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PML, a Growth Suppressor Disrupted in Acute Promyelocytic Leukemia

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The nonrandom chromosomal translocation t(15;17)(q22;q21) in acute promyelocytic leukemia (APL) juxtaposes the genes for retinoic acid receptor α (RARα) and the putative zinc finger transcription factor PML. The breakpoint site encodes fusion protein PML-RARα, which is able to form a heterodimer with PML. It was hypothesized that PML-RARα is a dominant negative inhibitor of PML. Inactivation of PML function in APL may play a critical role in APL pathogenesis. Our results demonstrated that PML, but not PML-RARα, is a growth suppressor. This is supported by the following findings: (i) PML suppressed anchorage-independent growth of APL-derived NB4 cells on soft agar and tumorigenicity in nude mice, (ii) PML suppressed the oncogenic transformation of rat embryo fibroblasts by cooperative oncogenes, and (iii) PML suppressed transformation of NIH 3T3 cells by the activated *neu* oncogene. Cotransfection of *PML* with *PML-RARα* resulted in a significant reduction in PML's transformation suppressor function in vivo, indicating that the fusion protein can be a dominant negative inhibitor of PML function in APL cells. This observation was further supported by the finding that cotransfection of *PML* and *PML-RARα* resulted in altered normal cellular localization of PML. Our results also demonstrated that PML, but not PML-RARα, is a promoter-specific transcription suppressor. Therefore, we hypothesized that disruption of the *PML* gene, a growth or transformation suppressor, by the t(15;17) translocation in APL is one of the critical events in leukemogenesis.

Acute promyelocytic leukemia (APL) represents a clonal proliferation and expansion of the hematologic precursors at the promyelocyte stage of myeloid differentiation. A nonrandom chromosomal translocation, t(15;17), can be found in over 95% of patients with APL (3, 42), suggesting that this translocation plays a critical role in leukemogenesis. In recent attempts to explore this role further, genes involved in this translocation have been cloned and characterized (4, 7, 17, 37, 48). The t(15;17) breakpoint occurs within the second intron of the retinoic acid (RA) receptor α (RARα) gene and within two major sites of the PML (or MYL) gene (1, 8, 10, 28, 47). The chimeric *PML-RARα* and *RARα-PML* genes transcribe fusion transcripts and presumably encode fusion proteins. The PML-RARα fusion protein consists of the essential functional domains of PML and RARα and is able to act as an RA-dependent transcription factor (18, 37, 38, 48).

Because patients with APL treated with all-*trans* RA rapidly achieve remission, it has been suggested that disruption of the *RARα* gene may play a role in APL pathogenesis. However, it was shown by Robertson et al. (55) that RA inducibility of HL-60 cells can be substituted for by any other forms of RAR or retinoic X receptor (RXR), indicating that disruption of the *RARα* gene by the t(15;17) translocation may not be important for APL pathogenesis. Furthermore, it was recently reported that the fusion protein PML-RARα is able to form a heterodimer with PML but not RARα (38), thus supporting the hypothesis that PML-RARα may be a dominant negative inhibitor of PML (37).

The importance of the PML-RARα fusion protein in the pathogenesis of APL was recently demonstrated by Perez et al. (49) and Grignani et al. (32), who showed that PML-RARα can efficiently form heterodimers with RXR and so result in sequestration of its normal function. It was also shown that PML-RARα can inhibit binding of the vitamin D3 receptor (VDR) to its target sequence and prevent vitamin D3-dependent activation of VDR-responsive genes. Furthermore, it was shown that transfection of *PML-RARα* into U937 human myeloid leukemia cells inhibits its ability to respond to vitamin D3 and transforming growth factor β1 and so induce differentiation. These results strongly suggest that the fusion protein encoded from the breakpoint of the t(15;17) translocation plays a critical role in APL pathogenesis. Recently, Doucas et al. (19) reported that PML-RARα, instead of being an inhibitor, is an RA-dependent activator of the transcription factor AP-1. However, since, as Kastner et al. (38) report, most of the PML-RARα fusion protein remains in the cytoplasm, it is not known how significantly this change actually affects transcription regulation of AP-1 in vivo.

In any case, the biological function and etiologic implications for the *PML* gene are not known. The structural features of the predicted PML protein suggest that it may function as a transcription factor (18, 37). Furthermore, it would appear that this protein belongs to a new family of zinc finger DNA-binding transcription factors (2, 25), which includes the recombinase-activating *RAG-1* gene and the major histocompatibility complex *RING-1* gene. Immunofluorescence staining of the PML protein has indicated a nuclear speckled pattern similar to that of the splicing factor small nuclear ribonucleoprotein or SC-35 (15, 38). PML-RARα is found mainly in the cytoplasm or in the nucleus in a micropunctate pattern. In our cotransfection experiments and in APL-derived NB4 cells in which both PML and PML-RARα proteins are expressed, the observed PML-RARα staining pattern suggested the dominance

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of PML-RARα over normal PML. These results, in turn, strongly suggest that PML-RARα can be a dominant negative inhibitor of normal PML function. Therefore, elucidating the functional role of PML is critical for understanding APL pathogenesis.

In the study reported here, we demonstrated that PML suppresses anchorage-independent growth in soft agar and tumorigenicity of APL-derived NB4 cells in nude mice. PML also suppresses transformation of rat embryo fibroblasts (REF) and NIH 3T3 cells by oncogenes. These results strongly suggest that PML is a growth suppressor. Furthermore, cotransfection of *PML* with *PML-RARα* inhibited the transformation suppressor function in vivo, supporting the hypothesis that PML-RARα is a dominant negative inhibitor of PML. Together, the results of our studies indicate that disruption of PML by t(15;17) translocation in APL is critical for the development of APL. On the basis of these results and those of others, we present a model for APL pathogenesis.

