Deconstructing a Disease: RARα, Its Fusion Partners, and Their Roles in the Pathogenesis of Acute Promyelocytic Leukemia

By Ari Melnick and Jonathan D. Licht

IN THE LATE 1980s and early 1990s, the elucidation of the molecular basis of acute promyelocytic leukemia (APL) emerged as a paradigm for the connection between the bench and bedside. At that time, it became apparent that APL was, among the forms of acute myeloid leukemia, uniquely sensitive and bedside. At that time, it became apparent that APL was, emerged as a paradigm for the connection between the bench and bedside. At that time, it became apparent that APL was, among the forms of acute myeloid leukemia, uniquely sensitive and bedside.

The retinoic acid receptor (RARα) gene to the promyelocytic leukemia (PML) gene on chromosome 15, yielding the fusion protein PML-RARα. These data suggested that disruption of RARα function was the major cause of APL. According to this line of reasoning, retinoic acid in pharmacological doses could then overcome this pathology, leading to in vivo differentiation and clinical remission. Although this hypothesis is essentially correct, 7 years of intense investigation of the APL model have begun to uncover a more complicated picture.

APL is now associated with four different gene rearrangements, fusing RARs to the PML, promyelocytic leukemia zinc finger (PLZF), nucleophosmin (NPM), or nuclear matrix associated (NuMA) genes (Fig 1), leading to the formation of reciprocal fusion proteins (N-RARα and RARα-N). This again highlights the importance of retinoid metabolism, but also suggests that partner genes with RARα could also play important roles. In this review, we will deconstruct the APL problem by evaluating the role of RARα in normal and neoplastic myeloid development. We will examine each of the genes fused to the RARα in APL, searching for similarities and differences among the four partner proteins that may explain the distinct clinical outcome some patients with variant forms of APL.

Finally, we will reconstruct the disease of APL and examine the leukemogenic functions of the RARα fusion proteins in cell culture models, animal models, and patients. We will also examine how the recent explosion of knowledge in APL has led to the development of new therapeutic agents such as arsenic trioxide and sodium butyrate.

The Retinoic Acid Receptor

Retinoids may be key for myeloid differentiation. Vitamin A-deficient mice and humans were noted to have defects in hematopoiesis and retinoids can preferentially stimulate granulopoiesis. In the early 1980s, it was noted that retinoic acid (ATRA) could induce differentiation of myeloid cell lines such as HL60 and of primary cells from patients with APL. The cloning of the RARs and other members of the nuclear receptor superfamly allowed for further detailed studies into the mechanism of action of ATRA. Among the genes encoding RARs (reviewed previously), RARα is identified with myeloid development.

Transcriptional Function of RARα

RARα structure parallels that of other nuclear receptors and is divided into 6 evolutionarily conserved domains (A through F; Fig 2). The most highly conserved domain among nuclear receptors and retinoid receptors is the C domain, which contains two Cys2 zinc finger motifs (reviewed in Chambon). Through this domain, RARα binds to retinoic acid response elements (RARE) located in the promoters of many genes, including those of RARα, RARβ, and RARγ. RAREs consist of a direct repeat (A/G)(G/T)GACA separated by 2 or 5 nucleotides. RARα binds as a heterodimer to this site along with the related retinoid X receptor protein (RXR),. Heterodimerization is mediated both by the DNA binding and ligand binding domain of RARα. RXRα is a ligand-dependent transcription factor stimulated by ATRA, whereas its partner, RXR, responds to ATRA or 9-cis retinoic acid. RARα and other nuclear receptors contain two domains, AF-1 (A/B domains) and AF2 (E domain), which can cooperate to activate transcription, AF-1, contained within the N-terminal A/B domain, is a ligand-independent transcriptional activation domain that works in a promoter context-dependent manner. Through alternative promoter usage, the RARα protein can have two different A domains (A1 or A2). The C-terminal E domain of RARα contains the AF2 ligand-dependent transcriptional activation domain as well as a dimerization interface for RXR.

RARα modulate transcription through interaction with cofactors. The AF-2 domain of the protein associates with corepressor molecules in the absence of ligand. These corepressors, N-CoR and SMRT, were recently shown to be part of a multiprotein repressor complex also containing the Sin3A corepressor and histone deacetylases (Pazin and Kadonaga and references therein). This suggests that RARα may silence certain promoters by alterations in chromatin configuration. Structural studies of RARα and RXRα indicate that, in the presence of ligand, the AF2 changes its conformation, making new residues available to bind to coactivator proteins. Nuclear receptor coactivators include TIF1 and related to the PML protein associated with t(15;17)-associated APL (see below), TIF1/Sug1, TIF2, ACTR, Srg-1, TAF1, and CBP. The functions of these coactivators are beginning to be elucidated. TIF1 interacts with the TATA binding protein.

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Submitted April 14, 1998; accepted December 29, 1998.

Supported by Grant No. ROI CA 59936 (J.D.L.) and American Cancer Society Award DHP 160 (J.D.L.). J.D.L. is a scholar of the Leukemia Society of America. A.M. is supported by Grant No. K08 CA73762.

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0006-4971/99/9310-0049$3.00/0
Molecular Pathogenesis of APL

(TBP), TBP-associated factors (TAFs), and the basal factor TFIIE. Murine and yeast Trip1/sug1 have DNA helicase activity, which could unwind DNA, whereas the CBP and ACTR cofactors have histone acetylase activity and associate with P/CAF, another histone acetylase. It is believed that histone acetylation leads to alterations in the conformation of chromatin and stimulation of gene transcription. Hence, the ligand-activated RAR can best be imagined as a multiprotein complex bound to DNA in association with RXR and a number of coactivator proteins. The ligand bound complex might then stimulate transcription through interaction with basal factors, alteration of chromatin, and unwinding of DNA.

The use of synthetic ligands specific for RAR and RXR indicate that RAR/RXR complexes that stimulate gene transcription are responsible for the pro-differentiation effect of ATRA, whereas RXR/RXR complexes cannot induce differentiation of APL cells. RARs can also repress transcription through cross-talk with other transcriptional activators, including the AP1 family of activator proteins, probably due to competition for limiting coactivators such as CBP. However, the most important action of RAR in myeloid differentiation is its ability to activate transcription through RAREs, because artificial ligands that inhibit AP1 activity but fail to stimulate RARE-mediated transcription fail to induce myeloid differentiation. Which key genes are stimulated to affect myeloid differentiation remain to be determined.

RARα AND MYELOID DIFFERENTIATION

The importance of RARα in myeloid differentiation was underscored when Collins et al. developed a HL60 cell line resistant to differentiation by ATRA. This cell line harbored a dominant negative mutant RARα with a truncation within the C-terminal AF-2 domain. Differentiation of these cells under the influence of ATRA was restored by infection with a retrovirus expressing wild-type RARα, RARβ, or RARγ. Furthermore, RXRα expression in the resistant cells restored
myeloid differentiation, suggesting that the mutant receptor may have acted in a dominant negative mode by heterodimerizing with wild-type RXR and forming an inactive transcriptional complex. Overexpression of RXR overcame this block, perhaps by recruiting other isoforms of RAR to mediate the transcriptional response required for differentiation.

RARα may help program normal hematopoietic development. Erythroid induction of multipotent FDCP mixA4 cells by erythropoietin was correlated with complete downregulation of RARα expression, whereas myeloid differentiation induced by granulocyte colony-stimulating factor (G-CSF) was correlated with upregulation of RARα, particularly the RARα2 isoform.81 Introduction of an RARα mutant, with a deletion in the ligand-binding domain, into a multipotential hematopoietic cell line resulted in a switch in cell fate from the granulocyte/macrophage lineage to the erythrocytic lineage.82 Granulocyte-macrophage colony-stimulating factor (GM-CSF)–mediated myeloid differentiation of these cells was blocked at the promyelocyte stage, an effect that could be overcome by high doses of ATRA.83 Although truncation of the RARα within the ligand binding domain has a profound effect on myeloid differentiation, this type of mutation was not identified in a series of 118 specimens of human cancer, including a number of fresh APL specimens.84 In leukemia, the RARα gene is only disrupted by the formation of chromosomal translocations yielding fusion genes (see below). The notion that the dominant negative RARα functions by sequestration of RXR into inactive complexes was supported by the finding that overexpression of wild-type RARα in murine bone marrow cultures85 led to the accumulation of promyelocytic colonies. Upon addition of ATRA, the RARα-expressing marrow colonies consisted mainly of more differentiated granulocytes. Hence, overexpression of wild-type RARα, C-terminal truncated forms of RARα and fusion proteins consisting of partners fused to the N-terminus of RARα (eg, PML-RARα; see below) can all lead to the blockade of myeloid differentiation at the promyelocyte stage when cells are grown at physiological levels (~10⁻⁸ mol/L) of ATRA. Only pharmacological levels of ATRA (10⁻⁷ to 10⁻⁴ mol/L) can overcome this block. How might this blockade occur at the molecular level? The fact that wild-type RARα as well as mutant forms of RARα can cause the block suggests a squelching mechanism.86 At low ATRA concentrations, coactivators may bind loosely to nuclear receptor/DNA complexes and be easily sequestered by high levels of free normal or aberrant receptor in the nucleoplasm. Only at pharmacological ATRA concentrations would the corepressors be drawn to target genes along with RARα and the basal transcriptional machinery. In support of this notion, in vivo footprinting of the RARβ promoter shows occupancy of the RARE only under pharmacological ATRA concentrations.87 It might be predicted that forced expression of RXR and/or RARα coactivators would rescue the block by dominant negative RARα and allow differentiation to proceed at physiological ATRA concentrations.

**RARα TARGET GENES**

ATRA treatment of myeloid precursor cells and other cells drives the expression of multiple classes of genes (Table 1) expressed either immediately after ATRA treatment or after some delay. ATRA-induced changes in myeloid gene expression are accompanied by inhibition of cell growth and induction of terminal differentiation and production of a mature cell ready to fight infection. The retinoic acid syndrome encountered during treatment of APL with ATRA, characterized by an increase in leukocyte count, fever, and pulmonary infiltrates, may be due to the increased adhesive characteristics of the differentiating granulocytes and secretion of cytokines.88 ATRA also downregulates the expression of procoagulants found in the undifferentiated promyelocyte, explaining the ability of differentiation therapy to treat APL without inducing hemostatic disorders (reviewed in Barbut et al89). The initial waves of leukocytes found in APL patients are derived from the malignant clone90 and function normally in vitro to kill pathogens,91 despite some abnormalities of secondary granules involved in immune function.92,93

<table>
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<th>Class of Genes</th>
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<td>Regulators of the cell cycle</td>
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dynamic manner. Enforced expression of the hox genes as leukemia-associated translocations is associated with altered myeloid growth and differentiation. RAREs were identified in the promoters and enhancers of hoxb1, hoxa1, hoxa, and hoxd4. Furthermore, specific RARs differentially regulate the homeobox genes. In embryonic carcinoma cells null for RARα, ATRA fails to induce the hoxb1 whereas RARγ null cells fail to express hoxa1. This information suggests that in APL the disruption of RAR function may alter expression of a subset of ATRA-inducible genes critical for myeloid differentiation.

Recent data indicate an interplay between retinoic acid and interferon (IFN)-mediated signaling. ATRA can rapidly induce transcription of the IFN regulatory factor-1 (IRF-1) gene. IRF-1 expression is associated with the expression of IFN and IFN-inducible genes, cessation of cellular growth, and induction of apoptosis. Thus, IFN may potentially mediate some of the antiproliferative effects of ATRA. ATRA induction of the IFN-1 promoter is mediated by a GAS (γ-IFN activation sequence) rather than a RARE, signifying a role for retinoic acid in the STAT (signal transducer and activator of transcription) pathway for IFN signaling. ATRA rapidly induces the expression of STAT1α at the mRNA level and increases tyrosine phosphorylation of STAT1α, together leading to a large increase in DNA binding activity of the STAT1α complex to an IFN-responsive element (IRE). RARs and STAT1α synergized to stimulate transcription from an IRE-containing reporter plasmid, whereas PML-RARα did not, suggesting that cross-talk between the two signaling pathways may be aberrant in APL and play a role in disease pathogenesis.

Recently C/EBP-ε, a newly identified basic-zipper transcription factor that recognizes CCAAT DNA sequences, was found to be rapidly upregulated during ATRA-mediated differentiation. C/EBP-ε is the only member of this family of transcription factors expressed in the APL cell line NB4, suggesting that the gene may play a role in the promyelocyte stage of differentiation. HL60 cells engineered to express PML-RARα downregulate C/EBP-ε expression in the absence of retinoic acid; the C/EBP-ε gene is then upregulated when pharmacological doses of ATRA are added to the cell. Hence, C/EBP-ε may be a model target gene of the PML-RARα fusion protein that is inhibited in expression at ambient physiological concentrations of ATRA and stimulated in expression when cells are treated with ATRA.

ATRA is known to alter cell cycle kinetics, because it induces the differentiation of APL and other myeloid cells. ATRA treatment is associated with G1 arrest and the accumulation of hypophosphorylated forms of the retinoblastoma protein. ATRA induces the expression of the p21WAF1/Cip1 cyclin-dependent kinase inhibitor in myeloid cell lines, and ATRA-mediated p21 induction may also depend on the PML-RARα protein disrupted in t(15;17) APL. RARα in combination with RXR binds to an imperfect RARE within the human p21 promoter and RARs can activate the p21 promoter in a ligand-dependent manner. Therefore, p21 meets criteria for a bona fide RARα target gene whose expression could decrease leukemic cell proliferation. Potentially relevant to the treatment of APL, the cytosolic retinoic acid binding protein II (CRABPII) promoter contains an RARE and can be transcriptionally induced by ATRA. CRABPII may sequester ATRA, contributing to therapy resistance. Tissue glutaminase II, which plays a role in differentiation and apoptosis, is induced rapidly by ATRA and contains functional RAREs in its promoter.

