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The PML–retinoic acid receptor α translocation converts the receptor from an inhibitor to a retinoic acid-dependent activator of transcription factor AP-1

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ABSTRACT **We report here that the fusion of PML, a nuclear protein defined by the t(15;17) chromosomal translocation in acute promyelocytic leukemia, with retinoic acid receptor α (RARα) changes the RARα from a retinoic acid (RA)-dependent inhibitor to a RA-dependent activator of AP-1 transcriptional activity. The PML–RARα chimera cooperates with c-Jun and, strikingly, with c-Fos to stimulate the transcription of both synthetic and natural reporter genes containing an AP-1 site. Stimulation is dependent on the concentration of RA and its dose–response curve is comparable to that for activation by RARα of transcription on RA-responsive genes. Further, in the absence of RA, a circumstance in which RARα has no effect on AP-1 activity, PML–RARα is an inhibitor. Deletion of the dimerization, transactivation, or DNA-binding domains of c-Jun and removal of the PML dimerization domain in the PML–RARα hybrid abrogates their transcriptional cooperativity. In view of the association between AP-1 activity and hemopoietic differentiation, we suggest that these properties of PML–RARα could contribute to the leukemic phenotype and its response to RA.**

The t(15;17)(q22;q12–21) translocation is exclusively associated with acute promyelocytic leukemia (APL) and is often the only visible karyotypic aberration present (1). This translocation is detected in as many as 90% of APL patients and has become the definitive marker of the disease (2). It fuses retinoic acid receptor α (RARα) and a nuclear protein, PML (3–8). Administration of high doses of retinoic acid (RA) to APL patients overcomes, for a time, the oncogenic defect and restores myelocytic differentiation (9–11). We wish to know how the PML–RARα protein may contribute to the leukemic phenotype and to the ability of RA to provoke differentiation of the cells and clinical remission.

The pleiotropic effects of RA are thought to be mediated through the ligand-dependent activation of transcription factors that are members of the steroid/thyroid superfamily (12–20), and RARα is one such factor. PML, the partner of RARα in the chimera, is part of a newly recognized family of zinc-finger proteins including transcription factors, DNA modifiers, and oncoproteins (3–7, 21). These gene products are thought to bind DNA, suggesting an analogous function for PML. Computer analysis of the PML sequence predicts, in the center of the protein, an α-helical region defining a leucine zipper similar to the c-Fos dimerization domain.

When compared with the wild-type receptor, the PML–RARα hybrid exhibits altered transactivating properties, although the type of alteration (repression or superactivation) depends upon both the nature of the RA target gene and the cell type (3, 4, 7). A second property of activated RARs is their ability to inhibit AP-1 transcriptional activity mediated by c-Jun homodimers or c-Jun/c-Fos heterodimers (22–24). Although the mechanism is not clear, direct binding of the RARs to the AP-1 site is excluded and the functional interference may reflect direct protein–protein interactions (23, 24). We report that the PML–RARα chimeric protein has this inhibitory activity in the absence of RA, but in its presence it becomes a potent activator of AP-1 activity.

EXPERIMENTAL PROCEDURES

Cell Culture. MCF-7 (25), NIH 3T3, HeLa, and CV-1 cells were maintained at 37°C, 7% CO_2 in Dulbecco's modified Eagle's medium with 7% fetal bovine serum. At least 4 days before each experiment, the cells were washed with phosphate-buffered saline, and medium lacking phenol red and containing glutamine (0.6 mg/ml) and 7% fetal bovine serum treated with activated charcoal (26) was added.

Transfection and Chloramphenicol Acetyltransferase (CAT) Expression. Cells were grown in the absence of retinoids for at least 4 days, seeded onto 6-cm Petri dishes, and transfected (27) when they reached about 70% confluence. Typically, 1.8 μg of reporter plasmids and 1 μg of Rous sarcoma virus (RSV) promoter-driven β-galactosidase expression plasmid as an internal control for transfection efficiency were used. Sonicated denatured salmon sperm DNA was used to adjust the total transfected DNA to 10 μg. As an internal control for transfected promoters we used the RSV-luciferase expression plasmid (28) at concentrations equal to those used for the other expression plasmids under RSV promoter regulation (c-*jun*, c-*fos*, c-*jun* mutants, etc.) and pSG5 (Stratagene) in place of expression plasmids under simian virus 40 promoter regulation. The cells were exposed to the DNA precipitate for 16–20 hr and washed before incubation for 24 hr in fresh medium with the appropriate additions. Cells were collected for CAT assay, and percent conversion of chloramphenicol was calculated after correction for transfection efficiency by use of the β-galactosidase activity. Experiments were repeated several times. The CAT chromatograms given are from a typical experiment.