MATERIALS AND METHODS

Plasmid construction. The full-length *PML* cDNA clone was obtained by screening a cDNA library constructed from poly(A) RNA of a patient with acute myeloid leukemia with the *PML* cDNA probe as described in our previous article (7). The DNA sequence of the entire coding region of the cDNA was determined and found to be identical to the reported *PML* sequence, except for the 3′ portion of the cDNA. This *PML* cDNA consists of the 144-bp exon 4 as a result of alternative splicing (23); the 3′ portion of this cDNA consists of the nucleotide sequence GAGGCAGAGGAACGCGTTGTGGT GATCAGCAGCTCGGAAGACTCAGATGCCGAAAACT CGCCCTCAGTCTGAGGTTCTGTATTGGAAAGTGCAT GGAGCCCATGGAGACCGCCGAGCCACAGTCCTCCC CAGCCCACTCCTCGCCAGCCCACTCCTCGCCAGCCC ACTCCTCGCCAGTCCAGTCTCTGCTGAGAGCACAA GGAGCCTCCAGCCTGCCCTGTGGCACATACCACCCC CCAGCTTGGCCTCCCCACCAGCCCGCTGAGCAGGC TGCCACCCCCGATGCTGAGCCTCACAGCGAGCCT CCTGATCACCAGGAGCGCCCTGCCGTCCACCGTGG GATCC. The underlined 29-base sequence is a result of alternative splicing, which generates a termination codon shortly after the 5′ spliced site. The 2.03-kb *PML* cDNA insert comprising the translation initiation and the termination codons was excised by *Bam*HI-*Bgl*II digestion and subcloned into the expression plasmid pMAMneo (Clontech, Palo Alto, Calif.) at the *Bam*HI site (pMAMneoPML) and into the pSG5 expression vector at the *Bam*HI site (pSG5PML). The out-of-frame mutant of PML was created by digesting pSG5PML with *Kpn*I and was trimmed with a high concentration of S1 nuclease (2500 U/ml) under high-stringency conditions (50 mM sodium-acetate [pH 4.8], 2 mM $ZnCl_2$, 0.4 M NaCl). A mutant with a 50-bp deletion between nucleotides 745 and 795 (7) was identified by DNA sequencing. A *PML* cDNA variant with a different 3′ end and the expression plasmid *PML-RARα* (MylRAR-A) were obtained from P. Chambon (Strasbourg, France) (38). The full-length cDNA of RARα (pHK1), a gift from R. E. Evans (San Diego, Calif.) (26), was excised by *Eco*RI digestion and subcloned into the pMAMneo vector (pMAMneoRARα).

Gene transfection. The expression plasmid was transfected into NB4 cells by electroporation. In brief, NB4 cells, at a concentration of 5×10^6/0.5 ml in phosphate-buffered saline (PBS) containing 25 μg of the plasmid and 250 μg denatured salmon DNA, were electroporated with a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.). Electroporation was performed twice at a capacitor setting of 500 μF and 200 V with a pulse length of 15 to 20 ms. Transfected cells were transferred to RPMI 1640 containing 20% fetal bovine serum and then cultured in a CO_2 incubator at 37°C for 24 h.

The expression plasmids were transfected into SW13 cells, NIH 3T3 cells, and REF by calcium phosphate coprecipitation as described by Chen and Okayama (9). REF were isolated from 12- to 14-day-old rat embryos. Gene transfer experiments were performed with REF at passages 3 and 4. Total protein was isolated from the transfected cells approximately 48 h posttransfection.

To study the expression of PML-RARα and PML protein in NIH 3T3 cells (see Fig. 2B), high-efficiency transfection of the expression plasmids was achieved by using lipofectamine-mediated gene transfer (GIBCO/BRL, Gaithersburg, Md.), according to the manufacturer's recommended procedure.

For retrovirus-mediated gene transfer, *PML* cDNA was subcloned into plasmid pLXSN (45), and the recombinant retroviral plasmid pLPMLSN was transfected into the ecotropic cell line GPE86 cells (44) by electroporation. G418-resistant transfectants expressing the PML protein were selected, and high-titer recombinant viral stock was prepared and used to infect the amphotropic packaging cell line PA317. Infection of NB4 cells by pLPMLSN was performed by a cocultivation procedure described elsewhere (21, 22).

Clonogenicity and focus-forming assays. NB4 cells transfected with the expression plasmids were cultured at 37°C in a humidified CO_2 incubator for 24 h. Clonogenicity was assayed by plating 4×10^3 viable NB4 cells on each soft-agar (0.367%) plate containing 50% conditioned medium as described elsewhere (11). After being cooled to 4°C, the plates were incubated in a humidified CO_2 incubator at 37°C for 14 days. Five different plates were spread in each electroporation experiment. Macroscopic colonies were visualized by staining with 0.1% *p*-iodonitrotetrazolium violet (Sigma Chemical Co., St. Louis, Mo.) after incubation for 10 to 14 days in a CO_2 incubator at 37°C. Focus-forming assays of REF and NIH 3T3 cells transfected with the expression plasmids were performed as described previously (33, 65). Foci were visualized after 10 to 14 days by staining with 1% crystal violet in 25% methanol. Transfection efficiency was monitored by cotransfection with pHβApro-1-neo and selection for G418 (500 μg/ml)-resistant colonies. In cotransfection experiments with PML, the number of G418-resistant colonies was significantly reduced. This observation is likely the result of the growth suppressor effect of PML.

CAT assays. For each cotransfection experiment, approximately 10^6 SW13 cells were plated and then cotransfected with 10 μg of pSG5PML and with 5 μg of the applicable promoter-chloramphenicol acetyltransferase (CAT) construct (9). CAT activity was determined 40 h after transfection (58) by using whole-cell extracts and then normalized to protein. Extracts were incubated with [^{14}C]chloramphenicol in the presence of acetyl coenzyme A for about 2 h at 37°C. Reaction products were then extracted with xylene and counted.

Immunoprecipitation and Western blotting. Monoclonal antibodies against mutant p53 (Ab-3) and Ha-ras were obtained from Oncogene Science, Inc. (Uniondale, N.Y.). Anti-peptide antibody against PML was raised in rabbits by using a synthetic peptide comprising amino acids 352 to 366 of PML (7). The synthetic peptide was coupled to keyhole limpet hemocyanin according to the method of Reichlin (53) and inoculated subcutaneously into multiple sites on the back of each rabbit. High-titer antibody was collected and used directly for Western blotting (immunoblotting). The antipeptide antibody was affinity purified on a peptide-coupled Affi-Gel 10

column (Bio-Rad) prepared according to the manufacturer's instructions.