Identification of further direct target genes of RARα relevant to normal and malignant myelopoiesis has accelerated, using techniques such as subtractive cloning, differential screening, differential display (DD), and representative difference analysis (RDA). These studies focused on genes induced 24 hours after ATRA treatment and tended to identify indirect targets of ATRA action. These included the IFN-inducible RIG-I and the related RIS8 gene, RIG-E, encoding a GPI-linked cell surface molecule, and Jem1, a basic/leucine zipper transcription factor gene. In contrast, using RDA, a calcium/calmodulin kinase was isolated from ATRA-treated murine promyelocyte MPRO cells harboring a dominant negative RARα molecule. This gene was activated within a few hours after ATRA treatment in a cycloheximide-resistant fashion and therefore is a reasonable candidate as a direct target of RARα. Another report also using the MPRO cell line isolated, by subtractive hybridization, several genes rapidly induced by ATRA treatment. One transcript induced threefold by ATRA treatment was identical to that encoding LAPTM5, a lysosomal protein expressed preferentially in hematopoietic cell lines. The LAPTM5 promoter contained RAREs, and an LAPTM5-luciferase reporter gene was inducible by retinoic acid. The importance of this gene in differentiation is unknown. Current efforts towards the identification of ATRA target genes are using microarrays of immobilized cDNAs or oligonucleotides, which can monitor expression of thousands of genes simultaneously.

Table 2 summarizes some of the salient points regarding myeloid biology, RARs, and APL.

| PML | PML structure. The t(15;17) rearrangement affecting the PML gene on chromosome 15q22 is the molecular basis for approximately 98% of all cases of APL. The PML gene locus spans 35 kb and contains 9 exons encoding mRNAs of 4.6, 3.0, and 2.1 kb. Alternative splicing of C-terminal exons yields up to 20 different isoforms of the protein; however, most cell lines express a similar pattern of isoforms. | Evidence for Involvement of RARα in Myeloid Development

| Vitamin A deficiency results in abnormal hematopoiesis. | Retinooids preferentially stimulate granulopoiesis. | ATRA is expressed preferentially in myeloid cells. | HL60 cells undergo differentiation with ATRA via RAR/RXR heterodimer stimulation. | Wild-type and dominant negative RARα overexpression results in a differentiation block at the promyelocyte stage. | RARα targets myeloid genes, of which C/EBP-ε is a model target. (and see Table 1) | ATRA upregulates RARα. | RARα upregulates p21 and decreases cell growth. | Cross-talk between retinoid and IFN system to induce differentiation and halt cell growth. |
cDNA open reading frame encodes a 560 aa polypeptide with a predicted molecular weight (MW) of 70 kD. The PML protein has a modular structure with several domains (Fig 3). These include the following:

1. A cystein-rich region (aa 57-222) composed of three zinc-finger like structures. The first is a RING (Really Interesting New Gene) finger, a Zn$^{2+}$ binding motif with the configuration C$_3$HC$_4$ (aa 57-91). The following two are called B box zinc fingers (aa 140-161 and aa 189-222). The RING finger motif is found in more than 80 proteins involved in various cellular processes. The B box zinc fingers are involved in protein-protein interactions and transcriptional regulation.

2. A coiled-coil domain (aa 299-360), which is involved in dimerization and tetramerization. The coiled-coil structure is a repetitive alpha-helix that allows proteins to form long, rigid rods.

3. An N-terminal nucleolar localization signal (NLS) (aa 476-490), which targets proteins to the nucleolus, a site of ribosome biogenesis.

4. A serine-proline rich region (aa 540-560), which contains motifs that are involved in protein-protein interactions and nuclear localization.

In all patients, the RING finger, B boxes, and at least the first two coiled coil domains of PML are included in the fusion protein. Heterogeneity in the breakpoint within the PML gene leads to a long and short form of PML-RAR$\alpha$ depicted, as well as the rarer intermediate form (not shown). The RAR$\alpha$-PML fusion is detected in the majority of cases, but no evident function can be ascertained from its structure.
in oncogenesis, regulation of gene expression, mRNA processing, and DNA recombination and repair (reviewed previously). RING finger/B Box proteins often are linked to a coiled-coil domain and comprise a subfamily of RING proteins (RBCC for RING-B-Box-Coiled-Coil).

The RING finger/B-box region is involved in localization of PML into distinct nuclear domains known as nuclear bodies (NB), presumably through interactions with protein partners (see below). Biophysical studies, including 2-D NMR, showed that the RING finger structure is spherically organized around several loops of protein extending from two Zn ions that coordinately bind cysteine and histidine residues. This positively charged structure precludes DNA binding, implying that the RING domain is required for protein-protein interactions. PML with mutations in critical RING finger cysteine residues loses its characteristic nuclear body localization and its biological activity as a growth suppressor (see below), but other charged residues on the surface of the globular RING structure may also affect NB formation.

The B box domains also bind zinc ions and any mutation of the B-box cysteine residues disrupts NB formation. Neither the RING finger nor the B-box motifs are required for PML to self-associate, suggesting that interactions with other proteins, through the Cys-rich motifs guide PML into the multi-protein complex of the nuclear body (see below).

(2) A helical coiled-coil region (aa 229-360) consisting of eight heptad repeats with hydrophobic amino acids at the first and fourth positions. This region is responsible for multimerization of PML and heterodimerization with PML-RARα and plays a role in NB localization. This region also interacts with partner proteins and is required for the growth suppression activity of PML as well as the ability of PML-RARα to block differentiation. All PML isoforms contain the RING Finger/B Box domains as well as at least the N-terminal coiled-coil motifs.

(3) An N-terminal proline-rich sequence (aa 1-46) that can bind the Arenavirus Z proteins involved in viral genome synthesis but is not required for growth suppression by PML.

(4) A basic sequence, containing a nuclear localization signal (aa 476-490) required for the biological activity of the protein. However, exact nuclear localization in NBs also requires the RING finger/B box and coiled-coil motifs.

(5) An acidic C-terminal Ser/Pro-rich domain of unknown function, highly variable in length due to alternative splicing and rich in potential phosphorylation sites.

The PML protein. PML, when expressed after transfection into cells, is detected as a series of 90- to 100-kD protein bands as well as a set of bands in the 70 kD range, as predicted from the amino acid sequence. Endogenous PML is detected as a 90-kD species along with a variety of other protein species due to alternative splicing and covalent modifications. The PML sequence contains potential casein kinase II and proline-directed kinase sites, and labeling studies demonstrated that PML is phosphorylated on serine and to a lesser extent on tyrosine residues. Some of the sites are constitutively phosphorylated and others may be cell cycle dependent. In this regard, PML was found to be a substrate for phosphorylation by Cyclin A/cdk2 in vitro.

PML expression. PML mRNA was widely expressed in all cell lines tested. The pattern of PML protein expression in tissues is complex and controversial, possibly due to differences in techniques and antibodies used for immunodetection. In addition, PML mRNA and protein expression are often not concordant, suggesting posttranscriptional regulation. There are a few observations that should be highlighted.

First, PML is highly expressed in inflammatory diseases such as psoriasis and hepatitis, in inflammatory cells surrounding epithelial cancers and Hodgkin’s disease, in inflammatory lesions of graft-versus-host disease, and in activated fibroblasts. This suggests induction of PML expression by soluble factors, probably IFNs (see below). Second, some, but not all, groups found a correlation between the level of PML expression and degree of dysplasia in atypical breast hyperplasia and cervical intraepithelial neoplasia cells. Interestingly, when the breast tumors became invasive, PML expression decreased again. Some investigators found a correlation between the rate of proliferation of normal tissues and PML expression, with postmitotic cells tending to express higher levels of PML, whereas others suggested that PML levels were better correlated with cellular activation by cytokines and rates of protein synthesis. Third, PML delocalization can be associated with neoplasia. Hepatic carcinoma in particular was associated with mislocalization of PML in the cytoplasm rather than the nucleus. Fourth, PML and probably other proteins of the nuclear body are induced by hormones such as estrogen and cytokines such as IFN. In lymphoid cells, PML is expressed mainly in postmitotic mature T and B cells and not the germinal center or in proliferating cortical thymocytes. Hence, PML may play a broad role in mature immune cells.

NUCLEAR BODIES AND PML EXPRESSION

One of the most striking features of PML is its speckled localization to discrete nuclear domains termed PODs (PML Oncogenic Domains), ND10 (Nuclear domain-10), or NB. These structures, originally described more than 35 years ago, came under new scrutiny when NB proteins, including PML, were detected by human autoimmune antisera. Nuclear bodies vary in both size and number in different cell types. Their presence is roughly proportional to the rate of protein synthesis and inversely proportional to differentiation. Strikingly, PML is delocalized from the NBs to a microspeckled nuclear pattern in APL cells and relocates to the NB after ATRA treatment. Cells generally contain 10 to 20 doughnut-like or spherical 0.3-0.5-µm NB structures. Electron microscopy showed that, in the NB, PML and other proteins surround an electron dense core that may contain ribonucleic acid. NBs are associated with the nuclear matrix, which plays a role in trafficking of molecules and organization of chromatin within the nucleus. In initial studies, the NBs did not overlap with spliceosomes, centromeres, or sites of RNA transcription.
sequence-specific transcription factors such as the glucocorticoid receptor and E2F nor a general factor such as TFIH were concentrated within the NB, which appeared to exclude a transcriptional role for the NB. This notion must be seriously re-examined in light of new data from the lab of Ron Evans, who, using refined techniques, found the presence of nascent mRNA in the center of the NB structure. Furthermore, the transcriptional coactivator CBP was found colocalized in the NB with PML. This, together with recent data indicating the presence of PML in the AP1 DNA binding complex, suggests that the NB may play a role in stimulating transcription. Whether the NB might be a site of transcriptional initiation, elongation, or processing of the new mRNA transcript is unknown. However, data that PML can repress transcription and the finding of HP1, a heterochromatin component, within the NB suggest a role for PML and/or other NB components in downregulation of transcription.

PML-containing nuclear bodies do not colocalize with sites of nascent DNA, except during mid-S phase, when they are found adjacent to replication sites, potentially indicating a role for the NB in this process. Although PML-containing nuclear bodies are distinct from the coiled body, involved in mRNA splicing, the two structures were often found adjacent to each other, suggesting a functional interrelationship. PML was also found in the interchromatin granules, which are thought to be sites of RNA splicing, and can be found in the nucleoplasm in a diffuse staining pattern as well as in a cytoplasmic granular pattern. Although the exact site and mechanism of PML action is still uncertain, nuclear expression of PML seems to be essential for its biological function, because PML mutants lacking the NLS were unable to suppress oncogenic transformation. However, certain spliced isoforms of PML, devoid of the C-terminal NLS, are found exclusively in the cytoplasm, but other isoforms that do contain the NLS can be found in both the nucleus and cytoplasm of transfected cells. Hence, a cytoplasmic role for the protein is not ruled out and neither is the possibility that PML may shuttle between the nucleus and cytoplasm. The partition of PML between the nucleoplasm and the NBs may be controlled by processes such as differential phosphorylation and conjugation of PML to a ubiquitin-like molecule, sentrin.

The presence of the RING finger, B box motifs, and coiled-coil motifs are all necessary for PML to properly localize in the NBs. Mutants lacking these structures sequestered normal PML from the NBs in a dominant negative manner. In addition, forms of PML with mutations in critical cysteine residues required for normal protein folding failed to localize in the NBs. PML expression and NB structure are dynamic. Augmenting the cellular levels of PML either by transfection with a PML expression vector or by treating cells with IFNs increased the size and number of nuclear bodies, possibly due to deposition of PML and recruitment of other proteins to these sites. In contrast, PML-RARα caused disappearance of nuclear bodies from the nucleus as determined by staining with antibodies directed against PML and the SP100 NB protein.

The pattern and intensity of PML expression changes across the cell cycle. Cells in G0 exhibit few nuclear bodies and show weak staining for PML. As cells are stimulated into the cell cycle, the number of NBs and their intensity of staining with PML antibodies increases. As cells progress through G0 to G2 phase, PML disperses to multiple smaller dots and gradually fades, with two or three of these residual structures left in mitosis. Prolonged amino acid starvation or induction of cell senescence induces the coalescence of the NBs into 2 to 3 large structures. When amino acid-starved cells were rescued by addition of fresh nutrients, the normal pattern of 10 to 20 smaller NBs reappeared. The pattern of PML expression also responds to cell stresses such as heat shock, γ-irradiation, and viral infection (see below). How these changes relate to the function of the nuclear body is unknown.

PML and transcription

Findings related to transcriptional properties of PML have been contradictory but intriguing. PML specifically suppressed transcription of the MDR and EGF-receptor (EGF-R) promoters. However, PML enhanced transcription of the CD18 promoter as well as the promoter for the gene encoding the major histocompatibility complex (MHC) class I transporter molecule TAP-1. Furthermore, PML increased the transcriptional activity of the progesterone receptor (PR) in the presence of hormone up to 20-fold and also stimulated transcription mediated by the mineralocorticoid, glucocorticoid (GR), and androgen receptors, but not the RARs. Deletion analyses indicated that both activation domains (AF1 and AF2) of PR as well as the Cys-rich and coiled-coil domains of PML were required for the PML effect on transactivation. PML did not increase the affinity of PR for progesterone, increase DNA binding by PR, or coprecipitate with PR. This does not preclude PML as a transcriptional cofactor or adaptor to the basal transcriptional machinery. Alternatively, PML might sequester a negative regulatory factor from the PR, indirectly stimulating its activity. The physiological relevance of this interaction must be tempered by a report that PML does not colocalize with the GR.