Plasmids. The SVPML–RARα "type L" form was described (3). The Δ271–331 and Δ216–331 mutants were produced by deletion, using *Bss*hII at positions 725, 891, and 1071 of the PML–RARα nucleotide sequence. RSVcfos, RSVcjun, and the Δ2–168, Δ284–311, Δ2–220, Δ132–220, and

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Abbreviations: APL, acute promyelocytic leukemia; CAT, chloramphenicol acetyltransferase; RA, retinoic acid; RAR, RA receptor; RSV, Rous sarcoma virus; TRE, tetradecanoylphorbol acetate response element.

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Δ318–333 c-Jun mutants were described (29). The c-Jun mutants Leu-62,72 and Leu-62 have leucines in place of serines whose phosphorylation potentiates c-Jun activation (30). B.D.1 and B.D.2 correspond to mut 14 and mut 12 (31). The collagenase (−517)–CAT construct was described (32). PK3-CAT contains three AP-1 sites upstream of the thymidine kinase minimal promoter linked to the CAT gene (29).

RESULTS

PML–RARα-Mediated Activation of AP-1. To assess the cross talk between steroid or vitamin hormone receptors and AP-1 transcriptional activity, we first transfected the human breast carcinoma cell line (25) with the PK3-CAT reporter plasmid containing three AP-1 binding sites [or tetradecanoylphorbol acetate response elements (TREs)] upstream of the thymidine kinase promoter of herpes simplex virus 1. CAT activity was strongly stimulated by cotransfection of plasmids expressing c-Jun and c-Fos (Fig. 1, lane 7), and more weakly by c-Jun or c-Fos alone (lanes 3 and 5), in agreement with previous studies (29). The stimulation of CAT activity was greatly reduced by activating the endogenous RARs in MCF-7 cells with 0.1 μM RA (Fig. 1, lanes 4, 6, and 8), again consistent with previous findings (23, 24). When a parallel set of dishes were cotransfected with a plasmid expressing the "L" form of PML–RARα (3) alone or with the various c-Jun and c-Fos combinations, we observed a further stimulation of CAT activity that was particularly marked for c-Fos (compare lanes 6 and 14) but also was seen for c-Jun (lanes 4 and 12), and the c-Jun/c-Fos heterodimer (lanes 8 and 16). This interaction was dependent on the presence of RA (Fig. 1, lanes 11–16) and the stimulation significantly exceeded that observed in the absence of RA and PML–RARα (e.g., compare lanes 3 and 12). The activation of the reporter by PML–RARα and RA in the absence of exogenous c-Jun or c-Fos (lane 10) probably reflects the potentiation of endogenous Jun and Fos proteins (see below). Finally, the activity obtained by cotransfection of c-Jun, c-Fos, and PML–RARα vectors in the presence of RA exceeded the level obtained with saturating amounts of c-Jun and c-Fos in the absence of RA (Fig. 1, lanes 7 and 16). This last point shows that the activation we observe is not due to induction of the endogenous Jun or Fos in the transfected cells.

Cooperation between PML–RARα and c-Jun/c-Fos was not observed when the reporter carried two copies of a mutated site (TAACTCA for TGACTCA) which does not bind AP-1, but it was unaffected by varying either the number of sites (between one and three) or their relative orientation (data not shown). A minimal promoter containing one or three AP-1 sites upstream of the TATA box and initiator elements of the adenovirus major late promoter (33) was also responsive, but not if the AP-1 sites were replaced with sites for the factors Sp1 and USF (data not shown).