Immunoprecipitation was performed with monoclonal antibodies against mutant p53 and Ha-ras. REF transfected with the expression plasmids were cultured in a CO_2 incubator at 37°C for 48 h and metabolically labeled with 100 μCi of [^{35}S]methionine per ml (1,200 Ci/mmol) (NEN, Boston, Mass.) for 3 h in methionine-free medium containing 5% dialyzed fetal calf serum. The protein fraction was then isolated and incubated with protein A-agarose and monoclonal antibodies in Eppendorf tubes. The tubes were rotated overnight at 4°C; then, the immunoprecipitated proteins were collected by centrifugation and resuspended in the electrophoresis sample buffer. The proteins were electrophoresed in a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel, vacuum dried, and finally autoradiographed. Western blotting was performed according to the method of Sambrook et al. (56) using the antipeptide antibody against PML.

In vitro transcription and translation of *PML* cDNA. In vitro transcription of the pSG5PML expression plasmid (1 μg) was performed with T7 RNA polymerase and the RNA transcription kit obtained from Stratagene (La Jolla, Calif.). In vitro translation of PML RNA was performed by [^{35}S]methionine (1,200 Ci/mmol) (NEN) incorporation using the rabbit reticulocyte lysate in vitro translation system obtained from GIBCO/BRL. The in vitro-translated PML protein was electrophoresed in an SDS–10% polyacrylamide gel, dried, and autoradiographed.

RESULTS

PML suppression of clonogenicity of APL-derived NB4 cells. In APL, since chromosomes 15 and 17 are involved in the t(15;17) translocation, expression of the normal *PML* and *RARα* mRNA transcripts is reduced significantly (14, 17). Consequently, to investigate whether reduced expression of *PML* or *RARα* is important for APL pathogenesis, cDNAs of *RARα* (a gift from R. E. Evans) (26) and *PML* were subcloned into the expression vector pMAMneo. The expression plasmids were then transfected into APL-derived NB4 cells (41) by electroporation. Transfectants were selected by plating on soft agar containing 0.4 mg of G418 per ml. Many G418-resistant NB4 clones transfected with pMAMneo and pMAMneoRARα were selected. Southern blot analysis of the genomic DNA isolated from five of the transfectants demonstrated the presence of *RARα* cDNA in each transfectant (data not shown). Morphologically, these cells appear similar to the NB4 cells. However, only a few G418-resistant clones, morphologically similar to NB4, were found among those transfected with pMAMneoPML. Three of those clones were expanded, and the presence of the transfected cDNA was analyzed by PCR amplification of the total DNA isolated from those cells. Single base pair deletions at nucleotides 255 and 386 (38) resulted in a frameshift, found in two clones. A small deletion of about 400 bp close to the 5′ end of the cDNA was found in the other clone. This result suggests that these transfectants lost the normal function of the transfected *PML* cDNA.

We reasoned that the failure to select a functional pMAMneoPML transfectant could be the result of a loss of clonogenicity of the NB4 cells induced by PML. To investigate this possibility, *PML* cDNA without *neo* was subcloned into the expression vector pSG5 (31) and analyzed in a soft-agar clonogenic assay. In this experiment, expression plasmids pSG5PML, pMAMneoPML, and pSG5 (negative control) were transfected into the NB4 cells by electroporation. We found that transfection of pSG5PML and pMAMneoPML into

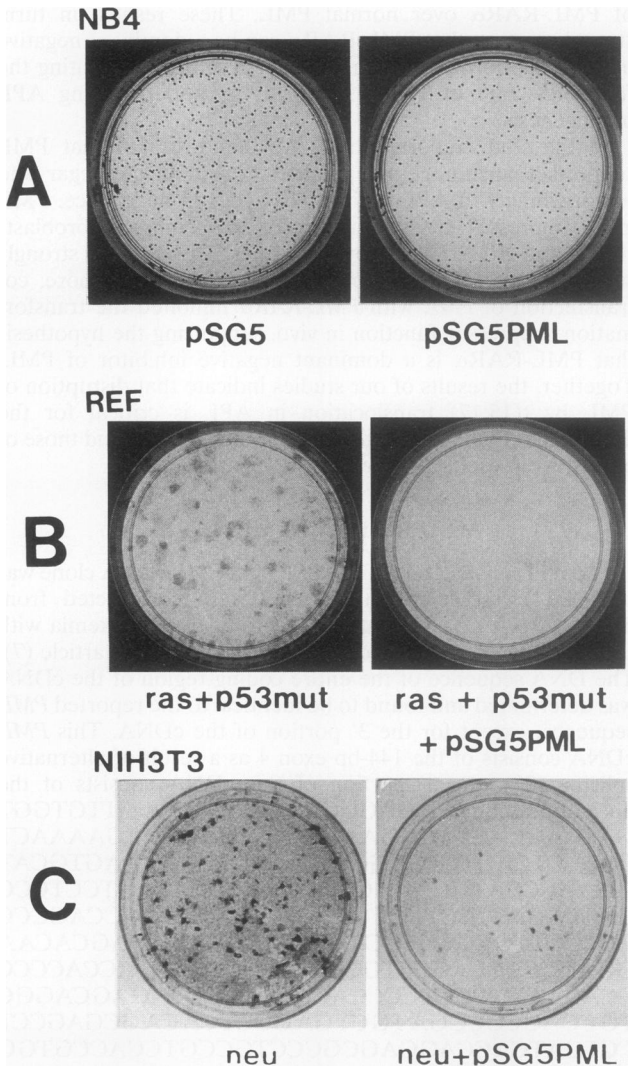

FIG. 1. PML-induced suppression of anchorage-independent growth and focus formation. (A) PML-induced suppression of anchorage-independent growth of APL cells. *PML* expression plasmid pSG5PML (an expression vector, pSG5, containing *PML* cDNA and driven by the SV40 early promoter) and a negative control (pSG5) were transfected into NB4 cells by electroporation and analyzed as described in Materials and Methods. (B) PML-induced suppression of focus formation in Ha-*ras* and mutant *p53* (*p53*mut)-transformed REF. REF were transfected with 10 μg each of plasmids pT24 (activated Ha-*ras*), LTRp53cG (*p53*mut with an Ala→Val substitution at amino acid 135) (22), and pSG5PML. (C) Effect of PML on oncogenic transformation of NIH 3T3 cells transfected with 10 μg each of plasmids c-*Neu*104 (activated *neu*) (56) and pSG5PML by calcium phosphate coprecipitation.

NB4 cells significantly reduced the number of colonies growing on soft agar (Fig. 1A and Fig. 2), indicating that PML can suppress the clonogenicity of NB4 cells. No significant changes were observed when NB4 cells were transfected with pSG5, with a pSG5PML mutant containing a frameshift created by a 50-bp deletion, or with the expression plasmid carrying the mutant PML (PML-RARα) (a gift from P. Chambon). These experiments were performed independently at least three times, and the results were quantitatively consistent (Fig. 2).