GAL4-PML fusions were found to repress a reporter gene containing GAL4 binding sites, although an earlier publication did not note such an effect. The repression domain mapped to the coiled-coil region and the C-terminal serine-rich region but not to the RING finger; the magnitude of repression was cell-type and promoter specific. Mutational analysis indicated that the coiled-coil, not the RING finger motif was required for repression and that localization to the NB was not required. Rather, the nucleoplasmic fraction of PML may be responsible for this effect. It is tempting to speculate that PML, like PLZF (see below), interacts with histone deacetylase corepressors to repress transcription. Another mechanism of action of PML was suggested by the finding that PML-mediated repression of the EGF-R promoter was mediated through Sp1 sites. PML was then found to inhibit the DNA binding by Sp1 protein. These data suggest that, under certain circumstances and probably not when localized to the nuclear body, the PML protein could modulate gene expression by direct interaction with specific transcription factors. This function might be abrogated by PML-RARα, which, when coexpressed with GAL4-PML, leads to simulation of transcription rather than repression. Although PML might be a transcriptional corepres-
sor, a recent report also indicated that PML can stimulate transcription mediated by fos and jun through AP1 sites.203

PML PARTNER PROTEINS

The matrix association of PML makes it difficult to study the interactions of PML with putative partners, because aggressive purification procedures required to extract PML from the nuclear matrix can disrupt protein-protein interactions.216 It is interesting to note that two other RARα translocation partners, NPM and, in particular, NuMA, are also closely associated with the nuclear matrix. In addition, the remaining fusion partner, PLZF, is also found in (the matrix-associated) nuclear bodies. Together, these observations indicate that disruption of nuclear matrix function may be a common theme in APL pathogenesis, perhaps further disturbing normal patterns of gene expression.

Nearly 20 natural components of the nuclear body have been identified to date (Table 3). The diverse nature of these proteins and the fact that they often localize both within the nuclear body and other subnuclear patterns suggests that the NBs may not be a site of active cellular metabolism but rather a storage site for nuclear proteins whose temporal and spatial expression must be tightly controlled. However, several of these proteins are induced by IFN, including PML, SP100, and PLZF,216-219 pointing to an alternative hypothesis that the nuclear bodies may play a role in cellular proliferation and/or the antiviral response.

Only a few proteins are known to actually bind directly to PML. The first of these, sentrin, originally called PIC1 (also called SUMO-1 and UBL-1), was identified in a yeast two hybrid screen.220 Both cotransfected and endogenous PML and sentrin colocalized in the NB with variable overlap. In the APL NB4 cell line, sentrin was partially delocalized to PML-RARα microspeckles. Sentrin is a ubiquitously expressed 11.5-kD peptide containing a ubiquitin homology (UbH) domain.220 Two other groups independently cloned sentrin by its association with the rad51 DNA repair protein221 and the ranGAP protein of the nuclear pore complex, 222 whereas a third group found that sentrin interacted the tumor necrosis factor (TNF) receptor. This group found that sentrin covalently binds to PML in the RING finger motif, the B box domain, and the more C-terminal finger motif, suggesting that the molecule might play a role in protein turnover.223 Furthermore, this group found that overexpression of sentrin protected cells from fas/apo- and TNF-induced apoptosis.224 Given the role of PML as a growth suppressor (see below), it is possible that PML sequesters sentrin, promoting apoptosis and balancing the function of sentrin. However, it is more likely that sentrin modifies the function of PML and other components of the nucleus. Newer data show that sentrin covalently binds to PML in the RING finger motif, the B box domain, and the more C-terminal nuclear localization signal of PML.277,180,225,226 This association was exclusively found in nuclear localized PML, particularly with PML associated with the NBs,225,226 suggesting that the sentrin modification targets PML to the NB. However, sentrin was not associated with PML in mitotic cells, suggesting that the modification is cell cycle dependent. It was reported that PML-RARα is not modified by sentrin180,226 and that sentrinization was not restored to this protein after ATRA treatment. This was surprising given the moieties modified by sentrin are present in PML-RARα, and another group found that, when expressed in U937, PML-RARα but not PLZF-RARα became conjugated to sentrin, suggesting that the molecule might play a role in the degradation of PML-RARα after ATRA treatment.227

The PLZF protein may be a key interaction partner of PML. The PLZF protein, originally characterized by its fusion to RARα in t(11;17)(q23;q21)-associated APL, colocalizes with PML both in myeloid and in transfected nonhematopoietic cells.216 Colocalization was not restored to this protein after ATRA treatment.227 The PLZF nuclear dots and PML NBs also

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<td><strong>Proteins</strong></td>
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<td>PIC1/SUMO-1/Sentrin</td>
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<td>BAX</td>
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<td>KIP1/p27</td>
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<tr>
<td>Glyceraldehyde-3-phosphatedehydrogenase (GAPDH)</td>
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<td><strong>Viral</strong></td>
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<td>E1A-E1B adenoviral early proteins578,579</td>
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appeared to be in different functional compartments, because expression of the adenovirus E4 protein in KG1 cells delocalized PML from the NB (see below) but did not affect the expression pattern of PLZF. In semistable transduced cells, several days were required for the PML and PLZF proteins to sort within the cell and become colocalized, suggesting complex regulation of this association.²¹⁶ Nevertheless, interaction between PML and PLZF was demonstrated by biochemical assay, and the association was found to be mediated by the coiled-coil domain of PML and the first two zinc fingers of PLZF (A. Zelent, personal communication, December 1998). PLZF could be delocalized to a microspeckled pattern in NB4 cells and reverted to the NBs upon treatment with retinoic acid. These provocative findings suggest that PML may be involved in the transcriptional modulation mediated by PLZF or, conversely, that PML regulates availability of PLZF by sequestration within the NBs.²¹⁶ Thus, a common mode of leukemogenesis may exist in APL, based on disruption of a pathway that contains both PLZF and PML.

New data indicate that the retinoblastoma (Rb) protein interacts with PML. PML could be coimmunoprecipitated with a low percentage of the underphosphorylated form of Rb but not the related p107 and p130 proteins. As seen by confocal microscopy, the speckled fraction of Rb in the cell overlapped the PML NB. PML-RB interaction required the pocket domain of Rb and the first two coiled-coil motifs of PML, with the RING finger motif, stabilizing the complex. In addition, a PML isoform with an extended serine-rich C-terminal domain bound significantly less strongly to Rb, suggesting that different PML isoforms could differentially bind Rb.²²⁹ PML-RARα also bound to Rb and delocalized Rb from its usual speckled pattern into the microspeckled pattern characteristic of PML in APL. The functional consequences of this interaction in growth control are uncertain, because both PML and PML-RARα are able to inhibit the growth of nonhematopoietic cells devoid of functional Rb. However, it remains fascinating to postulate that one of the mechanisms through which PML inhibits cell growth is by limiting accessibility of Rb to the cyclin-dependent kinases that phosphorylate it during G1 phase.

Finally, a novel function for PML was suggested by its association with the ribosomal P proteins P0, P1, and P2.²³⁰ These proteins form part of the large ribosomal subunit, are localized in both the nucleus and cytoplasm, and are involved in the process of translation,²³¹,²³³ suggesting that PML may have a role in translational control. This is supported by the findings that PML interacts with the L7 leucine zipper protein and EF-1,²²⁰ also implicated in translation, and that PML partially localizes to the cytoplasm.²¹⁷ It is intriguing to note that the NPM protein, fused to RARα in t(5;17) APL,²³⁴ is involved in ribosome biogenesis and shuttling ribonucleoproteins between the nucleus and cytoplasm.²³⁵,²³⁷ This places both PML and NPM in a similar functional axis.

PML, IFN, AND VIRAL INFECTION

PML is an IFN-responsive gene that may be important for its normal immunity. In hematopoietic NB4 and nonhematopoietic cell lines, PML and PML-RARα mRNA were induced fivefold to 30-fold in response to IFN in a dose-dependent manner.²⁸⁷,²⁸⁸,²⁸⁹ Actinomycin D blocked this effect, whereas cycloheximide did not, indicating that PML is a primary target of IFN action. Other cytokines that induce the JAK/STAT pathway did not cause this increase. The PML promotor contains an ISRE (IFN-stimulated response element) as well as a GAS (γ activation site), both of which bind to STAT proteins.²⁵⁸ After IFN treatment, different PML isoforms become visible with increases in the intensity of staining and number of nuclear bodies. STATs are involved in upregulating multiple components of the NB, including Sp100, whose promotor also contains a GAS and ISRE.²¹⁶ Because PML seems to be a growth suppressor, it may be an attractive target for the antiproliferative and antiviral effects of IFN.²⁵⁸ Finally, ATRA can cooperate with IFN to synergistically induce PML expression through induction and activation of STATs acting through the GAS element in the PML promotor.²¹⁸ Induction of wild-type PML could thus play a role in the therapeutic effect of ATRA in APL.

It is apparent that one of the earliest events of both lytic and latent viral infections is the targeting of viral products to NBs, often resulting in the reorganization of PML localization and NB architecture. Some of these results are summarized.

Adenovirus. Adenovirus infection causes PML to undergo dynamic spatial redistribution. As the virus progresses through its life cycle, the NBs disappear and become part of the viral nuclear inclusions.²⁰⁶ Mutational analysis showed that the adenovirus E4-ORF3 gene product was responsible for NB redistribution to fibrous structures or tracks. Overexpression of PML blocked this redistribution, an effect that was lost when PML deleted for the coiled-coil domain was used.²³⁸

HSV-1. ICP0, an immediate early gene, encodes a RING finger protein that is required for both the lytic and latent herpes virus life cycles. ICP0 activates viral gene expression, targets the NBs, colocalizes with PML, and modifies NB architecture.²³⁸ HSV-1 replication sites, with attachment of viral DNA to the nuclear matrix, appear to coincide with NBs as well,²⁴⁰ suggesting that NB components may be involved in viral DNA replication. In latent infections, in which HSV genes are partially expressed from episomes, viral genomes may also be associated with NBs. Stress, which disperses the NBs, also allows the virus to come out of latency.²⁴⁰

Epstein-Barr virus (EBV). In contrast to the viruses listed above, EBV tends to transform lymphocytes by latent infection rather than lysing target cells. EBNA 5 is an early protein critical for transformation and latency. This protein is colocalized with PML in interphase NBs, with PML coating the outside of the structure and EBNA found within.²⁴¹,²⁴² In contrast to the other viral infections, the NB structure is not disrupted by EBV infection.

Arenavirus (LCMV, Lhassa). The arenavirus RING finger containing Z protein binds to the N-terminal proline-rich region of PML, resulting in its delocalization to the cytoplasm.²¹² The fact that the Z protein targets P ribosomal proteins that are partners of PML suggests viral hijacking of the cellular translational machinery, of which PML may be a component.²³⁰ Alternatively, PML may be moved to the cytoplasm to prevent it from promoting apoptosis (see below), thus enabling survival of virus-laden cells and allowing the virus to establish chronic infections.²⁶⁸,²³⁰ This list is by no means complete, because...
cytomegalovirus (CMV)\textsuperscript{243,244} and papilloma virus\textsuperscript{245} also target the NBs.

To test whether PML plays a role in the antiviral effect of type I IFN, PML\textsuperscript{−/−} embryonic fibroblasts were infected with a number of DNA viruses, including herpes simplex and vesicular stomatitis virus, in the presence or absence of IFN.\textsuperscript{207} There was no difference in the efficacy of IFN in decreasing resultant viral titers; therefore, the absence of PML did not confer increased susceptibility to viruses. Engineered expression of PML in 3T3 cells did not confer resistance to HSV, VSV, EMCV, or Adenovirus.\textsuperscript{207} However, another group found that in HepG2 cells, overexpression of PML inhibited adenovirus growth, perhaps by delaying or blocking NB protein recruitment to viral replication domains.\textsuperscript{238} A third group found that PML overexpression selectively inhibited replication of influenza virus but not of encephalomyelitis virus. In contrast, overexpression of the Sp100 NB protein had no significant antiviral effect.\textsuperscript{246}

Together, it can be concluded that, although PML itself is not absolutely required for the activity of IFN, it may confer some of the antiviral activity of IFN.