PML–RARα Stimulates AP-1 in a RA Concentration-Dependent Manner. Transcription of the CAT reporter gene in the presence of PML–RARα and c-Fos was dependent on the concentration of RA in the range 0.1 nM to 0.1 μM, with maximal stimulation (50-fold) at 0.1 μM (Fig. 2). The dose–response curve was comparable to that for activation of transcription by RARα on RA-responsive genes (34, 35). When the quantity of transfected plasmid was varied for PML–RARα, or for c-Jun or c-Fos, a saturable increase in CAT activity was observed, at a level at least 50–100 times greater than that in the absence of RA (data not shown).

PML–RARα Represses AP-1 Activity in the Absence of RA. Fig. 1 indicates a second activity of PML–RARα in relation to AP-1, and this is shown in more detail in Fig. 3. When MCF-7 cells were transfected with PK3-CAT and plasmids expressing c-Jun, c-Fos, or both, the stimulation of CAT activity (Fig. 3, lanes 2, 4, and 6) was significantly decreased

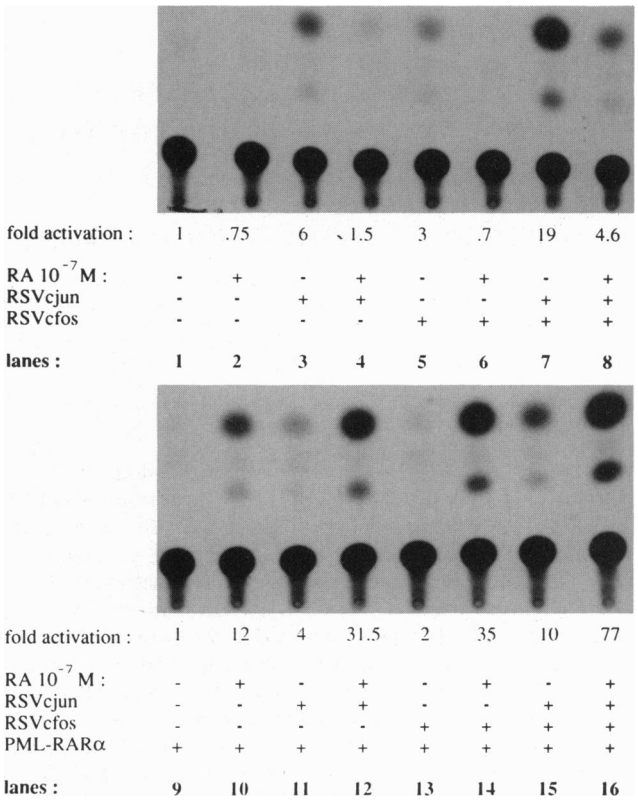


FIG. 1. PML–RARα fusion protein stimulates AP-1 activity in the presence of RA. MCF-7 cells were transfected with PK3-CAT and various expression constructs as indicated and cultured for 36 hr in the absence (−) or presence (+) of 0.1 μM all-*trans*-RA (Sigma) prior to analysis of CAT activity. Lanes: 1 and 2, RSV-luciferase; 3 and 4, RSVcjun; 5 and 6, RSVcfos; 7 and 8, RSVcjun and RSVcfos. For transfection of MCF-7 cells in 6-cm dishes, 1.8 μg of PK3-CAT reporter, 0.8 μg of RSVcjun, 0.2 μg of RSVcfos, and 1.2 μg of pSVPML-RARα were used. Lanes 9–16 correspond to the addition of the PML–RARα expression plasmid to the transfection mixtures of lanes 1–8. CAT activity was measured as the percent conversion of substrate (lane 1, 0.24; lane 9, 0.13 arbitrary unit). Fold activation relative to the basal promoter level is indicated.

by cotransfection of the PML–RARα plasmid in the absence of RA (lanes 3, 5, and 7). This is unlikely to reflect competition by the different promoters for limiting cellular factor(s), since the transfection mixtures were routinely balanced for promoter content, and the repression was dependent on the presence of the AP-1 site (data not shown). Note that addition of RA to the cells again produced a marked stimulation of AP-1 activity in cells transfected with c-Fos and PML–RARα (Fig. 3, lane 8). In summary, PML–RARα is a repressor of AP-1 transcriptional activity in the absence of RA and is a strong activator in its presence.