Since PML suppresses the clonogenicity of NB4 cells, we

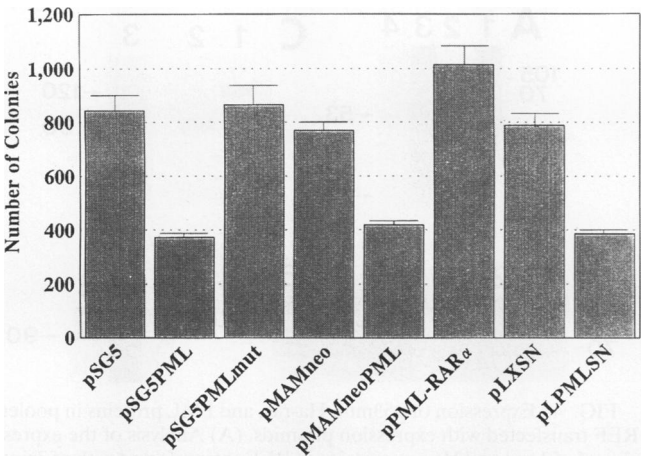

FIG. 2. Effect of PML on clonogenicity of APL-derived NB4 cells. APL-derived NB4 cells were transfected with various expression plasmids by electroporation and retrovirus-mediated gene transfer as described in Materials and Methods. Plasmid pSG5PMLmut carries a 50-bp deletion of *PML* cDNA between nucleotides 750 and 800 (7). Plasmid pPML-RARα carries the full-length cDNA of *PML-RARα* in the pSG5 vector (38). Plasmid pMAMneoPML carries the full-length cDNA of *PML* subcloned into the expression vector pMAMneo. Plasmids pLXSN and pLPMLSN represent the mock control, and the retroviral vector carries the full-length *PML* cDNA. Dexamethasone (1 μM) was included in all soft-agar assays for NB4 cells transfected with pMAMneo-derived plasmids. The data are means with their standard errors for three independent experiments with three plates each.

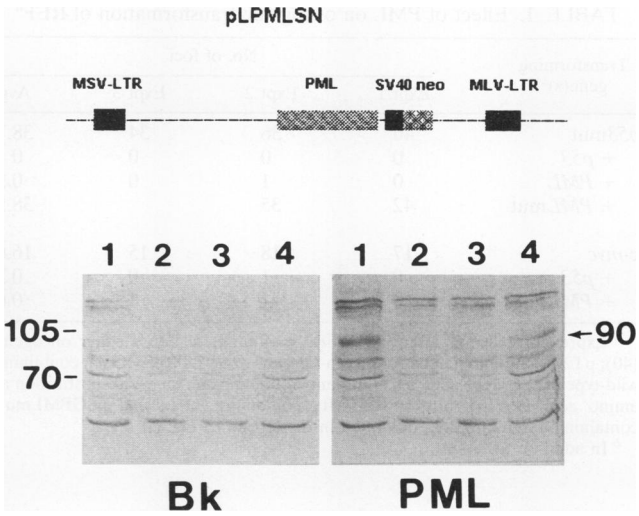

FIG. 3. Construction of retroviral expression plasmid pLPMLSN and expression of the PML protein in the recombinant retrovirus-producing cell line. The structural features of the recombinant retroviral construct pLPMLSN are shown at the top. MSV-LTR and MLV-LTR, murine sarcoma virus and murine leukemia virus long terminal repeats, respectively. The expression of the 90-kDa PML protein in two individual clones is shown below. Lanes 1 and 4, two individual clones in the PA317 host; lanes 2 and 3, protein samples isolated from the pLXSN-infected mock control and the uninfected PA317 host cells. Western blot analyses were performed by using the PML antipeptide antibody and preincubating the same antibody with the specific peptide (Bk). The positions of the 90-kDa PML protein bands are indicated.

have been unable to isolate a stable transfectant expressing the PML protein encoded from the transfected cDNA. Therefore, to increase transfection efficiency of NB4 cells, *PML* cDNA was subcloned into the retroviral vector pLXSN (45) to create the recombinant *PML* cDNA clone pLPMLSN. High-titer recombinant PML retrovirus stock expressing the 90-kDa PML protein (Fig. 3) was prepared by infecting the amphotrophic cell line PA317 and using it to infect NB4 cells. Transfection efficiencies of pLXSN and pLPMLSN retrovirus stocks were determined to be about 38% by colony-forming assay in the presence or absence of 400 μg of G418 per ml and by immunofluorescence staining of the PML protein. We found that NB4 cells transfected with PML recombinant retrovirus (pLPMLSN) could also significantly reduce its clonogenicity in a soft-agar assay (Fig. 2).

Transfection efficiency by electroporation, under our experimental conditions, was about 10%, as determined by transfection with pHβApro-1-neo and selection for G418-resistant colonies. Transfection efficiency by retrovirus-mediated gene transfer was about 38%. Our results (Fig. 2) showed that the efficiency of PML suppression of colony formation in NB4 cells transfected with PML was significantly higher than the transfection efficiency in each experiment. This indicates that expression of PML in NB4 cells may have resulted in the release of a negative growth control factor(s), which in turn, may have suppressed growth of the nontransfected cells in the soft-agar assay. To investigate this possibility, conditioned medium obtained from NB4 cells transfected with pSG5PML and cultured for 24 h was used in a soft-agar clonogenic assay. Significant suppression of clonogenicity (about 40%) of the NB4 cells was found. No suppression was seen when the conditioned medium was preincubated at 65°C for 30 min. In addition, dialysis of the conditioned medium did not remove the suppression effect (unpublished data). These results suggest that a negative growth control factor(s) released from the cells induced by PML may be involved in the suppression of clonogenicity in NB4.

These experiments demonstrated that PML can inhibit the clonogenicity of NB4 cells, resulting in a loss of anchorage-independent growth on soft agar. Conversely, the mutant form of PML in the APL cells (PML-RARα) lost its ability to suppress clonogenicity in NB4 cells. Our results thus suggest that PML is able to function as a growth suppressor.