These findings suggest that NBs could be carefully controlled organelles that store regulatory factors involved in viral transcription or replication.\textsuperscript{239} Alternatively, NBs may be an intranuclear defense system. This notion is supported by the localization of infecting virus genomes to the vicinity of the NB, the IFN induction of NB proteins, and the growth-suppressive properties of PML.\textsuperscript{240} Perhaps more plausibly, viruses may target and disrupt the nuclear body to abrogate an apoptotic program (see below), allowing the virus to promulgate freely in the cell population. The delocalization of NB proteins caused by PML-RAR\textalpha expression could mimic activation of the viral program, possibly resulting in uncontrolled cellular proliferation.\textsuperscript{238}

PML, GROWTH SUPPRESSION, AND APOPTOSIS

The growth-suppressive actions of PML were suggested by the finding that NB4 cells selected for expression of exogenous PML, harbored mutations in PML, suggesting that the wild-type protein was not tolerated.\textsuperscript{210,247} Infection of NB4 cells with a retrovirus harboring PML suppressed the ability of the cells to form colonies in soft agar. In addition, conditioned medium from these cells suppressed colony formation of wild-type NB4 cells, suggesting the release of negative growth control factors.\textsuperscript{210} Furthermore, PML-overexpressing NB4 cells, when injected into nude mice, yielded smaller tumors that appeared with a longer latency than vector-expressing cells.\textsuperscript{210} Attempts to circumvent the toxic effect of PML led another group to create an episomal transfection system in which it was demonstrated that even low levels of PML were toxic to NB4 cells. Because RAR\textalpha overexpression was also growth suppressive to these cells, these investigators concluded that, in t(15;17) APL, the reduced dosage of PML and RAR\textalpha could contribute to uninhibited cell growth.\textsuperscript{247}

It was further shown that PML inhibited transformation of rat embryo fibroblasts expressing Ha-ras and mutant p53 or Ha-ras and c-myc.\textsuperscript{210} However, PML did not inhibit all oncogenic pathways. PML suppressed foci formation in NIH 3T3 cells transformed by the neu oncogene, but not by the ras oncogene. PML-RAR\textalpha acted in a dominant negative manner to prevent suppression of transformation by PML, sequestering PML in the cytoplasm, whereas PML-RAR\textalpha itself did not stimulate the formation of foci. The neu oncogene-transformed NIH-3T3 cells mentioned above were reverted by retrovirus-mediated transfer of PML, resulting in restitution of wild-type morphology, contact inhibition, and anchorage-dependent growth. There was no major difference in cell cycle distribution of PML-expressing cells, suggesting that PML suppressed growth not by cell cycle inhibition but by altering cell survival and apoptosis. Although PML expression led to decreased expression of the neu protein, the cell cycle profile and morphologic response of cells to expression of PML differed from that of cells treated with an anti-neu antibody, which arrest in the G2 phase of the cell cycle. This suggests that PML interferes with downstream targets of the neu signal transduction molecule, perhaps reflective of the normal physiologic function of PML.\textsuperscript{248}

However, in another system, PML did affect the progression of the cell cycle. When overexpressed in HeLa cells, PML inhibited cell growth, colony formation in agar, and tumor growth in nude mice, as in the models described above, but also caused an accumulation of cells in G1\textsuperscript{249} and a delay of cell entry into S phase, correlated with decreased expression of cyclin E and cdk2. Similarly, expression of PML in breast cancer cell lines blocked cells in G\textsubscript{1} associated with a decrease in cyclin D1 and CDK2 and accumulation of hypophosphorylated Rb.\textsuperscript{250} This is intriguing given recent data that hypophosphorylated Rb and PML can complex.\textsuperscript{229} However, PML can suppress growth even of Rb-deficient cells, suggesting that complexes with other Rb-like proteins could play a role as well.

de Thé’s group stably expressed PML in HeLa, CHO, and NIH 3T3 cells and observed a twofold to fivefold decrease in growth rate, decreased colony formation, and inhibition of tumor formation in nude mice.\textsuperscript{244} Growth suppression by PML was further augmented by IFN, perhaps by stimulating expression of other nuclear body proteins.\textsuperscript{184} A structure/function analysis of the PML protein indicated that deletion or mutation of the RING finger motif of PML abrogates both NB formation and growth suppression.\textsuperscript{166-168,170} Deletion of the coiled-coil motif yielded a diffuse nuclear pattern of expression and no growth suppression, whereas deletion of the NLS of PML or expression of a splice variant of PML leads to a granular cytoplasmic pattern of expression and also abolished growth suppression.\textsuperscript{251} The proline-rich region at the N-terminus of PML and the serine proline variable region at the C-terminus of the protein were dispensable both for NB formation and growth suppression.\textsuperscript{167} Thus, PML expression in the nucleus is required for growth suppression, although a recent report questions whether expression in the NB per se was required for growth suppression.\textsuperscript{251} In support of this observation, infection of NIH 3T3 cells with LCMV virus rapidly relocalized PML into the cytoplasm and delayed apoptosis after serum starvation.\textsuperscript{168} Similarly, treatment of cells with antisense PML oligonucleotides delayed death after serum starvation. These results suggest that the correct localization of PML plays an important role in apoptosis and cell growth.

Recent data suggest that PML may promote cell death by novel mechanisms. PML interaction with the P0 ribosomal protein\textsuperscript{230} could target the 28S rRNA for cleavage, an important step during apoptosis.\textsuperscript{230} PML expression yields cell death that
is not associated with the usual chromatin condensation or activation of caspase 3 the major effector of apoptosis.\textsuperscript{252} Paradoxically, zVAD a caspase inhibitor accentuated PML-mediated apoptosis and increased PML expression levels in the NB, as did arsenic (see below). PML was found to recruit the pro-apoptotic BAX protein to the NB as well as the cell cycle inhibitor p27\textsuperscript{KIP1}; the importance of this recruitment remains unclear, because it is still not certain what processes occur in the NB. Use of PML\textsuperscript{+/−} animals yielded somewhat different results, with a 50% decrease in apoptosis of T cells after γ-irradiation and a great reduction in fas-induced apoptosis.\textsuperscript{253} PML null mice had no significant difference in expression of multiple apoptosis mediators, with the exception of caspase 1 and 3, whose activities, required for radiation and fas-mediated apoptosis, were markedly reduced. Furthermore, PML null animals were resistant to ceramide, TNF, and IFN-mediated apoptosis. Together, these data indicate a critical role for PML in multiple apoptotic pathways. High-level expression of PML may induce apoptosis in the absence of caspase action, whereas physiological levels may be required for the normal activation of caspases.\textsuperscript{254}

It is certain that PML is growth suppressive when overexpressed, but whether it is a tumor suppressor is uncertain. Mutations of PML have not been reported in other forms of cancer; even in t(15;17) APL, patients would not be expected to be null for this protein. However, new data from experiments with targeted disruption of the PML gene support a tumor-suppressor function for the protein.\textsuperscript{132,190} PML−/− mice were viable and fertile but highly susceptible to fungal or bacterial infections despite a strong inflammatory response,\textsuperscript{132} suggesting a functional defect in the inflammatory cells. Circulating and bone marrow mature myeloid cell counts were modestly decreased in these animals, indicating that, although PML was not required for the formation of the myeloid lineage, it may be involved in efficient terminal differentiation of these cells. Early death of these mice from infection prevented the long-term follow-up required to detect spontaneous tumor formation. However, the skin of PML−/− animals treated with the chemical tumor initiator DMBA formed twice as many papillomas as that of wild-type animals. In addition, animals treated with DMBA systemically formed twice the number of lymphomas as wild-type animals. PML replete animals. PML null murine embryonic fibroblasts proliferated more rapidly than wild-type fibroblasts and more readily formed colonies in soft agar. Whereas ATRA suppressed the growth of wild-type fibroblasts, there was little effect on the PML−/− cells. Whereas IFN inhibited the proliferation of normal marrow precursors as determined by clonogenic assays, α-IFN or γ-IFN had no myelosuppressive effect on PML−/− marrow.\textsuperscript{132}

PML may inhibit tumor growth in other ways as well. In a remarkable new study, PML was found to stimulate expression of MHC class I antigens and the transporters responsible for moving peptides to the cell surface in association with class I antigens.\textsuperscript{211} Hence, disruption of PML expression, whether by tumor viruses such as adenovirus or by the PML-RAR\textsubscript{α} fusion protein, might lead to decreased presentation of viral or oncoprotein antigens and defective immune surveillance for tumors. A recent study implicates PML in growth control by p53. PML expression was induced fivefold to 10-fold at the posttranscriptional level by ionizing radiation\textsuperscript{255} or a DNA-damaging agent. Overexpression of p53 in HeLa cells was also correlated with PML induction and G1 arrest, suggesting that PML might be considered a GADD (growth arrest and DNA damage) gene.\textsuperscript{256}

These exciting data suggest that PML can inhibit cell growth and may be required to mediate some of the physiological actions of the IFNs. To study potential effects of PML on RAR\textsubscript{α} signaling, an important target gene of RAR\textsubscript{α} in differentiation, p21\textsuperscript{WAF1/CIP1}, encoding a cyclin-dependent kinase inhibitor, was studied. Interestingly, p21\textsuperscript{WAF1/CIP1} could not be upregulated in the PML−/− fibroblasts. Thus, PML might be required for certain pathways of retinoid signaling. Therefore, PML-RAR\textsubscript{α} might also disrupt RAR\textsubscript{α} function in APL by blocking the ability of wild-type PML to cooperate with RAR\textsubscript{α} to stimulate myeloid differentiation. The L7 leucine zipper protein, a PML partner,\textsuperscript{220} modulates the transcriptional activity of nuclear receptor signaling complexes.\textsuperscript{257,258} It is therefore conceivable that this might mediate the effects of PML on retained nuclear receptor function.

In view of the emerging evidence, it is reasonable to describe PML as a tumor-suppressor protein involved in the growth suppression, differentiation, and immune response pathways of certain cytokines such as IFN. The mechanism by which PML encourages growth arrest is unclear, although interaction with Rb and related proteins offers an intriguing avenue. PML induces apoptosis in the absence of new protein synthesis \textsuperscript{252} and may act in both a caspase-dependent and -independent manner,\textsuperscript{254} suggesting that its role in transcription may be secondary to its role in growth control. Apoptosis by PML is intimately related to its localization in the NB organelle. The NB may be a repository for growth and apoptosis regulators, released or sequestered according to environmental cues, and could be a key component of the cell’s defense system against viral infections. Delocalization of PML by PML-RAR\textsubscript{α} may be a critical step in the pathogenesis of APL. Supporting this notion, PML-RAR\textsubscript{α} inhibited fas-mediated suppression of myeloid growth. When expressed in the PML+/− background, PML-RAR\textsubscript{α} further inhibited apoptosis, suggesting that PML-RAR\textsubscript{α} works in part by subverting normal PML function.\textsuperscript{253} In opposition to this view, the other PML-RAR\textsubscript{α} transgenic and PML null mice. APL could indeed develop very rapidly in the PML null background. More slowly in PML−/− mice, and with the longest latency in PML+/− mice.\textsuperscript{259}
mements of PML and RARα.12,260-264 PML-RARα cDNA was cloned from libraries derived from leukemic blasts of APL patients.11,13,15,170,170 Comparison of the cDNA structures obtained by multiple groups showed variation in the amount of PML sequences included in the fusion protein. The RARα portion was invariant, containing the DNA-binding and ligand-binding motifs (B-F domains).265 The PML sequence variation seen among patients was generated by heterogeneous breakpoint cluster regions as well as by alternative splicing.265-269 The most frequent breakpoint, bcr3, fuses PML exons 1 through 3 of PML, encoding the RING, B-boxes, and coiled/coil domains to RARα exon 3, encoding the B domain of the receptor. This breakpoint yields short PML-RARα fusion proteins [PML(S)-RARα]. Bcr1 is more 3′ within the PML gene and includes sequences from PML exons 5 and 6. This fuses up to 554 amino acids of PML to RARα and has C terminal serine-rich sequences of PML that are putative sites of phosphorylation [PML(L)-RARα]. Breakage in bcr2 involves sites in and around exon 6 of PML and leads to an intermediate length of PML sequence [PML(V)-RARα]. In general, 70% of patients exhibit PML(L)-RARα, 20% PML(S)-RARα, and 10% PML(V)-RARα.270,271 with PML(S)-RARα and PML(L)-RARα representing extremes of contiguous PML sequence fused to RARα. Internal splicing of portions of PML exon 3 led in one patient to a small PML-RARα fusion protein that contained the RING fingers, B boxes, and the first two portions of the α-helical coiled-coil domain, representing the minimal PML moiety required for oncogenicity.265 Each t(15;17) APL patient exhibits a unique set of PML-RARα fusion products indicative of a single breakpoint with alternative splicing, highlighting the clonal nature of the disease. Detection of the PML-RARα fusion transcript by reverse transcription-polymerase chain reaction (RT-PCR) is a sensitive272 and specific test for the diagnosis of APL and can be used to measure minimal residual disease after chemotherapy, differentiation therapy, and bone marrow transplantation.273,274 Reappearance of PML-RARα transcripts in the marrow often precedes a frank leukemic relapse.5,275-277 Initial studies indicated that patients treated with ATRA who harbored the PML(S)-RARα had a high likelihood of early death or relapse.278,279 One in vitro study indicated that blasts from APL patients with the PML(V)-RARα isomorph had decreased ATRA sensitivity.280 There was an association between the PML(S)-RARα, PML(L)-RARα, and PML(V)-RARα isoforms and more primitive morphology and secondary cytogenetic abnormalities, suggesting a biological difference between the isoforms.271 possibly due to abnormalities of DNA repair or cell cycle control. Despite the potential differences among the PML-RARα isoforms, numerous studies reported consistently good clinical outcomes in all APL patients.271,282,283 probably due to the highly effective nature of current therapy.

The mechanism by which the t(15;17) translocation occurs is not known. It may be that many illegitimate recombinations occur during normal cell division and are eliminated by DNA repair systems.284,285 Recent analysis found short stretches of identity between the PML and RARα genes in the breakpoint regions.286 It was proposed that random cleavage of the RARα and PML genes is followed by limited pairing of short stretches of homologous DNA, repair of the breaks, and joining of the loci. Those clones that contain the PML-RARα transcript survive and have a growth advantage.