Functional c-Jun and the Potential α-Helical Region of PML–RARα Are Necessary for Their Cooperativity. MCF-7 cells were cotransfected with the PK3 reporter, the PML–RARα plasmid, and a plasmid expressing c-Jun or various c-Jun mutants (29–31). This analysis (Fig. 4*A*) indicated that deletion of the dimerization domain (Δ284–311) or the transactivation domain (Δ2–168, Δ2–220) or mutation of the basic domain involved in DNA binding (B.D.1, B.D.2) resulted in loss of the transcriptional cooperativity with PML–RARα. The transactivation function of c-Jun is known to require phosphorylation of Ser-62 and -72 (30), and mutation of these residues also led to loss of the functional interaction. On the other hand, deletion of residues 132–220, a region required for the functional interaction between c-Jun and the estrogen receptor (29), did not abolish totally the functional interaction

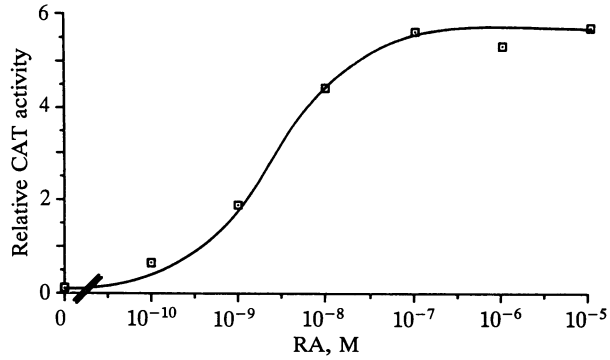


FIG. 2. Activation of AP-1 activity by PML–RARα is RA-dose dependent. MCF-7 cells were transfected with 1.8 μg of PK3-CAT reporter and 1.4 and 0.2 μg of PML–RARα and c-Fos expression vectors, respectively. The cells were cultured in the absence or presence of various concentrations of RA. In the absence of RA, PK3-CAT had a relative CAT activity of 0.11 arbitrary unit. Fold activation of PK3-CAT, based on its relative activity in the absence of RA, was 6, 17, 40, 50, 48, and 51 times for RA concentrations from 10^{-10} to 10^{-5} M. Values are the mean of two experiments.

with PML–RARα, whereas deletion of residues 318–333 slightly increased the stimulation, possibly due to the overexpression of this c-Jun protein (29). Mutant Δ2–220, which lacks all transactivating sequences, and B.D.1, which fails to bind DNA (31), decreased the CAT activity obtained with PML–RARα and RA alone. They may do so by forming inactive dimers with endogenous Fos and Jun proteins, since their suppressive effect was relieved by overexpression of c-Fos (data not shown). We checked in these experiments that the point mutations introduced in the phosphorylation sites or in the DNA-binding domain did not affect the amount of c-Jun synthesized in the transfected cells, as revealed by Western blots, or their nuclear localization (results not shown). Previous experiments showed that deletions in the transactivation domain (e.g., Δ2–168; Δ132–220) did not modify the nuclear localization of these truncated Jun proteins or strongly decrease their yield as judged by immunofluorescence intensity (29). However, deletion of the leucine repeat (Δ284–311) did modify the nuclear localization of the resulting protein. For this reason we cannot exclude formally that the absence of functional cooperation between PML–RARα and this last mutant is caused by distinct nuclear compartimentalization. This initial analysis of c-Jun mutants indicates that a functional c-Jun protein with DNA-binding, dimerization, and transcriptional activation domains is required for the cooperation with PML–RARα.