PML suppression of transformation of early-passage REF by cooperative oncogenes. To verify that PML is a transformation suppressor, we investigated its ability to suppress the transformation of early-passage REF. Similar experiments were performed to establish the tumor suppression function of *p53* (24). The results of these studies are summarized in Table 1. As predicted, transfection of REF with Ha-*ras* plus *p53*mut or Ha-*ras* plus c-*myc* induced focus formation (Table 1). Cotransfection of a wild-type *p53* expression plasmid (LTR p53cGala) with Ha-*ras* plus *p53*mut or with Ha-*ras* plus c-*myc* suppressed focus formation, as previously observed (24). As shown in Fig. 1B and Table 1, cotransfection of *PML* with Ha-*ras* plus *p53*mut or Ha-*ras* plus c-*myc* also completely suppressed focus formation. A single focus was found in repeated experiments (Table 1) using *p53*mut and Ha-*ras* with *PML*. Together, these experiments clearly showed that *PML* can suppress the transformation of REF by cooperative oncogenes. When we repeated the above cotransfection experiment using various concentrations of pSG5PML, *PML* dose-dependent suppression of focus formation was noted (data not shown). These results strongly suggest that PML is a transformation suppressor. A splicing variant of *PML* (a gift from P. Chambon) (38) used in separate cotransfection exper-

TABLE 1. Effect of PML on oncogenic transformation of REF[a]

Transforming gene(s)[b]	No. of foci			
	Expt 1	Expt 2	Expt 3	Avg
*p53*mut	46	36	34	38.7
+ *p53*	0	0	0	0
+ *PML*	0	1	0	0.3
+ *PML*mut	42	35		38.5
c-*myc*	17	18	15	16.6
+ *p53*	0	1	0	0.3
+ *PML*	0	1	1	0.6

[a] Expression plasmids used: pSVc-*myc*, containing a mouse c-*myc* oncogene (40); pT24, containing an activated Ha-*ras* gene (24); LTRp53cGala, containing wild-type *p53*; LTRp53CG, a *p53* mutant containing an Ala→Val substitution at amino acid 135 (*p53*mut); pSG5PML, containing *PML*; and pSGPMLmut, containing a mutant *PML*, described in the legend to Fig. 2.

[b] In addition to Ha-*ras*.

iments was also found to suppress transformation of REF by oncogenes in much the same way as pSG5PML (data not shown).

To confirm the expression of the proteins used in these assays, Western blotting and immunoprecipitation were performed on transiently transfected cells. A high level of *p53*mut was found in the transfected cells with or without the pSG5PML (Fig. 4A). This band was not detectable in the REF control (data not shown). Similarly, a 21-kDa Ha-ras protein was detected in both transfected cells at very low levels. Expression of the 90-kDa PML protein was confirmed by Western blotting (Fig. 4B). An unknown 70-kDa protein was detected in normal REF and in samples transfected with the pSG5 or pSG5PML plasmid. We found that this band was not PML specific, since a second antipeptide antibody against PML developed in our laboratory did not recognize this 70-kDa band in REF or NIH 3T3 cells in a Western blot analysis (Fig. 4C and D). Expression of a 90-kDa protein was detected in cells transfected with pSG5PML only. Since the predicted size of the PML protein encoded by pSG5PML is about 70 kDa, we investigated the molecular mass of the in vitro-translated protein. We found that pSG5PML translated a 90-kDa protein in vitro (Fig. 4C). In addition, our antipeptide antibody detected single bands of 120 and 90-kDa in NIH 3T3 cells transfected with the PML-RARα and pSG5PML expression plasmids, respectively (Fig. 4C). The 90-kDa PML protein was also detected in the nuclear extracts of K562 and HeLa cells in our laboratory (unpublished data), indicating that the *PML* gene encodes a major protein of 90 kDa in vivo. In addition, the size of the PML protein detected by Weis et al. (63) (Fig. 4) was also significantly larger than 70 kDa. The fact that *PML* encodes a 90-kDa protein was also noted by Kastner and Chambon (37a). Though differences in the predicted size of the PML protein could have been the result of its acidic nature (pKI = 5), our results nevertheless confirmed that the expression plasmids were able to express their respective proteins in REF.

Suppression of oncogenic transformation of NIH 3T3 cells by PML. To examine the ability of PML to suppress oncogenic transformation of NIH 3T3 cells by oncogenes, we cotransfected expression plasmid pSG5PML with c-*Neu*104 (activated *neu* oncogene) (65) and with Ha-*ras*. As shown in Fig. 1C and 5, PML significantly suppressed focus formation caused by the *neu* oncogene but not focus formation caused by Ha-*ras* in NIH 3T3 cells.

PML-RARα inhibition of the transformation suppressor function of PML. As reported previously by Kastner et al. (38),

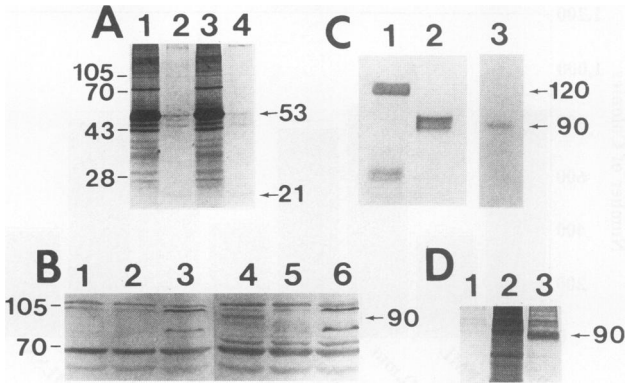

FIG. 4. Expression of p53mut, Ha-ras, and PML proteins in pooled REF transfected with expression plasmids. (A) Analysis of the expression of p53mut and Ha-ras proteins in REF cotransfected with *p53*mut, Ha-*ras*, and *PML* by immunoprecipitation using the p53mut-specific antibody (Ab-3) and anti-v-Ha-*ras* antibody. Lanes 1 and 2, samples isolated from REF transfected with *p53*mut and Ha-*ras*; lanes 3 and 4, samples transfected with *p53*mut, Ha-*ras*, and *PML*. The 53-kDa p53mut protein (lanes 2 and 4) and the 21-kDa Ha-*ras* protein (lanes 1 and 3) are indicated. The positions of the protein size markers are indicated in kilodaltons on the left. (B) Analysis of the expression of PML protein in REF transfected with Ha-*ras*-, *p53*mut-, and *PML*-carrying plasmids. Proteins (100 μg) isolated from transfected cells were analyzed by Western blotting using either the antipeptide antibody against PML (lanes 4 to 6) or the peptide-preincubated antibody (lanes 1 to 3). Lanes 1 and 4, samples isolated from REF transfected with Ha-*ras*, *p53*mut, and pSG5PML; lanes 2 and 5, samples isolated from REF transfected with *p53*mut and Ha-*ras*; lanes 3 and 6, normal REF controls. (C) Lanes 1 and 2, expression of PML-RARα and PML in NIH 3T3 cells transfected with 10 μg of the PML-RARα or pSG5PML expression plasmid. High transfection efficiency was achieved by using lipofectamine-mediated gene transfer. Total protein was isolated, and 20 μg of the protein was loaded on each lane. Western blotting was performed by using the affinity-purified PML antipeptide antibody. Lane 3, result of in vitro transcription and translation of plasmid pSG5PML. PML (90 kDa) and PML-RARα (120 kDa) are indicated. (D) Western blot in panel B repeated with a different PML antipeptide antibody. Lanes 1, 2, and 3 correspond to lanes 6, 5, and 4, respectively, of panel B. Protein fractions were isolated from the transfected cells 48 h posttransfection.