The PML(L)-RARα fusion transcript yields proteins of 110 and 120 kD and PML(S)-RARα species of 103 and 90 kD,170,174,287 possibly resulting from alternative start codons.11,13,15,170,171 The fusion proteins of transfected cells or NB4 cells are about 10 kD larger than proteins generated by in vitro translation, suggesting the presence of posttranslational modifications.170,287 In APL cells, PML-RARα is present in great excess over wild-type RARα, making it the predominant retinoid receptor in those cells.265,287 Protein-protein interactions. PML-RARα oncoprotein, an aberrant retinoid receptor with altered DNA binding activity,11,13,15,170,171 can bind RARs as a homodimer,171,287 whereas wild-type RARα cannot.288 Homodimerization requires the coiled-coil domain of PML and not the E/F ligand binding/dimerization moiety of RARα (Fig 3). The first 2 (of the 4) hydrophobic clusters of the coiled-coil region also mediate PML-RARα/PML association. The smallest PML-RARα protein identified contained only clusters 1 and 2 (Fig 3).265 PML-RARα homodimers can be detected as a distinct DNA-binding species in nuclear extracts from NB4 cells287 and display weaker affinity for certain artificial and natural RARE sites than RAR/RXR heterodimers.287 When combined with RXR, PML-RARα forms multimeric complexes on the RARE,171,287,289 even in a 1:1 molar ratio of in vitro translated RXR to PML-RARα favors the formation of PML-RARα/RXR heterodimers.171,289 Hence, the existence of the PML-RARα homodimer complex in NB4 extracts probably reflects the high level of expression of PML-RARα relative to wild-type RARα and RXR in APL cells. When bound to RAREs along with RXR, PML-RARα displays the same binding site preference as wild-type RARα. The PML-RARα/RXR interaction does not require the DNA binding domain of the RARα moiety within PML-RARα,171 but can occur through the E/F domain of RARα. Multimeric complexes from transfected cells may reflect the ability of PML-RARα/RXR heterodimers on one DNA binding site to associate through the PML coiled-coil domain with heteromers on other sites. In the cell, this could reflect the ability of PML-RARα to efficiently bind to RXR and sequester it from wild-type RARα. These multimeric PML-RARα/RXR complexes were also seen on EMSA in extracts derived from NB4 cells.285 In addition, PML-RARα, detected by size exclusion chromatography by its ability to bind [3H]-ATRA, elutes with an apparent MW of 600 to 1,300 kD, further supporting the idea that PML-RARα multimerizes with itself and/or other proteins.290,291 Reinforcing these studies, confocal microscopy showed that PML-RARα draws RXR from its usual subnuclear localization into the compartment occupied by PML-RARα.170

Taken together, these data suggest that PML-RARα may affect ATRA-mediated signaling through several mechanisms: (1) binding of PML-RARα homodimers to a novel set of target genes, (2) binding of PML-RARα as homodimer or heterodimer with RXR to RARα target genes in competition with RARα, and (3) high levels of PML-RARα in APL cells could sequester RXR and/or other RARα cofactors in a novel nuclear and/or cytoplasmic compartment.
TRANSCRIPTIONAL ACTIVITY OF THE PML-RARα FUSION PROTEIN

The PML-RARα protein has altered transcriptional properties. Many groups observed, in the absence of ATRA, PML-RARα represses transcription from RAREs to a greater extent than RARα.13,170,292 This may be the most important quality of PML-RARα. There were conflicting reports regarding transcriptional activation by PML-RARα. In some reports, both PML-RARα (S) and (L) stimulated ATRA-mediated transactivation more strongly than RARα,13,15 whereas others found that PML-RARα activated weakly or not at all.170 Some of these differences may have been due to the use of different cell types or reporter genes. In general, when coexpressed with RARα, the fusion proteins behaved in a dominant negative fashion, reducing activation to the level of PML-RARα alone.157,292 RARα and PML-RARα, although activating transcription to different extents, had a similar ED50 for ATRA. In an allosteric manner.287 The mechanism of this activity is obscure, but this finding underscores the pleiotropic effects of PML-RARα. PML-RARα, when coexpressed with fos and jun, stimulates transcription from an AP1 binding site containing reporter gene in the presence of ATRA.295,296 Whereas PML could not be shown to directly interact with fos or jun, PML could be detected in an AP1 DNA-protein complex.203

PML-RARα also affects other transcriptional pathways important for myeloid differentiation. ATRA and RARα can inhibit transcriptional activation by the AP1 protein, possibly by a competitive effect between the ligand-engaged receptor and either fos or jun for a limiting amount of a common coactivator protein p300 and/or CBP.73 Somewhat paradoxically, PML-RARα, when coexpressed with fos and jun, stimulates transcription from an AP1 binding site containing reporter gene in the presence of ATRA.295,296

Distillation of these studies shows several points. (1) The transcriptional activity of PML-RARα varies depending on cell type and target promoter. (2) PML-RARα tends to suppress transcription of RARα target genes at physiological concentrations of ATRA to a greater extent than wild-type RARα. This effect, due to aberrant interactions with corepressors, may be critical for differentiation block in APL. (3) On some promoters, PML-RARα can activate transcription to a similar or greater
extent than wild-type RARα at 10^{-6} mol/L ATRA. (4) PML-RARα can suppress ATRA-mediated transcription by endogenous RARs as well as transfected wild-type receptors. This may be due to competitive binding by PML-RARα, which on some promoters displays intrinsically lower trans-activation potential, as well as competition with RARα for coactivators. (5) The synergistic effects of butyrate or TSA and ATRA on transcription by PML-RARα and in differentiation are likely due to inhibition of histone deacetylase and alterations of chromatin stimulating activation of RARα targets. (6) PML-RARα also effects the transcriptional function of other nuclear receptors as well as other transcription factors such as AP1 and STATs.

PML-RARα AND RETINOID RESISTANCE

ATRA-resistant APL cell lines derived by x-ray mutagenesis^{302} were found to lose expression of the PML-RARα protein, although the fusion gene and mRNA remained.^{302} This was due to a protease activity that degrades exogenous PML-RARα and is blocked by chemical inhibitors of the proteosome.^{303} This highlights the importance of the PML-RARα protein both in generating the APL phenotype and in mediating the unique sensitivity of the disease to ATRA. These data also imply that there are secondary changes in APL cells that maintain the transformed phenotype even in the absence of stable expression of the protein.

Resistant APL cells were also generated by prolonged culture of NB4 cells^{121} in the presence of ATRA.^{304,305} One such resistant cell line expressed the PML-RARα protein but had an abnormal retinoic acid binding profile^{305} and failed to upregulate tissue glutaminase II expression in response to ATRA. This was due to a missense mutation in the E domain of PML-RARα,^{306} which abolished its ability to bind ligand and mediate trans-activation by ATRA. These data support the notion that PML-RARα has a critical effect in blocking gene expression at low doses of ATRA and further underscores the fact that therapeutic response to ATRA in APL is dependent on the ability of the chimeric protein to activate transcription in the presence of ligand, reversing the blockade of target genes. In this and other resistant cell lines, at least one gene, CD18, continued to be induced by ATRA. This indicates that the remaining endogenous RARs within the cell can activate a subset of target genes, but the genes most critical for cell differentiation continue to be inhibited by PML-RARα.

These types of mutations are clinically relevant, because recent studies showed mutations in the ligand binding domain or adjacent AF-2 region in nearly 15% of patients both de novo but particularly after prolonged ATRA treatment,^{307,308} indicating that ATRA treatment puts a strong selective pressure for clones with defects in PML-RARα.^{308} However, the sequence of PML-RARα in cell lines derived from resistant patients and in primary patient specimens is most frequently normal, indicating that another mechanism must play a major role in ATRA resistance, such as loss of the PML-RARα fusion protein due to accelerated degradation or activation of novel oncoproteins.^{303,308,309}

PML-RARα AND THE NUCLEAR BODY

Whereas PML is localized in 6 to 30 large nuclear bodies/cell measuring between 0.2 and 0.3 μm^{191} in APL, it is delocalized to greater than 100 small (0.1 μm) microspeckles due to the ability of PML to heterodimerize with PML-RARα though the coiled-coil motif.^{170,191} PML-RARα draws other nuclear proteins, including SPI1,^{198} PLZF,^{218} RXR,^{192} and Rb,^{229} into the microspeckled structure as well. These microspeckles have no evident structure and colocalize with nascent RNA, signifying the transcriptional function of PML-RARα.^{191,201} In at least one set of studies, a large proportion of PML-RARα fusion was localized in the cytoplasm rather than the nucleus.^{173} This is consistent with the notion that PML-RARα, under low physiological concentrations of retinoids, acts as a dominant negative receptor drawing critical factors away from loci controlled by RARα to a new set of loci or to a transcriptionally inactive compartment.

ATRA treatment of APL cells relocates the PML protein into the wild-type nuclear body configuration.^{173,191,197,198,310} This is largely due to degradation of PML-RARα^{95,96,191,311,312} through the action of the proteosome.^{303} likely by the induction of a caspase 3-like activity after ATRA treatment.^{311} The specific cleavage of PML-RARα occurs C-terminal to the the RING, B-boxes, and coiled-coil motifs of PML, yielding a product recognized by RARα antibodies that contains residual PML sequences.^{95,96} The resulting protein could be predicted to be unable to bind wild-type PML, which would then be released and free to form its usual macromolecular complex in the NB. The remaining truncated PML-RARα protein might function in a similar fashion as wild-type RARα, activating its target genes and no longer sequestering other proteins critical for cell differentiation through the N-terminal PML moiety. However, early after ATRA treatment, reactivity to RARα antibodies is detected in the large nuclear bodies, suggesting that the PML-RARα protein itself undergoes some conformational shift or novel protein association after ATRA treatment, which then allows it to colocalize with wild-type PML in the NBs.^{173,197,198} It should also be noted that the PML(S)-RARα isoform does not contain the sequences required for caspase cleavage and does not undergo degradation after ATRA, yet these patients respond to ATRA therapy. In addition, in a model system, ATRA could induce differentiation even in the presence of caspase inhibitors, suggesting that the degradation of PML-RARα may not be essential for therapeutic response. The relatively slow reorganization of the NB is accelerated by other agents, such as cyclic AMP, which also increase the rate of differentiation, suggesting that altered phosphorylation of the PML-RARα fusion changes its rate of degradation and/or relocalization. Thus, PML-RARα relocalization is highly correlated with induction of differentiation in APL cells, suggesting that disruption of some component of the nuclear body other than PML plays a key role in this process.

Other agents promote normalization of the nuclear body structure through different mechanisms. Recent studies indicate that arsenic trioxide (As2 O3), a component of traditional Chinese medicine, induces complete clinical remission of APL in ATRA resistant patients. Whereas reaggregation of the nuclear body takes 1 to 2 days of ATRA exposure, treatment with As2 O3 leads to rapid formation of wild-type pattern nuclear
bodies within 6 hours, followed by loss of PML staining after 24 hours. This effect was observed both in NB4 and other cell lines and was enhanced by IFN. During this process, both PML and PML-RARα are targeted to the nuclear body and then rapidly degraded, although there is no effect on other NB proteins such as SP100 and relatively little degradation of endogenous RARα. Furthermore, As2O3 increased the transfer rate of PML from the nucleoplasm to the nuclear matrix and increased PML levels within the NB, accelerating apoptosis. It was recently shown that As2O3 induces the phosphorylation-dependent covalent linkage of PML to the ubiquitin-like molecule sentrin, perhaps targeting the protein for degradation. The C-terminal portion of PML was required for its targeted degradation. Whether sentrin binds PML-RARα is controversial and whether sentrinization or ubiquination of PML-RARα is required for its degradation is not yet certain. As2O3 induces degradation of PML-RARα even in APL cell lines resistant to ATRA. Both in vitro and in cells derived from patients undergoing As2O3 treatment, this correlates with only partial differentiation of the malignant promyelocytes and predominantly the induction of apoptosis. Simultaneous treatment with ATRA and As2O3 enhanced differentiation and apoptosis of NB4 cells and enhanced survival of animals harboring APL (H. de Thé, personal communication, December 1998). However, this was not the case in fresh human APL cells treated in vitro, making it not yet certain whether ATRA and arsenic might best be used concomitantly or as sequential agents in the treatment of APL. The organic arsenical melarsoprol may also provide effective treatment of APL and other hematological malignancies. As2O3 and melarsoprol induce apoptosis of an APL cell line without detectable PML-RARα as well as PML−/− murine fibroblasts. However, others have found a dependence of PML expression for arsenic induced apoptosis (H. de Thé, personal communication, December 1998), and one group found that a cell line harboring PLZF-RARα could not be induced to undergo apoptosis with arsenic, suggesting that the extreme sensitivity of APL cell lines to arsenic may be due to their dependence on PML-RARα for continued growth and the prevention of apoptosis. Intriguingly, antimony, a metal in the same column of the periodic table as arsenic, can also induce the degradation of PML and induction of apoptosis of APL cells, suggesting a common mechanism of covalent modification of critical cellular proteins by these heavy metals.

These results lead to a model in which treatment of APL can occur by two different mechanisms, each rescuing the disrupted nuclear body and perhaps restoring a growth control mechanism to the cell. In both cases, degradation of PML-RARα releases the complete block on cell differentiation. After both ATRA and As2O3 treatment, an initial hyperleukocytosis is noted. However, the complete lack of PML-RARα after As2O3 treatment leads to apoptosis, whereas the residual PML-RARα fragment present after ATRA treatment, in combination with residual RARα, induces the genes critical for cell differentiation. How these agents cause the retargeting of PML-RARα to the nuclear body is unknown. As2O3 can react with sulphydryl groups and alters phosphorylation pathways, a fact supported by the finding that As2O3 treatment is associated with hyperphosphorylation of the RARα itself. ATRA may cause a conformational shift in PML-RARα, allowing new sets of proteins to interact with the ligand binding domain. RXR may play a role in this conformation shift as well. When treated with ATRA plus an RARα antagonist, APL cells did not differentiate or reorganize their NBs. However, when treated with the same RARα antagonist plus a RXR agonist, both NB reorganization and differentiation occurred. These experiments underline the complexity of retinoid signaling and highlight the importance of both components of the RAR/RXR heterodimer in myeloid differentiation and APL pathogenesis.

Although the disruption of the nuclear body in t(15;17) APL is one of the most dramatic features of this disease, it may not be absolutely required for the pathogenesis of APL, because PML is in the wild-type configuration in variant forms of APL. Hence, it may be the degradation of PML-RARα rather than changes in PML/NB function that may be most critical for the induction of differentiation in APL. Alternatively, a component of the NB other than PML may be sequestered by all of the RARα chimeras.