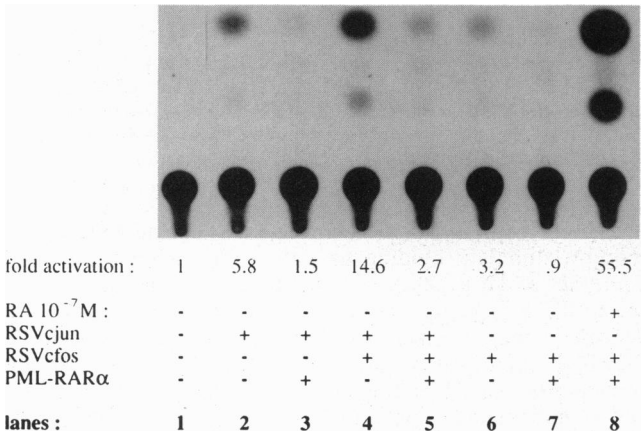


FIG. 3. PML–RARα inhibits the AP-1 activity of c-Jun and c-Jun/c-Fos proteins in the absence of RA. MCF-7 cells were transfected with PK3-CAT and various expression vectors as indicated and then were cultured in the absence (−) or presence (+) of 0.1 μM RA. CAT activity was measured as in Fig. 1 (lane 1, 0.11 arbitrary unit). Fold stimulation relative to the control (lane 1) is given. Lanes: 2, RSVcjun; 3, PML–RARα and RSVcjun; 4, RSVcjun and RSVcfos; 5, PML–RARα, RSVcjun and RSVcfos; 6, RSVcfos; 7, PML–RARα and RSVcfos; 8, PML–RARα and RSVcfos. Quantities of the various plasmids were the same as in Fig. 1.

As neither PML nor exogenous RARα alone altered the modulation of AP-1 activity by the endogenous RAR(s) (results not shown), it became evident that the fusion of PML to RARα generates a novel biochemical entity. In view of the potential relevance of the α-helical region in the PML for the interaction, we investigated the effect of a deletion of 60 amino acids with homology to c-Fos (Δ271–331) and a larger deletion (amino acids 216–331) removing this region entirely but maintaining the integrity of the third cysteine-rich cluster (3, 4, 7). Both these mutants of PML–RARα lost the ability to cooperate with c-Jun (Fig. 4*B*) or c-Fos (data not shown). Since these deletion mutants still code for the same N-terminally truncated RARα as the initial PML–RARα fusion protein, it is highly improbable that the activity change seen between the endogenous RAR and the fusion protein was caused by the truncation of RARα.

Collagenase Promoter Responds to PML–RARα in a Hormone-Dependent Manner. The human collagenase gene is strongly activated at the transcriptional level by tumor promoters such as phorbol 12-myristate 13-acetate ("12-*O*-tetradecanoylphorbol 13-acetate," TPA) (32). The first TPA response element (TRE) was identified in the promoter of this gene and was shown to be the target for transcriptional activation by c-Jun and c-Fos. As an example of a natural promoter that responds to AP-1, its interaction with PML–RARα and RA was of interest. The promoter responded to overexpression of c-Jun and c-Fos (Fig. 5, lanes 1 and 2) and this activity was repressed by 0.1 μM RA (lane 3) (23, 24). Cotransfection of the collagenase promoter with the PML–RARα expression vector showed a weak stimulation of transcription in the presence of RA. In parallel, cotransfection of the chimera in the presence of c-Jun and c-Fos expression vectors repressed the collagenase activity in the absence of RA (compare lanes 2 and 6), while RA treatment converted the chimera into a strong transactivator of AP-1 activity (compare lanes 6 and 7). In this case, the activity was even higher than that observed with saturating amounts of c-*jun* and c-*fos* in the absence of RA (compare lanes 2 and 7). Thus the cooperative interaction between PML–RARα and AP-1 obtained with synthetic promoters can also be observed with a natural AP-1-responsive gene.

When we tested PML–RARα in RAR-negative CV-1 monkey cells, human HeLa cells, and mouse NIH 3T3 cells it behaved as an activator of AP-1 in the presence of RA and a repressor in its absence (results not shown). However, the degree of stimulation or repression varied between the different lines. This may reflect variations in the concentrations of PML or other proteins that can interact with the transfected chimera.

DISCUSSION

RARs are potent inhibitors of AP-1 (Jun/Fos dimer) function in the presence of hormone. We show here that fusion of the PML moiety to the N-terminally truncated RARα radically alters this interaction. The fusion protein becomes a RA-dependent activator of AP-1 activity. It cooperates with the endogenous or cotransfected c-Jun and/or c-Fos in activating reporter plasmids including the human collagenase promoter or chimeric constructions containing one or several AP-1 binding sites. In contrast to its activating properties in the presence of hormone, PML–RARα is an inhibitor of AP-1 activity in the absence of RA.