PML-RARα can form a heterodimer with PML in cotransfection experiments. This observation supports the hypothesis that PML-RARα can be a dominant negative inhibitor of PML in APL cells (37). To investigate whether PML-RARα can inhibit the transformation suppressor function of PML in NIH 3T3 cells, *PML-RARα* expression plasmid was cotransfected with various quantities of *PML* (2, 5, and 10 μg) and the *neu* oncogene. Our results (Fig. 5) demonstrated that cotransfection with *PML-RARα* significantly increased the number of foci. Thus, it is possible that PML-RARα can inhibit the normal function of PML by forming a nonfunctional heterodimer. This notion is supported by our observation of immunofluorescence staining in the transfected cells. We found that cotransfection of *PML* and *PML-RARα* resulted in cytoplasmic retention with a pattern unlike the normal speckled nuclear pattern of PML (unpublished data). In a separate experiment, we showed that transfection of the *PML-RARα* expression plasmid alone did not induce focus formation in NIH 3T3 cells. This result demonstrated that PML-RARα alone is insufficient to cause oncogenic transformation. Thus, an increase in focus number, as shown in our experiment, is more likely a result of inhibition of the PML suppressor

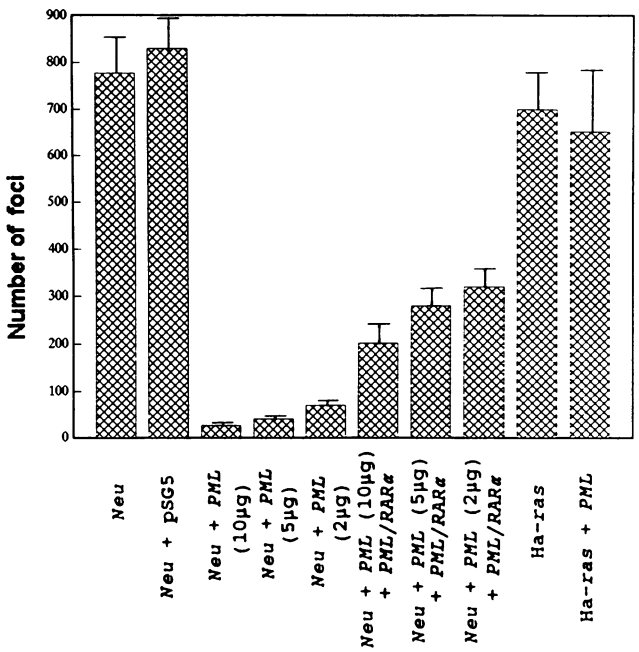

FIG. 5. Effect of PML-RARα on transformation suppressor function of PML in *neu*-transformed NIH 3T3 cells. Expression plasmids used: a plasmid containing the activated *neu* oncogene (c-*Neu*104) (65); pT24, containing an activated Ha-*ras* gene (24); expression vector pSG5, containing *PML* cDNA, driven by the SV40 early promoter; and pSG5, containing *PML-RARα* cDNA (38). The NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37°C in a humidified CO_2 incubator. Cells (5×10^5) were transfected with 10 μg of each expression plasmid, unless otherwise specified, by calcium phosphate coprecipitation.

function than of PML-RARα-induced focus formation. This result supports the hypothesis that PML-RARα can be a dominant negative inhibitor of PML in vivo.

Transactivation function of PML and PML-RARα. To assess the putative functions of PML and PML-RARα as transcription factors, we examined their ability to transactivate promoter activity by cotransfecting the pSG5PML and PML-RARα expression plasmids with various CAT reporter plasmids fused to the human multidrug resistance (*MDR1*), epidermal growth factor receptor (*EGFR*), chicken β-actin, Rous sarcoma virus long terminal repeat, and simian virus 40 (SV40) early-gene promoters. When we transiently cotransfected human adrenocortical carcinoma SW13 cells with the reporter vectors and pSG5PML, we found that PML significantly suppressed the activities of MDR1 and EGFR (Table 2). However, cotransfection with *PML-RARα* did not suppress the activity of MDR1, and the suppression was significantly lower on the *EGFR* promoter. No significant changes were observed in the activities of the Rous sarcoma virus long terminal repeat and the SV40 early promoters. A modest repression of the β-actin promoter was consistently observed. Together these results showed that PML, rather than transactivating, acts to suppress the transcription activities of specific gene promoters. PML, in this sense, is similar to the tumor suppressors p53 (12, 27, 51) and Rb (39, 46, 54), both of which can also suppress or transactivate the promoter activities of a number of genes in a cell-type-specific manner. Our results showed that the mutant form of PML (PML-RARα) lost its transcription suppressor function on these specific promoters.

TABLE 2. Transactivation effects of *PML* and *PML-RARα* on various promoter-CAT fusions in SW13 cells

Construct[a]	% CAT activity[b]	
	PML	*PML-RARα*
MDRCAT	12.3 ± 0.6 (4)	94.8 ± 20.3 (4)
EGFRCAT	17.0 ± 3.7 (6)	67.5 ± 08.8 (6)
ACTINCAT	80.7 ± 3.7 (3)	91.5 (2)
SV_2-CAT	106.0 ± 4.0 (3)	123.5 (2)
RSVCAT	94.0 ± 4.0 (3)	103.3 ± 19.8 (3)

[a] MDRCAT, human multidrug resistance gene promoter-CAT fusion (12); EGFRCAT, human epidermal growth factor receptor gene promoter-CAT fusion (36); ACTINCAT, chicken β-actin promoter-CAT fusion (50); SV_2-CAT, SV40 promoter-CAT fusion (30); RSVCAT, Rous sarcoma virus long terminal repeat-CAT fusion (29).