CELLULAR MODELS OF PML-RARα FUNCTION

Cellular models of PML-RARα function have been hampered by the toxicity of the fusion protein, as underscored by studies in which the PML-RARα fusion protein could not be expressed after retroviral infection in nonhematopoietic cell lines and was expressed in only a few hematopoietic cell lines. PML-RARα retroviruses were difficult to generate due to the growth-suppressive effects of the protein on fibroblasts, including retroviral packaging cell lines. The most successful model of PML-RARα function in APL was constructed in the monocytoid U937 cell line. Cells stably or inducibly expressing PML-RARα failed to differentiate in response to ATRA or a combination of vitamin D3 plus transforming growth factor β (TGFβ). Under physiological concentrations of ATRA (10−9 mol/L), PML-RARα expression was associated with an increase in cell growth rate. However, when treated with 10−6 mol/L ATRA, PML-RARα expression was associated with markedly decreased cell proliferation and increased differentiation. The change in growth rate was due to an increase in apoptosis and not to alterations in the cell cycle. In addition, when grown under conditions of reduced serum, PML-RARα–expressing U937 cells proliferated, whereas control cells underwent apoptosis. PML-RARα also blocked apoptosis in response to TNFα. TNF resistance was due to a posttranscriptional downregulation of the TNF receptor, allowing the APL cell to escape an autologous growth inhibitory mechanism, because APL cells secrete high levels of TNFα. From these data it was proposed that PML-RARα might function to promote cell survival. This idea was supported by the fact that myeloid, G-CSF–dependent TF-1 cells expressing PML-RARα were protected from apoptosis induced by G-CSF withdrawal. In addition, recent data indicate that ablation of PML-RARα expression in NB4 APL cells either by homologous recombination or expression of a ribozyme induces apoptosis, even in ATRA-resistant cells. The fraction of cycling cells in APL is relatively low; thus, the persistence of cells due to the anti-apoptotic effects of PML-RARα could be critical. If PML-RARα prevents apoptosis when cells are grown under physiological conditions of ATRA, how does the protein encourage apoptosis when such cells are treated with pharmaco-
logical ATRA or As$_2$O$_3$? 311,315,318,319,333 One possibility is that PML-RAR$\alpha$ is degraded under these conditions, 30,311 removing the protective agent. Alternatively, these agents, through the action of PML-RAR$\alpha$, may activate novel target genes that play a role in cell cycle arrest and apoptosis. The p21 gene is such a candidate, being activated by both ATRA130 and arsenic. 319 As$_2$O$_3$-mediated apoptosis of APL cells is accelerated by agents that deplete cellular glutathione, a scavenger of free radicals. 334

Given recent data that p53 induces apoptosis through generation of reactive oxygen species, 335 there may be overlap between the p53 and As$_2$O$_3$ apoptotic pathways.

One of the drawbacks of the U937 model is that these cells undergo monocytic rather than granulocytic differentiation. One model, possibly more reflective of the pathophysiological role of PML-RAR$\alpha$, was created by transient expression of PML-RAR$\alpha$ in HL60 cells. This inhibited granulocytic differentiation induced by ATRA and vitamin D3 but not granulocytic differentiation induced by dimethyl sulfoxide (DMSO) or monocytic differentiation induced by phorbol ester. These results support a relatively specific mechanism of action for PML-RAR$\alpha$s upon nuclear receptor pathways. However, NB4 cells cannot differentiate in response to vitamin D3, 300,327 but when ATRA and D3 are added in concert or sequentially (ATRA first), marked differentiation and inhibition of proliferation occurs. In addition, NB4 cells are resistant to polar compounds such as sodium butyrate or HMBA, unless pretreated with ATRA for a period as brief as 30 minutes. 390 Thus, PML-RAR$\alpha$s may affect non-nuclear receptor differentiation pathways as well (see below). Recently, PML-RAR$\alpha$s was shown to enhance the proliferation of murine bone marrow progenitor cells after retroviral transfer, allowing these cells to be serially replated ex vivo. However, the cells remained growth factor dependent, suggesting that PML-RAR$\alpha$ on its own cannot completely transform the cell. The cell lines resulting from PML-RAR$\alpha$s expression were undifferentiated and could be induced to stop proliferation and undergo differentiation after ATRA treatment. 336

The U937 model was used to determine which structural features of the PML-RAR$\alpha$s fusion protein were critical for effects on cell growth and differentiation. 160 PML expression did not block differentiation induced by vitamin D3 and TGF$\beta$, whereas RAR$\alpha$s did slightly, perhaps due to sequestration of RXR. PML-RAR$\alpha$s was a more potent inhibitor of differentiation. Deletion analysis showed that the first coiled-coil motif of PML-RAR$\alpha$s was required for its ability to block differentiation, but deletion of this segment did not affect PML/PML-RAR$\alpha$s interaction, NB disruption, or RARE-dependent trans-activation. This suggests that disruption of PML within the nuclear body is not critical for the action of PML-RAR$\alpha$s and that the ability of PML-RAR$\alpha$s to interact with an unidentified factor, through the coiled-coil motif, may be critical for its function. In support of this hypothesis, deletion of coil 2 prevents PML-RAR$\alpha$s from delocalizing PML from the NBs, but still allows disruption of the nuclear body pattern of Sp100. This protein or another of the multiple proteins within the nuclear body such as Rb28 could represent the target protein of PML-RAR$\alpha$s. This is not likely to be RXR, because coexpression of RXR prevented differentiation block induced by wild-type RAR$\alpha$s but not the block mediated by PML-RAR$\alpha$s. The RING finger and B box motifs are also always found in the PML-RAR$\alpha$s fusion protein of APL patients, suggesting that the integrity of the RBCC unit is required for leukemogenesis. Furthermore, the ability of PML-RAR$\alpha$s to induce apoptosis in nonhematopoietic cells depends on the integrity of the RING finger/B-box motifs and the microspeckled localization of PML-RAR$\alpha$s. How this relates to APL pathogenesis by PML-RAR$\alpha$s is unclear. 326 Lastly, as noted, there are subtle differences in the PML(L)-RAR$\alpha$s and PML(S)-RAR$\alpha$s isoforms. In TF-1 cells, only the long PML isoform inhibited cell growth, whereas only the short isoform protected the cells from growth factor withdrawal. When cells were treated with ATRA, they underwent apoptosis, with the long isoform showing a more prominent effect. 314

Several major conclusions can be reached from these cellular models. (1) PML-RAR$\alpha$s does not activate cell growth or confer factor-independent growth to cells and in this regard is not a conventional oncogene. (2) PML-RAR$\alpha$s alters the cell setpoint for apoptosis. It inhibits apoptosis due to growth factor withdrawal in hematopoietic cells, but encourages it in the presence of ATRA or arsenic. In many nonhematopoietic cells, PML-RAR$\alpha$s is toxic and induces apoptosis. (3) PML-RAR$\alpha$s can inhibit differentiation mediated by nuclear receptor pathways and some non-nuclear receptor pathways. The mechanism of cross-talk with nonreceptor pathways is unknown. (4) PML-RAR$\alpha$s requires the first coiled domain of PML and the AF2 domain, which binds HDAC complexes, of RAR$\alpha$s to block differentiation. The second coiled domain, which delocalizes PML, is dispensable for differentiation block. The first coiled-coil motif of PML may contact a critical cofactor. (5) The PML(S)-RAR$\alpha$s isoform, which lacks phosphorylation sites, and the nuclear localization sequence of PML may have somewhat different biological properties from the PML(L)-RAR$\alpha$s isoform.

ANIMAL MODELS OF PML-RAR$\alpha$s FUNCTION

Several laboratories have tried to model APL in animals. Infection of avian bone marrow with a PML-RAR$\alpha$-containing retrovirus resulted in an undifferentiated form of leukemia that did not respond to ATRA. 337 Curiously, the PML-RAR$\alpha$s gene harbored in these leukemic clones bore two point mutations. The first, located in the RING finger, caused the fusion protein to lose the typical microspeckled pattern and have altered transcriptional properties. The second replaced a serine with an alanine residue in the coiled-coil domain, indicating that there may be two points of control by ATRA in hematopoiesis, one at the pluripotent progenitor stage and a second at the promyelocyte stage. 338 Why RAR$\alpha$s disruption clinically yields only the promyelocyte phenotype is uncertain.

The first transgenic mouse created that expressed the PML-RAR$\alpha$s fusion used the CD11b promoter, which was expressed relatively late in myeloid maturation. These mice did not develop APL or a preleukemic syndrome, but did show a defect in myeloid progenitor response to cytokines and profound neutropenia after sublethal irradiation, implying that the PML-
RARα protein impaired myeloid development.\textsuperscript{339} Another transgenic murine model expressed the PML-RARα transgene from the metallothionine promoter.\textsuperscript{340} The investigators had difficulty obtaining transgenic founders, likely reflecting the poor tolerance of PML-RARα by nonhematopoietic cells. One animal line expressed PML-RARα only in the liver and brain, which was induced by the addition of Zn\textsuperscript{2+} to the animals' drinking water. These animals developed liver pathology, including hepatocellular carcinoma after only 5 days of induction with zinc. ATRA treatment did not prevent the emergence of liver disease, which was associated with an increased proliferation rate and no change in spontaneous apoptosis. These experiments confirmed the oncogenic nature of PML-RARα and suggested that, under certain circumstances, the protein could accelerate cell proliferation. Two groups performed transgenic experiments with PML-RARα using the cathepsin G promoter.\textsuperscript{341,342} These mice developed a preleukemic syndrome characterized by an increase in immature myeloid forms in the bone marrow and splenomegaly due to extramedullary hemato poiesis,\textsuperscript{341} About 10\% to 30\% of the animals developed leukemia, with a median latency of 300 days; it was also associated with anemia, thrombocytopenia, and massive splenomegaly. A modest peripheral promyelocyte count of only 7\% was noted,\textsuperscript{342} with some cells harboring Auer rods. No bleeding diathesis was noted. Unlike human APL, many fully differentiated granulocytes were present in the peripheral blood before treatment with ATRA. ATRA treatment led to an initial increase in peripheral white blood cell count, reminiscent of the retinoic acid syndrome\textsuperscript{88} and consistent with the mobilization of cells in peripheral white blood cell count, indicative of the retinoic-acid syndrome\textsuperscript{88} and consistent with the mobilization of cells from the marrow. This was followed by a decrease in the leukocyte count and the appearance of differentiated neutrophils. However, after ATRA treatment, Grisolano et al\textsuperscript{341} found that promyelocyte counts decreased and the cells appeared to undergo apoptosis rather than differentiation. Therefore, whether ATRA induced differentiation of the promyelocytes or selectively killed the immature cells is not certain, because differentiated cells were present both before and after ATRA treatment. These animals therefore offer a somewhat imperfect model for differentiation therapy. Intriguingly, as in humans,\textsuperscript{7,8} all mice relapsed even with the continuation of ATRA, suggesting that other oncogenic lesions must be involved in APL development.\textsuperscript{299}

The mouse model of APL most similar to human disease was generated by use of the MRP\textsubscript{8} promoter, which is expressed at the promyelocyte to metamyelocyte stage and continues to be active in mature neutrophils,\textsuperscript{343} as opposed to the cathepsin G gene, which is expressed during a more narrow window of promyelocyte differentiation.\textsuperscript{344} These mice also developed a preleukemic phase, and about one third developed promyelo cytic leukemia with a median latency of 6 months\textsuperscript{85} (S. Kogan, personal communication, July 1998), accompanied by bleeding, anemia, thrombocytopenia, and a low leukocyte count, all characteristic of human APL. When these cells were placed into culture and treated with ATRA, differentiated neutrophils were observed. The transgenic mice also developed epidermal papil lomas, which further demonstrated the neoplastic activity of the PML-RARα fusion. When the animals were treated with ATRA, mature neutrophils appeared in the peripheral blood and marrow and splenomegaly was reduced, consistent with clinical differentiation. Highly purified, residual nonleukemic progenitor cells from APL patients are PML-RARα negative,\textsuperscript{346} suggesting that the PML promoter driving PML-RARα expression must function at a specific stage of myeloid differentiation. Therefore, a knock-in\textsuperscript{347} strategy for the creation of transgenic mice might yield the most physiologic model of t(15;17) translocation APL.

In all of these models, the delay in onset of leukemia suggests that a second, as yet uncharacterized, genetic hit is required for neoplastic transformation. Whether the leukemias that do develop in the mice are monoclonal or polyclonal has not yet been determined. A monoclonal origin would be consistent with a model of tumor promotion induced by PML-RARα, potentially by preventing apoptosis and blocking differentiation, followed by a second genetic lesion. Such a multistep pathway was modeled by the introduction of N-ras into the hematopoietic cells derived from mice harboring the PML-RARα fusion expressed from the CD11b promoter.\textsuperscript{348} As a result, there was a 10- to 100-fold synergistic increase in myeloid colonies. With this proof of principle, we can anticipate experiments in which PML-RARα mice could be crossed with mice expressing activated oncogenes or lacking tumor suppressors. The APL that develops spontaneously in PML-RARα mice could also be screened for secondary mutations required for pathogenesis.

**SUGGESTED MODEL OF PML-RARα ACTION IN APL**

Myeloid differentiation usually occurs at physiological levels of ATRA (10\textsuperscript{-8} mol/L), which activates key RARs target genes. These target genes may contain high-affinity RARs or bind factors that cooperate with the RARα to load the basal transcriptional machinery onto the promoter, even when only a fraction of cellular RARα is engaged by ligand. The highly expressed PML-RARα protein may functionally sequester RARα cofactors from the wild-type receptor or bind to critical genes in place of RARα. Even if a PML-RARα/RXR heterodimer bound to the critical genes, the increased affinity of PML-RARα for corepressors\textsuperscript{294,299} would make it a poor activator of RARα target genes. This would overwhelm the usual tonic effect of RARα to induce myeloid differentiation at low ATRA concentrations (Fig 5).