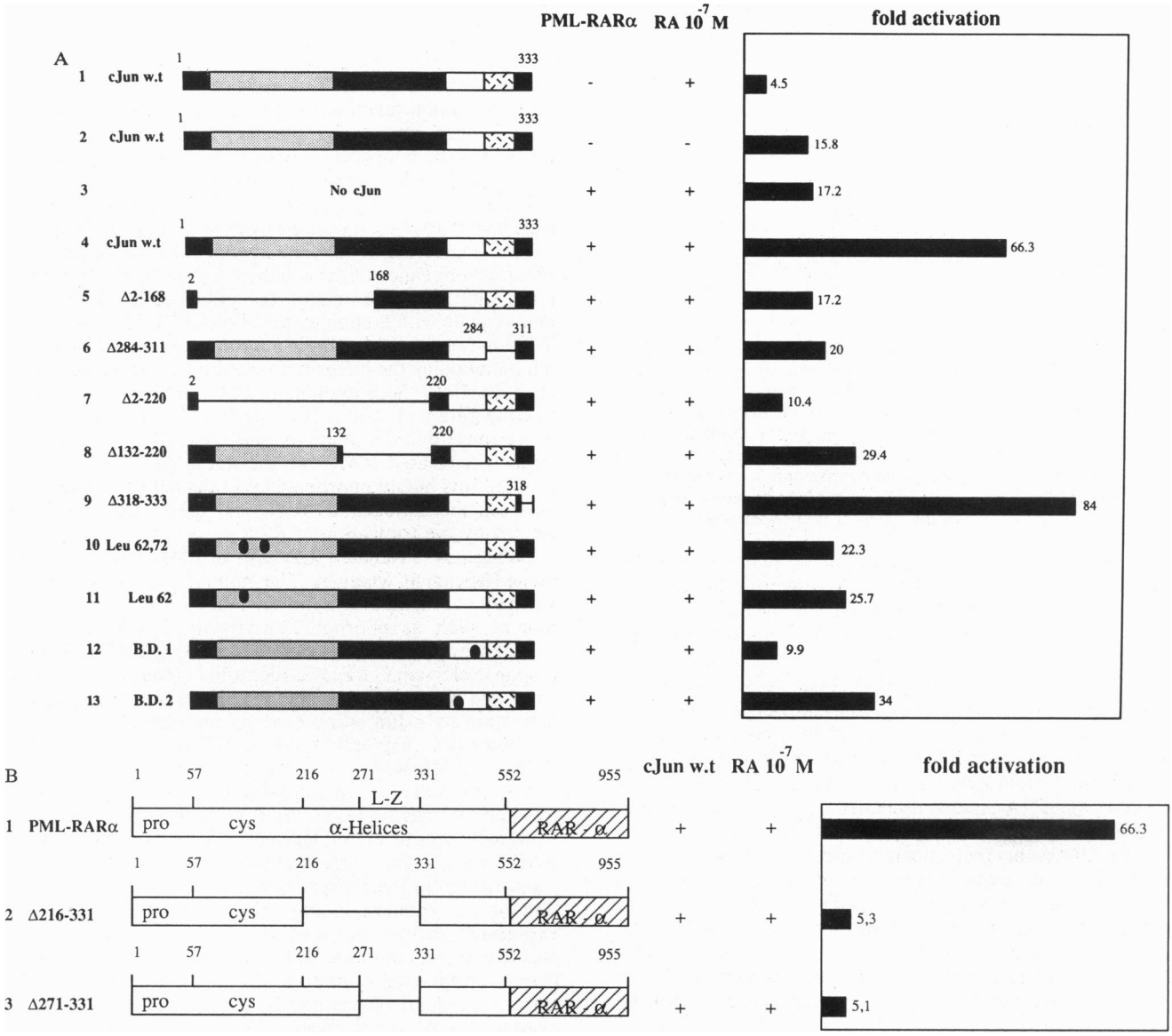
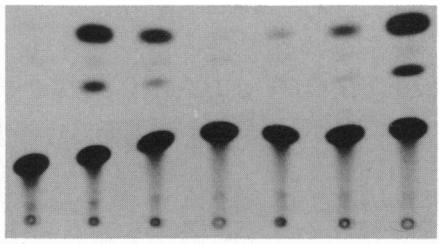


FIG. 4. Analysis of various c-Jun and PML–RARα mutants for their ability to cooperate in the presence of RA. (*A*) MCF-7 cells were transfected with PK3-CAT and various expression vectors and were cultured in the absence (−) or presence (+) of 0.1 μM RA. Structures of the mutants are at left. Note that mutant B.D.1 has a more drastic effect on DNA binding than mutant B.D.2 (31). Fold activation for each experimental point is indicated. Quantities of the various plasmids were as in Fig. 1. (*B*) MCF-7 cells were transfected as in *A*. Value given for bar 1 corresponds to the activation of PK3-CAT by PML–RARα and c-Jun (bar 4 of *A*). Amount of wild-type or mutant PML–RARα expression plasmid used was 1.2 μg. Structure of PML–RARα wild-type and mutant genes is shown at left. Length of the RARα part is not to scale. Values in *A* and *B* are the mean of at least two transfections.