[b] The control, whose CAT activity corresponded to 100%, received pSG5 or pSG5mut in the transfection. The number of independent transfections is given in parentheses.

PML suppression of tumorigenicity of NB4 in nude mice. To investigate whether PML can suppress the tumorigenicity of NB4 cells in nude mice, we injected NB4 cells transfected with the pSG5, pSG5PML, and PML-RARα expression plasmids subcutaneously into five groups of nude mice (Table 3). Mice injected with 1×10^6 and 2×10^6 pSG5-transfected NB4 cells developed tumors in 20 to 25 and 15 days, respectively. Mice injected with pSG5PML-transfected NB4 cells but not with *PML-RARα* developed tumors that were significantly smaller and took longer to grow. To confirm that tumors produced in the nude mice were derived from the NB4 cells, total RNA was

TABLE 3. Suppression of tumorigenicity of NB4 cells in nude mice by PML[a]

Plasmid and no. of cells (10^6)	Tumor vol (mm^3) the following no. of days postinjection:				
	15	20	25	30	35
pSG5					
1		12	240	960	1,680
1		8	150	360	864
1		0	50	280	770
1		0	0	0	0
2	12	240	1,170	2,240	3,520
2	12	270	960	2,760	4,488
2	8	252	810	1,920	3,740
2	8	180	720	1,350	1,936
pSG5PML					
1			12	129	640
1			0	8	60
1			0	0	0
1			0	0	0
2		8	175	480	1,050
2		12	280	594	1,440
2		4	252	360	864
2		12	240	539	2,178
PML-RARα					
1		24	380	1,085	2,080
1		16	252	960	1,750
1		8	180	810	1,908

[a] NB4 cells were transfected with pSG5, pSG5PML, or expression plasmid *PML-RARα* by electroporation as described in Materials and Methods. Transfected cells were incubated in a CO_2 incubator for 24 h. The number of viable cells was determined by trypan blue dye exclusion. After two washes in PBS, cells were resuspended in 0.2 ml of PBS and injected subcutaneously into athymic nude mice (*nu/nu*; 5 to 6 weeks old).

isolated from the tumors, and reverse transcription PCR was performed to amplify the fusion transcript RARα-PML, according to a method we reported previously (6). A predicted DNA fragment of about 400 bp was amplified, indicating that the tumors originated from the NB4 cells. This result demonstrated that PML but not PML-RARα can suppress the tumorigenicity of NB4 cells in nude mice.

DISCUSSION

Inactivation of PML, a growth suppressor, may cause uncontrolled growth in the APL cells. The biological function of PML, a putative zinc finger transcription factor involved in the t(15;17) translocation in APL, is completely unknown. Our studies reported here convincingly demonstrated that PML is a growth or transformation suppressor and that its mutant form, PML-RARα, loses its ability to suppress the clonogenicity and tumorigenicity of NB4 cells and to suppress promoter-specific transcription. The biological function of PML as a growth suppressor is supported by the following conclusions: (i) PML suppresses anchorage-independent growth of APL-derived NB4 cells on soft agar, (ii) PML suppresses the tumorigenicity of NB4 cells in nude mice, and (iii) PML suppresses the oncogenic transformation of REF and NIH 3T3 cells by oncogenes. Since the DNA sequence of *PML* shows that it may potentially function as a transcription factor on the basis of the presence of putative zinc finger and transactivation domains, we decided to test the function of *PML* in reporter CAT experiments. Results from these studies showed that *PML* can specifically suppress the promoters of MDR1 and EGFR genes but not the promoters of β-actin, SV40, and Rous sarcoma virus. Therefore, MDR1 and EGFR genes are potential targets for regulation by *PML*, and their expression is expected to be altered when *PML* is functionally mutated by its fusion with *RARα* in APL. No significant sequence homology was found in the promoter regions of these two genes. Recently, in collaboration with A. G. Rosmarin (Brown University), we found that cotransfection of pSG5PML with the CD18 promoter [CD18 (−900)/luc] resulted in at least a twofold increase in transcription activity in HL-60 cells (unpublished data). This observation, together with the results presented in Table 2, demonstrates that PML is capable of acting as a promoter-specific transcription activator and suppressor.

PML is a growth suppressor, as our present study shows. To elucidate PML's normal cellular location, we recently demonstrated that it is associated with the nuclear matrix (unpublished data), an insoluble nuclear skeletal framework that directs functional domains of the chromatin to provide sites for specific control of gene activities. Actively transcribed genes, sites of RNA processing, and transcription factors have been shown to be associated with the nuclear matrix. We also showed that PML is a phosphoprotein and that at least one of the sites is phosphorylated by a tyrosine kinase (unpublished data). These findings, in addition to the results presented in this article, suggest that PML has many properties similar to those in the tumor suppressors, e.g., Rb.

Our results showed that PML suppressed oncogenic transformation of NIH 3T3 cells by *neu*. Recently, using B104-1-1 cells (NIH 3T3 cells transformed by the activated *neu* oncogene) as a model, we established stable transfectants that constitutively express PML. We found that expression of PML could reverse phenotypes of B104-1-1 including morphology, contact-limiting properties in culture, and growth rate. PML also suppresses the clonogenicity of B104-1-1 in soft-agar assay and tumorigenicity in nude mice (unpublished data). These results further imply that PML is a transformation or growth suppressor since inactivation of a growth suppressor may result in uncontrollable growth. Just such an event may be directly involved in the development of APL.

PML-RARα is a dominant negative inhibitor of PML and RXR. As proposed by Kakizuka et al. (37), the fusion protein PML-RARα encoded from the t(15;17) breakpoint may be a dominant negative oncogene that suppresses the normal function of PML. This hypothesis is supported by the finding of Kastner et al. (38) that the PML-RARα fusion protein can form a heterodimer with the normal PML protein but not with RARα. In addition, the PML-RARα fusion protein is much more stable and more abundant in APL cells. Results from these studies also suggest that the fusion protein and the normal PML may form heterodimers. The formation of such nonfunctional heterodimers, however, may interfere with the normal function of PML in APL cells. Significantly decreased expression of the normal *PML* transcripts due to the t(15;17) translocation seems to support the above hypothesis. Our results demonstrated that cotransfection of *PML* with *PML-RARα* significantly reduced the transformation suppressor ability of PML. This observation, in turn, suggests that PML-RARα can form a nonfunctional heterodimer with PML in vivo, thereby suppressing the growth suppressor function of PML. This result also provides direct evidence that PML-RARα can be a dominant negative inhibitor of PML in vivo. In combination, these findings indicate that the t(15;17) translocation in APL inactivates a growth or transformation suppressor gene, a molecular event that likely contributes to the uncontrollable growth of the APL cells.

