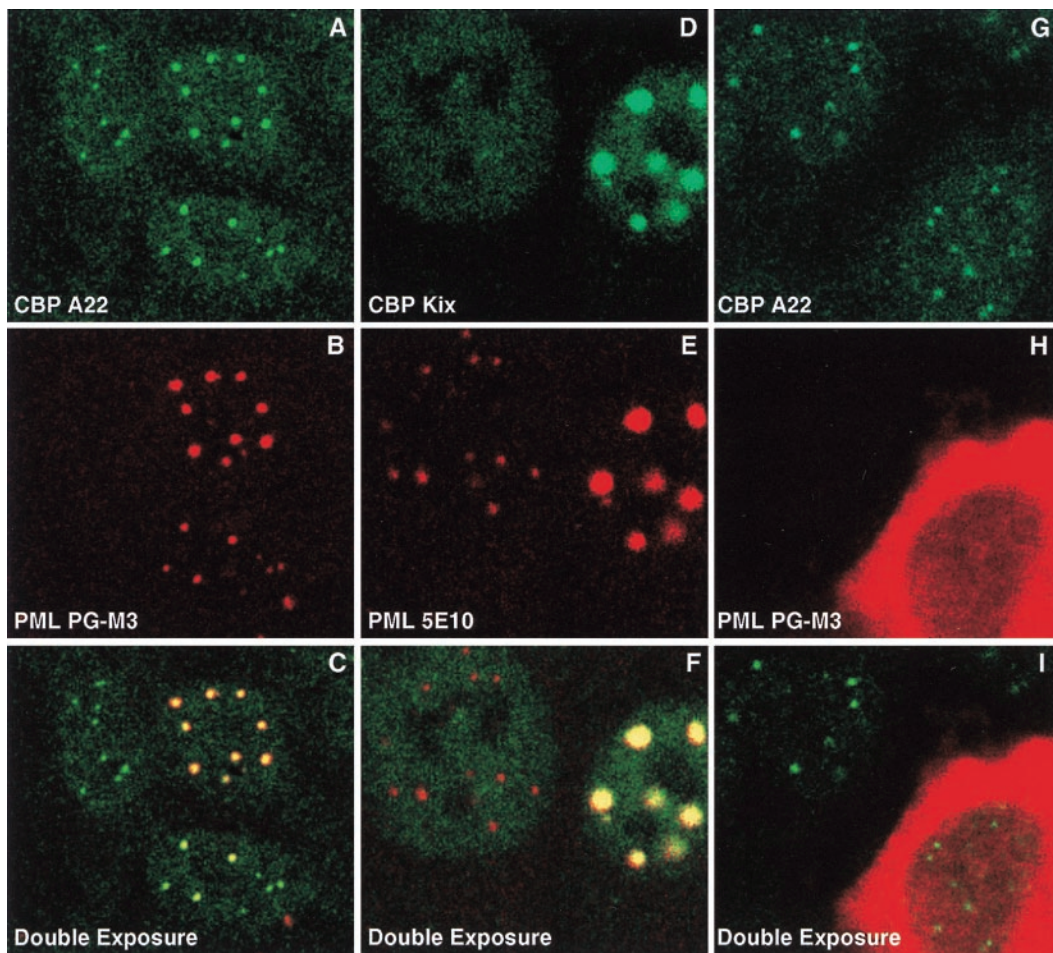

FIG. 3.   PML recruits CBP to PODs. (*A–I*) Double immunofluorescence of CV1 cells (6-well dishes) analyzed in confocal microscopy. Cells were transfected at 70% confluence with 2.5 μg pCMXPML expression vector (*A–C*); 1 μg of pCMXPML and 2.5 μg pCMXCBPm (mouse) (*D–F*); and 2.5 μg pSVPMLΔ216-331 (*G–I*). Primary Abs were used as indicated. Green corresponds to the CBP staining revealed with the FITC-conjugated secondary Ab, red corresponds to the PML staining revealed with the Texas red-conjugated secondary Ab, and the yellow color in the double-exposure image indicates the sites where PML and CBP colocalize.