The inhibition of AP-1 function in the absence of RA may reflect the effect of the PML partner in converting the ligand-dependent suppressive activity of RARα to one that is independent of ligand. This activity is believed to reflect a functional interaction with AP-1 components, but its exact nature is unclear. This inhibitory effect may be also related to the suppressive phenotype of the PML–RARα hybrid in the absence of RA observed by Kastner *et al.* (7). The RA-dependent stimulation of AP-1 function poses a particularly intriguing problem. Our mutational analysis of c-Jun, while not restrictive for mechanism, underlines the necessity for functional transactivation, dimerization, and DNA-binding domains. The mutational analysis of PML–RARα was limited to the region that was shown to contain a potential α-helical domain with homology to the leucine repeat domain of c-Fos (4, 7). Deletion of this region eliminated the functional interaction between the chimera and AP-1. It is possible that PML–RARα could form heterodimers with Jun or Fos through its leucine repeat and thereby stimulate binding to AP-1 sites. Alternatively, it could act as an accessory factor to assist dimerization between these proteins by stabilizing or activating them (36). We are inclined to reject these hypotheses at present because they all predict an increase in AP-1 DNA-binding activity of Jun and Fos proteins in the presence of PML–RARα and RA, yet we have failed to detect such an increase after mobility-shift assays with *in vitro* translated proteins or with nuclear extracts prepared from MCF-7 cells after cotransfection of the appropriate expression vectors (data not shown). This is reminiscent of other cases of transcriptional cooperation between Jun/Fos and steroid hormone receptors or other nuclear proteins where there was no evidence for an increase in DNA-binding activity (37–39).

Another example for modification of AP-1 activity in the absence of a direct effect on DNA binding concerns the cross talk between this factor and the glucocorticoid receptor.



fold activation :	1	40.9	16.7	1	3.1	10.8	107
RA 10^{-7}M :	-	-	+	-	+	-	+
RSVcjun	-	+	+	-	-	+	+
RSVcfos	-	+	+	-	-	+	+
PML-RARα	-	-	-	+	+	+	+
lanes :	**1**	**2**	**3**	**4**	**5**	**6**	**7**

FIG. 5. PML–RARα is an activator of the human collagenase promoter in the presence of RA and a repressor in its absence. MCF-7 cells were transfected with 1.8 μg of the collagenase (−517)–CAT reporter and various expression vectors as in Fig. 1. CAT activity was measured as percent conversion of substrate (lane 1, 0.11 arbitrary unit). Fold activation relative to the basal promoter is given.

König *et al.* (40) showed that the phorbol ester-induced binding of AP-1 to the collagenase TRE *in vivo* was not inhibited by glucocorticoids even though collagenase transcription was strongly reduced. We therefore favor models based on regulation of events occurring after DNA binding. The RA-activated PML–RARα could stabilize or participate in the complex formed between AP-1 proteins and potential coactivator(s) required for its interaction with the basal transcription machinery (41). Alternatively, it could relieve an inhibition at this level by resident vitamin or hormone receptors or other nuclear proteins, by interacting with them directly or with their required dimerization partners, such as members of the retinoid X receptor family (14–20).

Despite the uncertainties about mechanisms, the dual effects have clear implications for the function of PML–RARα. Since high AP-1 activity is associated with differentiation of leukemic cells in several contexts (42–44), the suppressive phenotype could be related to maintenance of the leukemic phenotype, whereas the stimulatory effect in the presence of RA could be relevant to its reversal by provoking differentiation. Previous discussions have focused on a potential interference of PML–RARα with RAR or PML function in APL leukemogenesis (3–7), but this study suggests that interaction with AP-1 is a strong candidate.

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