Recently, Perez et al. (49) reported that PML-RARα forms a heterodimer with RXR and that sequestration of normal RXR function may occur in the APL cells. This finding was supported by cotransfection experiments demonstrating that PML-RARα can inhibit RXR-dependent binding of the VDR to targeted sequences as well as vitamin D3-dependent activation of VDR-responsive reporter genes. Studies by Grignani et al. (32) also demonstrated that transfection of *PML-RARα* into cells from the human myeloid cell line U937 resulted in a loss of vitamin D3 and transforming growth factor β1 inducibility. In addition, expression of *PML-RARα* resulted in an increase in sensitivity to RA-induced differentiation and an inhibition of apoptosis (32). Together, these results suggest that increased expression of *PML-RARα* by gene transfection into myeloid cells can create a unique phenotype of APL. Since APL patients can be treated with all-*trans* RA and since over 95% of those so treated can achieve complete remission (5, 13, 34, 61), the above finding implies that PML-RARα may be responsible for RA inducibility. This notion is supported by the finding that NB4 cells that lose their RA inducibility also lose expression of the PML-RARα protein (16).

Molecular mechanism of APL pathogenesis. Carcinogenesis is a multistep event in most malignancies (35, 62). In APL, the t(15;17) translocation appears to be the only consistent chromosomal abnormality in APL and mutation of the *ras* and *p53* oncogenes is rare (43, 64). It is, thus, possible that inactivation of the growth suppressor function of PML and creation of PML-RARα represent two separate events arising from the t(15;17) translocation. In the first event, the translocation disrupts the *PML* gene in one chromosome and subsequently reduces its mRNA expression. This event in combination with the formation of a nonfunctional heterodimer with the PML-RARα fusion protein facilitates sequestration of PML. Inhibition of the function of PML, a growth suppressor, in APL may result in uncontrolled cell growth. In the second event, PML-RARα inhibits differentiation induction by hormones or growth factors (e.g., vitamin D3 or transforming growth factor

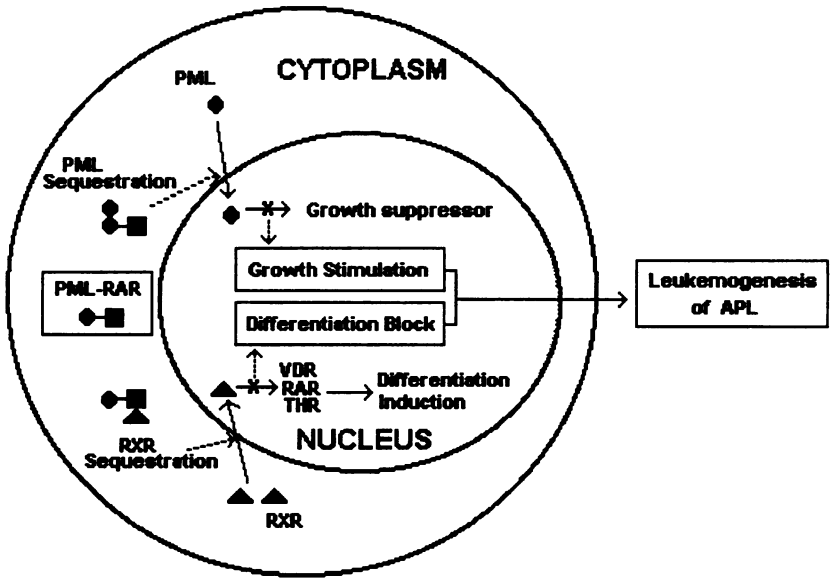

FIG. 6. Model of the molecular mechanism of APL pathogenesis. The t(15;17) translocation results in the creation of the fusion protein PML-RARα and inactivation of the growth suppressor PML. PML-RARα plays a central role as a dominant negative inhibitor for PML and RXR. Suppression of PML and RXR function leads to the stimulation of growth and to blocked differentiation at the promyelocyte stage. Pathogenesis of APL may then arise through (i) continuous proliferation caused by stimulation of growth as a result of PML sequestration and (ii) differentiation arrest at the promyelocyte stage caused by the inhibition of RXR function. THR, thyroid hormone receptor.

β1) (32, 49). Inhibition of differentiation induction by PML-RARα is likely a result of RXR sequestration by heterodimer formation (32, 49), which inhibits the activation of RXR-dependent vitamin D-responsive gene expression. Since vitamin D3 has been shown to induce differentiation in promyelocytes (52, 59), it is plausible that sequestration of RXR by PML-RARα can arrest differentiation at the promyelocyte stage of myeloid differentiation. Recently, strong evidence (reported by two different groups [20, 63]) demonstrated that the PML-RARα fusion protein forms a heterodimer with PML and RXR in APL cells in vivo. These observations confirm the hypothesis that PML-RARα is a dominant negative inhibitor of PML and RXR and that sequestration of these proteins is likely to occur in APL cells. Furthermore, it was demonstrated by Tsai and Collins that a dominant negative RARα is capable of blocking differentiation of normal bone marrow cells at the promyelocyte stage (60).

On the basis of such findings, the following model for multistep pathogenesis of APL can be proposed (Fig. 6). The t(15;17) translocation results in the creation of the fusion protein PML-RARα and inactivation of the growth suppressor PML. The fusion protein plays a central role as a dominant negative inhibitor for PML and RXR. Suppression of PML and RXR functions, in turn, leads to stimulation of growth and to blocked differentiation at the promyelocyte stage. Pathogenesis of APL may then arise through (i) continuous proliferation caused by stimulation of growth as a result of PML sequestration and (ii) differentiation arrest at the promyelocyte stage caused by the inhibition of RXR function. This model is in agreement with the hypothesis of Sawyers et al. (57) that abnormalities in both growth and differentiation are required for leukemogenesis.

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