PML dramatically increases immunofluorescence in the POD (17). To differentiate between endogenous and transiently expressed PML proteins in the immunohistochemistry studies, we used two different PML Abs (Fig. 3). In CV1 cells, the monoclonal PML PG-M3 Ab recognizes only the transiently expressed human PML protein. It was found that transient expression of human PML, with or without a CBP expression vector, increased both PML and CBP immunostaining in the POD by ≈3-fold (Fig. 3 *A–C*). This enhanced localization of CBP was revealed with either A22 or N-5729 Abs (Fig. 3 *A–C*; data not shown). When the Kix 5614 Ab was used, colocalization was clearly seen only on cotransfection of both PML and CBP expression vectors, but occasionally dramatic co-staining was seen (Fig. 3 *D–F*). In contrast, in untransfected cells, Kix 5614 Ab revealed only diffuse nuclear CBP, which is the typical pattern seen with this Ab (Fig. 3 *D* and *F*, left side of the image), suggesting that not all epitopes in the CBP protein are equally available in the compartmentalized state. Finally, as shown in Fig. 3 *G–I*, the coiled-coil domain of PML is required for the recruitment of CBP in the PODs because pPMLΔ216-331 does not affect CBP redistribution in the PML nuclear structures. This is consistent with the results of Fig. 2*C*. The above results lead to the conclusion that the balance between nuclear diffuse and punctuate CBP can be altered by increasing PML levels and suggest that the recruitment of CBP to the PML compartment is attributable to a direct interaction between these two proteins, which requires the PML coiled coil domain.

PML Functions as a Potent Transcriptional Activator of NRs. It has been shown that CBP directly interacts with NRs such as RXRα and GR to activate hormone-dependent transcription (24–26). Although PML does not directly associate with NRs, its interaction with CBP suggests that it may function as a CBP cofactor. When tested in a cotransfection experiment with the GR, PML was found to be a potent activator, although this effect is most dramatic at suboptimal hormonal stimulation (Fig. 4*A*). At the lowest doses (10^{-8} M dexamethasone), PML enhanced GR activity by >10-fold. As shown in Fig. 4*B*, in CV1 cells, PML also potentiated transcription of an apolipoprotein AI synthetic promoter by both the endogenous and cotransfected RXR proteins, activating RXR transcription by >200-fold (Fig. 4*B*, lanes 2–4). This dramatic effect of PML on RXR transactivation is all the more impressive when considering that these same cells typically require cotransfected RXR to obtain any response of the apolipoprotein AI synthetic promoter. Indeed, this stands as the single most potent effect of any identified cofactor on RXR function. The effect of PML on NR function was analyzed further using a modified one-hybrid assay based on transfection of a Gal-4 DNA binding domain/RXRα fusion (Gal-4 RXRαF). Although this construct possesses a relatively high basal activity, the activation (2- to 3-fold) again was stimulated by cotransfection of PML (Fig. 4*C*, lanes 1–4). Although the overall potentiation of the 9-*cis* effect is ≈10-fold, basal levels also are increased, and, thus, the inducibility ratio is not substantially affected. We presume that the effect on basal activity is attributable to the propensity of Gal-4 RXRα to activate even in the absence of 9-*cis* addition. Finally, as expected, a PML protein carrying a deleted coiled-coil domain, pPMLΔ216-331, did not affect transcriptional activation by the Gal-4 RXR fusion (Fig. 4*C*, lane 5). These results indicate that PML can function as a novel and unusually potent NR cofactor, and we propose that the recruitment of CBP to the PODs by PML may represent a critical regulatory step in transcriptional activation.

DISCUSSION

The data presented above provide evidence that endogenous CBP can be compartmentalized and support a model in which the nuclear bodies may contribute to transcriptional regulation. The direct association of CBP and PML, both *in vitro* and
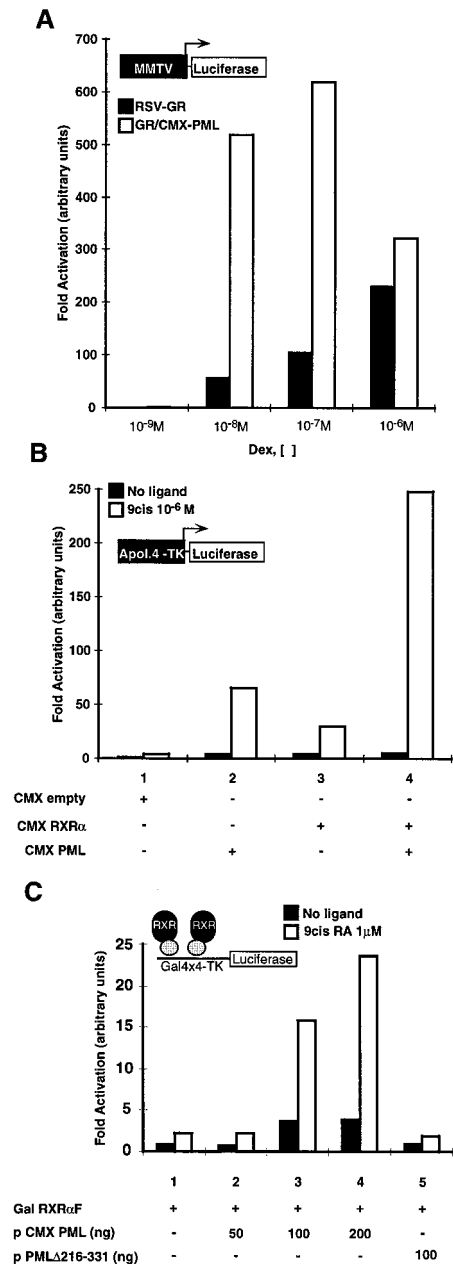