In the presence of pharmacological doses of ATRA, the PML-RARα fusion releases the corepressors and stimulates transcription of target genes that allow myeloid development to proceed. Furthermore, the PML-RARα protein is degraded\textsuperscript{95,96} and wild-type RARα is upregulated,\textsuperscript{94} shifting the balance of power of RARs in the cell from PML-RARα to RARα. However, the fact that PML-RARα can confer ATRA sensitivity to mutant HL60 cell lines without endogenous wild-type RARα suggests that PML-RARα does mediate at least part of the pro-differentiative\textsuperscript{149} effect. A PML-RARα/RXR heterodimer is probably the mediator of differentiation, because this process is synergistically stimulated by a combination of RARα- and RXR-specific ligands.\textsuperscript{68,76} In addition, brief treatment of NB4 cells or fresh APL cells with ATRA allows potent differentiation of APL cells to proceed in the presence of other agents such as hexamethylene bis acetamide (HMBA), cyclic AMP, and vitamin D3,\textsuperscript{300,350} suggesting that rapid transcriptional events before PML-RARα degradation mediate differentiation. These events might also include activation of other nuclear receptors, STATs
Fig 5.
and APL. In contrast, arsenic degrades PML-RARα fusion without RARα-mediated signaling. Modest differentiation might occur in this case by low level signaling through the endogenous RARα. A component in the pathogenesis of APL may include the delocalization of one or more key proteins from the NB. Current evidence points away from this being PML itself.

**RARα-PML**

The reciprocal RARα-PML fusion generated in t(15;17) is present in 70% to 80% of APL cases. Two transcripts can be generated from the alternative RARα promoters, of which RARα1-PML was the most common. As in the case of PML-RARα, several different forms of RARα-PML were also found due to alternative breakpoints within the PML gene. RARα1-PML and RARα2-PML transcripts from patients with breakpoints 5' in the PML gene encode RARα-PML proteins, whereas fusion transcripts derived from a more 3' breakpoint in the PML gene only encode a truncated peptide containing a portion of the RAR A domain. RARα-PML contains the A1 or A2 domain of the RARα protein fused to a variable portion of the PML protein, due to alternative splicing, including the serine-rich C-terminal domain. It is hard to predict the effects of RARα-PML, because the role of the C-terminus of PML is unknown. There were cases of APL associated with interstitial, nonreciprocal fusions of the PML and RARα genes that did not generate an RARα-PML fusion gene. Furthermore, there is no difference in ATRA sensitivity or clinical outcomes of patients who do or do not harbor the RARα-PML transcript. Patients with prolonged remissions of APL may express the RARα-PML transcript and not the PML-RARα transcript, suggesting that the small number of cells that harbor these genes may not have leukemogenic potential, although a sole APL patient was reported to possess the RARα-PML and not the PML-RARα transcript. In general, RARα-PML does not appear to be required for the pathogenesis of APL. Although transgenic mice harboring the RARα-PML fusion did not develop leukemia, when crossed with PML-RARα mice, leukemia developed with greater frequency. Hence, the RARα-PML may contribute to the disease process.

**PLZF**

The PLZF gene was initially identified by its rearrangement in an APL patient from Shanghai with translocation (11;17)(q23;q21). Eight confirmed cases of t(11;17)(q23;q21) APL fusing the PLZF and RARα genes were described, and a recent workshop identified a total of 8 cases around the world. Morphologic review of the original 6 patients and the 8 cases of the workshop showed features intermediate between M2 and M3 leukemia, with sparser granules, a lack of Faggot cells, and the absence of a bilobed nucleus. Strikingly, these 6 patients were resistant to differentiation therapy with ATRA as well as chemotherapy. Leukemic cells from these patients could not be induced to differentiate with ATRA in vitro. Translocation (11;17)(q23;q21)-APL is unique in its resistance to ATRA, suggesting a critical difference between PLZF and the other four fusion partners of RARα in APL. In ATRA, a critical difference between PLZF and the other four fusion partners of RARα in APL (Table 4). However, in opposition to this view, cells from 1 patient with t(11;17)(q23;q21) APL could be induced to differentiate when treated with a combination of ATRA and G-CSF but not ATRA alone, and this patient was successfully treated with a combination of ATRA and G-CSF. One other patient achieved a remission with concurrent ATRA and chemotherapy, suggesting that the resistance of this form of APL may not be absolute.

The PLZF gene, localized on chromosome 11q23, yields a 7-kb mRNA that codes for a zinc finger transcription factor. The PLZF gene is 1 Mb telomeric to the MLL/HRX gene, which is frequently rearranged in leukemia. The genomic structure of PLZF is incompletely known, but it is clear that the N-terminal portion is encoded on a single exon and that the C-terminal zinc finger motifs are encoded by a number of small exons. The PLZF gene codes for a DNA-binding protein of 673 amino acids with nine Krüppel-like C2H2 zinc finger domains with a predicted molecular weight of 74 kD (Fig 6) that migrates through polyacrylamide gels with an apparent MW of 80 to 90 kD. The N-terminal 118 amino acids encode a POZ (Pox virus and Zinc finger) or BTB (Broad Complex, tramtrack, Bric a Brac) domain. The POZ/BTB domain mediates protein self-association and heterotypic associations and acts as a transcriptional repression domain within zinc finger basic-zipper transcription factors. The POZ/BTB domain can be involved in chromatin remodeling and histone mobilization and transcriptional repression through interaction with histone deacetylase (see below). Physical characterization of the PLZF POZ/BTB domain found it to be a tight dimer in solution (Kd = 2 × 10^-7), with a high amount of helical structure. Characterization of a 1.9Å crystal structure of the BTB/POZ domain of PLZF confirmed these findings, showing a highly intertwined dimer with a large hydrophobic interface. The top portion of the dimer structure forms a groove exposed to solvent lined with conserved charged amino acids potentially representing a peptide binding site (Fig 7). Several missense mutations of conserved residues interfere with dimer contacts and disrupt the ability of the POZ domain to repress transcription, suggesting that dimerization and repression may be closely linked.
PLZF NUCLEAR LOCALIZATION

In transfected cells, PLZF is localized to the nucleus and is phosphorylated on serine and threonine residues (Shaknovich and Licht, unpublished data). MDS, a primitive leukemic cell line, expresses high levels of PLZF mRNA and PLZF protein when treated with the calcium ionophore A23187. Confocal microscopy of MDS cells showed that PLZF localized to approximately 50 small nuclear subdomains, with a lower

Fig 6. Functional domains of the PLZF protein and structure of the PLZF-RARα and reciprocal RARα-PLZF proteins generated in t(11q23;17) APL. PLZF-RARα always contains the N-terminal POZ/ BTB-self-association domain. Heterogeneity in the PLZF gene breakpoint can yield PLZF-RARα fusion proteins, including either the first two or first three zinc fingers of PLZF. The reciprocal RARα-PLZF protein can bind to PLZF binding sites and contains the last six or seven of the nine PLZF zinc fingers linked to the AF1 activation domain of RARα rather than the PLZF repression domains.

Fig 7. Crystal structure at 1.9Å of the PLZF BTB/POZ domain dimer as viewed from the side.
level of diffuse nuclear staining also noted, whereas only 10 PML-containing NBs were noted per cell. The speckled pattern of subnuclear expression of PLZF depends on the presence of the POZ/BTB domain as removal of this domain leads to the same or similar domains. In hematopoietic cells that naturally express both PML and PLZF as well as in transiently transfected nonhematopoietic cells, wild-type PML and PLZF could colocalize in nuclear body structures. However, this colocalization was not complete, indicating that PLZF may act in both the nuclear body and other subnuclear compartments. The PLZF-RARα fusion protein, either transcribed into cells or...
naturally expressed in blasts from a patient with t(11;17)(q23; q21) APL, did not colocalize with PML or delocalize PML from nuclear body structures. This critical fact indicates that delocalization of the PML component of the nuclear body is not required for the pathogenesis of APL. In contrast, in t(15;17) APL cells, PLZF is delocalized into a microspeckled pattern identical to PML-RARα. The coiled-coil region of PML, which is also responsible for PML self-association, was required for the PML-PLZF interaction. The first two zinc fingers of PLZF and not the POZ/BTB domain mediates the interaction of PLZF with PML (A. Zelent, personal communication, December 1998). Therefore, PML and other related RING finger proteins might be cofactors for PLZF function. Delocalization of PLZF might be a common and central theme in the pathogenesis of APL, because PLZF is also found in an abnormal microspeckled pattern and in t(5;17)-APL associated with the NPM-RARα protein.

PLZF mRNA is expressed in myeloid cell lines such as KG1, HL60, and NB4 as well as in the multipotent cell line FDCPMixA4. It is expressed at lower levels in more differentiated erythroleukemia, promyelocytic, and monocytic cell lines as well as in peripheral blood mononuclear cells, tissue macrophages (Fallon and Licht, unpublished data), and pro-B–cell lines. PLZF is also expressed in mature B-cell and chronic lymphocytic leukemia (CLL) specimens. PLZF is downregulated during ATRA-mediated differentiation of NB4 and HL60 cells (Chen et al and Chen et al, unpublished data) and during differentiation of FDCPMixA4. In contrast, PLZF is upregulated in the MDS cell line after treatment with calcium ionophore, perhaps recapitulating some aspect of monocyte development. In embryonic stem cells, PLZF levels increase as the cells are allowed to form embryoid bodies that presumably contain hematopoietic elements. Finally, CD34+ human progenitor cells could be immunostained with PLZF antisera in a distinct nuclear speckled pattern. When such cells were placed into culture and allowed to differentiate, PLZF levels transiently increased then declined (C. Labbaye, personal communication, December 1998). Incubation of human bone marrow with antisense PLZF oligonucleotides led to a decrease in the number of burst-forming unit-erythroid (BFU-E) and colony-forming unit–granulocyte-macrophage (CFU-GM) colonies (Shaknovich et al, unpublished data). Taken together, these data indicate that PLZF expression may be important for the maintenance or survival of hematopoietic stem cells and or early progenitors. Scheduled, regulated downregulation of PLZF may be required for normal hematopoietic differentiation and proliferation.

In the murine embryo, PLZF is expressed in the aorta, gonadal, mesonephros region (AGM), a zone containing hematopoietic precursors. Also, during mouse embryogenesis, PLZF is prominently expressed in the developing neural tube. Expression throughout the central nervous system (CNS) is initially uniform at day 8.5 postconception (pc). Subsequently expression is downregulated in rhombic segments 3 and 5 at the same time that genes such as krox-20 and hoxb2 are upregulated in these segments. A PLZF site was found in the hoxb2 5′ flanking region and PLZF could repress the hoxb2 promoter in cotransfection assays, suggesting that PLZF might directly regulate this gene. By 10 days pc, PLZF expression is restricted to the boundaries of the rhombomeres, perhaps acting to limit the expression of critical pattern formation genes. Early widespread expression of PLZF may initially repress developmental programs within the CNS, and the selective downregulation of PLZF could lead to segmental identities. Similarly, PLZF may repress the differentiated phenotype of myeloid cells, and its downregulation may allow differentiation to proceed (see below). It is reasonable to theorize that altered regulation of homeobox genes may be central to PLZF action in the development of both the CNS and hematopoietic system. Other sites of PLZF expression include neural crest cells, branchial arches, facial processes, and apical epidermal ridges of the developing mouse and chick limb buds (C. Tabin, personal communication, July 1998). The latter are sites of signaling between the epithelium and the underlying mesenchyme and suggest a role for PLZF in limb patterning. PLZF is also expressed in the mesonephros, a precursor kidney structure, and in the dilated structures found in autosomal dominant polycystic kidney disease (P. Wilson and J. Licht, unpublished data), which are felt to partially recapitulate early renal development. Murine PLZF expression is considerably upregulated in the liver, heart, and kidney in the perinatal period and shortly after birth. In mutant albino mice that have a defect in tyrosine metabolism PLZF, along with a number of liver-specific factors, including HNF-1, HNF-4, and C/EBPs, fail to be induced at birth, grouping these genes in a common regulatory pathway. Lastly, a search of the EST database (http://www.tigr.org) indicates that PLZF is expressed in both skeletal muscle and adipose tissue. In summary, although isolated by its involvement in APL, PLZF may play a role in nervous system development and limb patterning, renal development, hematopoietic development, and energy metabolism. Preliminary analysis of PLZF knockout animals found that they weighed up to 40% less than heterozygous littermates and have abnormally kinked tails and multiple skeletal defects, including foreshortening of the limb and fused digits. This suggests that PLZF, like RARα, might affect the expression of Hox genes involved in limb and body patterning. Additionally, PLZF may influence genes responsible for apoptosis limb and digit development. Homozygous PLZF null mice are sterile with testicular hypotrophy and impaired spermatogenesis. Disruption of other transcription factors, including CREM, RXRβ, and A-myb, has a similar phenotype, suggesting similar or overlapping functions. Intriguingly, cyclin A, the expression of which is affected by PLZF (see below), is expressed in a distinct developmental pattern in the developing testis. This suggests that disruption of PLZF expression might affect cell cycle regulators critical for spermatogenesis. PLZF null mice are viable, and, to date, these mice have not exhibited an obvious hematopoietic phenotype and neither have they developed leukemia or other tumors. This does not rule out a role for PLZF in hematopoiesis and could indicate the presence of redundant genes to partially compensate for the lack of PLZF during development.