FIG. 4. PML activates NR transcription. (*A*) PML potentiates GR-mediated transactivation of the mouse mammary tumor virus (MMTV) promoter. CV1 cells (48-well plates) were transiently transfected with 120 ng of reporter construct and 90 ng of CMXβgal. Transient transfection of GR at 60 ng induces MMTV-Luc transcription from 30- to 200-fold in a dexamethasone concentration-dependent manner. Coexpression of PML at 120 ng synergizes with GR and activates the MMTV transcription (600-fold) at 10 nM of ligand. The synergism of PML and GR is more dramatic in conditions at which lower concentrations of ligand and GR protein are tested. The open columns represent the points of PML and GR cotransfection, and the black ones are single transfected with GR expression vector, as indicated. (*B*) PML potentiates RXR-mediated transactivation of the Apol4-tk-Luc synthetic promoter. CV1 cells (48-well plates) were transiently transfected with 120 ng of reporter construct, 90 ng of CMXβgal, 120 ng of CMX-RXRα and CMXPML, as indicated. All of the transfection points were equalized with CMX empty vector at 240 ng. Transfected cells were treated with 9-*cis* retinoic acid at 1 μM for 8 h before the assay. (*C*) PML potentiates Gal-4 RXRαF-dependent transcription. Cells were transfected as before. Gal-4 RXRαF was transfected at 120 ng, and pCMXPML and pSVPMLΔ216-331 were used as indicated. Transfected cells were treated with 9-*cis* retinoic acid at 1 μM for 8 h before the assay. All points were performed in triplicate and varied by <10%. The presented values correspond to a representative experiment of at least four independent assays.

in vivo, suggests that PML may function as a novel CBP cofactor. PML is a remarkably effective coactivator, particularly in the case of the RXR homodimer. These findings are supported by recent data from a PML knockout study. Deletion of the mouse PML gene resulted in a reduction in RA-mediated myeloid differentiation and an apparent reduction in the RA-mediated transcriptional activation (13). The precise mechanism whereby PML modulates CBP function is as yet unknown, but the strong association of PML with nuclear bodies may lead to a new consideration as to the potential contribution of these structures to transcriptional control. The interaction of PML and CBP suggests that other PML-associated proteins also might be attracted to a larger coactivation complex and could directly participate in transcriptional regulation. Indeed, it already has been shown that PML and its associated factor SP100 are both covalently modified by a ubiquitin-like POD protein termed SUMO-1/Sentrin (refs. 42–44; D. Chen and R.M.E., unpublished work). The PML SUMO-1 modification sites have been shown to be required for targeting to the PODs and are thus, presumably, important for PML function (45). These results raise the intriguing possibility that CBP or other coactivator-associated proteins may be modified similarly.

Even though further analysis will be necessary to elucidate the role of the POD in transcriptional control, our data suggest that the differential recruitment of CBP by PML may represent a novel mechanism whereby the function of nuclear transcription cofactors can be modulated. When considered in the light of other recent studies on the function of the PML-RARα fusion protein in transcription (46, 47) these results suggest that the PML-RARα translocation may contribute to oncogenesis in multiple ways. Not only does the translocation event disrupt RARα function by increasing its association with the transcriptional corepressor SMRT, but, from the data shown above, we can speculate that the ability of PML to modulate CBP function and thus NR function also might be disrupted.

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