TRANSCRIPTIONAL FUNCTION OF PLZF

PLZF is a sequence-specific DNA binding protein that can recognize a TA-rich sequence derived from a pool of random-
ized oligonucleotides. A binding site for PLZF was fortuitously discovered in a yeast two hybrid screening experiment. PLZF fused to an acidic activation domain was isolated by its ability to activate a bacterial lex operator-containing reporter gene in yeast. The lex operator sequence actually has some similarity to the artificially selected PLZF site, a PLZF binding site within the cyclin A promoter, and a human genomic DNA fragment. With alignment of these sites, a relatively loose consensus sequence of GT (A/C)(A/C) AGT can be derived. The PLZF binding site derived from site selection can be recognized by the C-terminal seven zinc fingers retained in the RARα-PLZF fusion protein. Similarly, the lex operator site can be bound by proteins containing the last 7 or 5 or 4 zinc fingers of PLZF. The exact role of the first two zinc fingers, which are retained in PLZF-RARα in DNA binding, is not yet clear; they may play a role in associations with proteins such as PML. Given the relatively small length of the PLZF binding site, it is likely that the last 4 finger motifs may directly bind DNA, whereas others may either play a supporting role by interacting with the phosphatase backbone in a manner similar to the Gli zinc finger protein, whereas others might participate in protein interactions.

Reporter genes containing either the artificial PLZF binding site or the lex operator are repressed by coexpression of the PLZF protein. In contrast, the RARα-PLZF fusion protein can activate a reporter containing a TA-rich PLZF binding site, whereas PLZF-RARα has no effect on these genes. This information suggests that the RARα-PLZF protein could act in a dominant negative manner, binding and altering transcription of PLZF target genes. By fusing portions of PLZF to the heterologous GAL4 DNA binding domain, the PLZF protein was found to contain two separable transcriptional repression domains, one of which overlaps the POZ/BTB domain. Similarly, the POZ/BTB domains of the Bcl-6 and ZF5 proteins were found to mediate transcriptional repression. It is not certain whether the same portions of the POZ/BTB motif are required for PLZF repression, self-association, localization into subnuclear speckles, and association with cofactors. However, we recently created missense mutations in the POZ/BTB domain that abrogated repression and that will aid in the molecular characterization of the POZ/BTB domain. The mechanism of transcriptional repression by PLZF is rapidly becoming elucidated. PLZF was found to interact both in vitro and in vivo with the corepressors N-CoR and SMRT and Sin3A and HDAC1 (histone deacetylase 1). These interactions occur via the POZ/BTB domain of PLZF, although other regions of PLZF, including the zinc finger motifs, may contribute to binding. This correlates with the fact that there are two repression domains in PLZF, with one clearly mapping outside of the POZ domain. PLZF, in turn, binds to specific regions of N-CoR and SMRT and sin3a and sin3b. SMRT colocalizes with PLZF in nuclear speckles and is able to potentiate the ability of a GAL4-PLZF POZ/BTB domain fusion protein to repress transcription. Transcriptional repression by PLZF was potentiated by coexpression of the corepressors and only partially blocked by the HDAC inhibitor trichostatin A, suggesting additional mechanisms of repression other than alteration of chromatin. Other investigators showed that the Bcl-6 POZ/BTB domain also associates with SMRT and other members of the HDAC complex. Thus, it could be surmised that many POZ/BTB repressors work similarly by interacting with a multiprotein repressor complex that contains N-CoR, SMRT, sin3A/b, and histone deacetylases, leading to alterations of chromatin configuration. Additional mechanisms could be at play as well, because our group found that PLZF forms a DNA-protein complex with a molecular weight of nearly 600 kD that contained cdc2, which was implicated in transcriptional repression by phosphorylation of basal transcription factors.

GROWTH SUPPRESSION BY PLZF

PLZF is similar to PML in that both proteins can repress cell growth. Pools of the interleukin-3 (IL-3)-dependent, nontumorigenic murine myeloid 32DC13 cells overexpressing the PLZF protein were highly growth inhibited when cultured in IL-3, with their doubling time increasing from 18 hours to greater than 3 days. These cells were retarded in the G1 phase of the cell cycle and had a twofold to threefold increase in the spontaneous rate of apoptosis when grown in IL-3. Curiously, PLZF-expressing cells also secreted a negative growth factor into condition cell media that inhibited the growth of non–PLZF-expressing cells. PLZF expression was also associated with inhibition of myeloid differentiation induced by G-CSF or GM-CSF, upregulation of the early hematopoietic marker Sca1, and downregulation of the differentiated granulocytic marker Gr1. The molecular mechanism of action of PLZF on cell growth is beginning to be elucidated. Acute infection of myeloid cells with a PLZF-containing retrovirus was associated with growth arrest of cells in the S-phase of the cell cycle. Progress of cells from G1 into S phase is largely controlled by phosphorylation events mediated by cyclin A paired with CDK2. Fibroblasts expressing PLZF were growth suppressed and showed blunted induction of cyclin A when stimulated from G0 into the cell cycle by serum. PLZF can bind two sites derived from the cyclin A promoter and can downregulate the cyclin A2 promoter in cotransfection experiments. Furthermore, PLZF-expressing, growth-suppressed 32DC13 cells regain a normal rate of cellular growth when superinfected with a cyclin A-containing retrovirus. These data suggest that cyclin A is a bona fide target gene of PLZF and that PLZF can inhibit cellular growth in a variety of cell types by altering the expression of regulators of the cell cycle.

PLZF expression was associated with protection of 32D cells from apoptosis associated with IL-3 withdrawal, suggesting that PLZF might reset the set point between cell life and death, possibly by affecting the expression of bcl-2, bcl-x, bad, or other regulators of apoptosis. It is interesting to speculate that high-level PLZF expression plays a role in the quiescence and resistance to apoptosis exhibited by hematopoietic stem cells. Downregulation of PLZF during myeloid differentiation may be accompanied by cycles of committed cell division. It might be argued that PLZF is actually a tumor suppressor disrupted in t(11;17)(q23;q21)-APL. The resulting PLZF-RARα fusion proteins may then act as a dominant negative inhibitors of normal PLZF function (see below). Hence, t(11;17)(q23;q21) APL cells might be functionally null for PLZF. Disruption of PLZF function may also play a role in t(15;17)
APL, because PML-RARα delocalizes PLZF in these cells and may thus interfere in its function.216,299

**PLZF-RARα**

The t(11;17)(q23;q21) fusion yields two reciprocal transcripts (PLZF-RARα and RARα/PLZF)361,362 (Fig 6). The breakpoint within the PLZF gene occurs 3′ to the first translated exon. As a result of the fusion, the PLZF-RARα chimera contains the entire N-terminal transcriptional effector region of PLZF (including the POZ/BTB domain) as well as the first two zinc fingers of the protein. As in all forms of APL, the RARα gene is fused in the region corresponding to the B domain. In one case, a fusion of the N-terminus of PLZF and the first three PLZF zinc fingers (up to amino acid 484 of PLZF) was linked to RARα, indicating a breakpoint in the PLZF gene further 3′ within the gene. In 4 of 7 cases tested, a reciprocal RARα/PLZF transcript was detected, linking the A/AF1 ligand-independent transcriptional activation domain of RARα42 to the last 7 zinc fingers of PLZF.

In comparing PML-RARα and PLZF-RARα, some interesting contrasts and similarities can be defined. (1) Both fusion proteins can bind as homodimers to RAREs.171,289,383 In the case of PML-RARα, this is mediated by the coiled-coil motif,171 whereas in PLZF the POZ/BTB domain mediates self-association.383 When coincubated with RXR, both PML-RARα and PLZF-RARα form multiple different DNA-protein complexes. It is noteworthy that PLZF-RARα homodimers bound to a direct repeat of the sequence GGG TCA separated by 5 bp (Dr5T) with equal avidity as PML-RARα to a direct repeat of the sequence GGG TCA separated by 5 bp (Fig 6). The breakpoint within the PLZF gene occurs 3′ to the first translated exon. As a result of the fusion, the PLZF-RARα chimera contains the entire N-terminal transcriptional effector region of PLZF (including the POZ/BTB domain) as well as the first two zinc fingers of the protein. As in all forms of APL, the RARα gene is fused in the region corresponding to the B domain. In one case, a fusion of the N-terminus of PLZF and the first three PLZF zinc fingers (up to amino acid 484 of PLZF) was linked to RARα, indicating a breakpoint in the PLZF gene further 3′ within the gene. In 4 of 7 cases tested, a reciprocal RARα/PLZF transcript was detected, linking the A/AF1 ligand-independent transcriptional activation domain of RARα216 to the last 7 zinc fingers of PLZF.

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(2) Both PML-RARα13,15,157 and PLZF-RARα289,383,408 can act in a dominant negative manner to inhibit the activity of wild-type RARα and the vitamin D3 receptor (Perez et al 171 and Licht and English, unpublished data). PLZF-RARα is a relatively weak trans-activator, in some studies completely unable to activate transcription of coexpressed reporter genes408 and in our studies mediating ligand-dependent transcription, albeit at levels less robust than the wild-type RARα.289 The weakened transcriptional activity of PLZF-RARα might be due to inefficient binding by the protein. Alternatively, inclusion of the large PLZF moiety to the N-terminus of the RARα might produce steric hindrance between RARα and coactivators or basal factors. Deletion of the ligand-independent/AF1 activation domain of the RARα reduces activation by RARα, a promoter-dependent effect.42 Most importantly, PLZF-RARα interacts aberrantly with the SMRT and NCoR corepressors, Sin3A and HDAC1, both in vitro and in vivo.293-296,299,397 A key finding relates to the differential affinity of PLZF-RARα and PML-RARα for NCoR and SMRT in the presence of ATRA. Whereas PML-RARα could be released to the corepressors and HDAC1 in the presence of 10−6 mol/L ATRA, PLZF-RARα retained corepressors and HDAC1 even under these high ligand concentrations294-296,299,409 (Fig 8). PML-RARα association to HDAC1 and corepressors is mediated solely by the corepressor binding domain or CoR box of the RARα moiety. In contrast, PLZF-RARα binds to corepressors via the CoR box binds and the POZ/BTB domain. It is the latter association that is insensitive to ATRA even at high doses.295,296 This model is supported by several studies. (1) Mutation of the CoR box of PML-RARα but not PLZF-RARα results in loss of binding to HDAC1 and corepressors. (2) Mutation of the PML-RARα CoR box abolishes its ability of this protein to block differentiation, whereas the PLZF-RARα CoR box mutant inhibits differentiation, even in the presence of ATRA.296 (3) HDAC inhibitors, such as trichostatin A and sodium butyrate, were able to convert PLZF-RARα into an ATRA-responsive transcription factor, presumably by inactivating the remaining corepressor complex bound to the POZ/BTB domain. These inhibitors also allowed U937 cells transfected with PLZF-RARα to differentiate in the presence of ATRA.295,296

When coexpressed with wild-type RARα, PLZF-RARα inhibits reporter gene trans-activation by the wild-type receptor.293,383,408 This dominant negative effect of PLZF-RARα was partially relieved by overexpression of RXRα, consistent with the notion that the aberrant receptors block myeloid differentiation at least partly by limiting the ability of RARα to bind with RXR to its targets.289 Deletion mapping of PLZF-RARα protein also showed that dominant negative activity was dependent on the presence of the POZ/BTB domain.383 This region is also required for self-association of PLZF-RARα and for formation of multimers that could sequester RXR. Curiously, inhibition of wild-type RARα function was partially dependent on the presence of the first two PLZF zinc fingers, which are present in the fusion protein and which are also the binding site for PML. When the POZ/BTB domain and first two PLZF zinc fingers were deleted from the fusion protein, PLZF-RARα became an efficient activator of ATRA-mediated transcription.

The dominant negative effect of PLZF-RARα also suggests that it may work by sequestering RARα transcriptional coactivators such as TIF1 or CBP in an inactive conformation, drawing them from RARα target genes. This provides another explanation for why RXR only partially rescues the dominant negative effect of PLZF-RARα. However, the hypothesis that PLZF-RARα binds to RARα corepressors,45,410,411 inappropriately repressing RARα target genes in the absence of ligand is more likely. In keeping with this, PLZF-RARα, like PML-RARα, inhibited the activity of an RARE-containing promoter in the absence of exogenous ATRA.299 Finally, PLZF-RARα could, in theory, also affect the function of wild-type PLZF. In fact, PLZF-RARα and PLZF can preferentially heterodimerize over the formation of PLZF homomeric complexes.383 Hence, high-level expression of PLZF-RARα in t(11;17)(q23;q21) blasts might sequester PLZF from binding to its natural target
Fig 8. Potential mechanism of relative resistance of PLZF-RARα-associated APL to ATRA.

Fig 9. Functional domains of the NPM protein and structure of the NPM-RARα and reciprocal RARα-NPM proteins generated in t(5;17)-APL. A relatively short N-terminal portion of NPM containing the oligomerization domain of the protein is linked to RARα. In the index patient, a longer form of the fusion protein was identified, containing an additional sequences of uncertain origin. The reciprocal RARα-NPM protein could potentially interact with wild-type NPM and interfere with NPM functions.
genes and/or bind to limiting quantities of PLZF transcriptional cofactors. However, we have not yet observed this effect in transfection experiments (Li et al, unpublished data).

MODELS OF t(11;17)(q23;q21) APL

Cellular models are being developed to clarify the role of PLZF-RARα in APL. We found that PLZF-RARα was only transiently expressed in nontumorigenic 32DC13 cells after retroviral infection, suggesting a highly toxic or growth suppressive effect (Shaknovich and Licht, unpublished data). Pellici’s group found that PLZF-RARα could be stably expressed in HL60, TF1, and U937 leukemic cells and blocks their differentiation in response to a number of chemical inducers. The transformed state of these cells might have allowed them to escape the potent growth-suppressive effects of PLZF-RARα.

Consistent with the above results, PLZF-RARα, unlike PML-RARα, failed to increase the sensitivity of transduced cells to ATRA-mediated differentiation. HL60 cells lacking wild-type RARα transduced with PML-RARα showed increased expression of RARα target genes, whereas PLZF-RARα-transduced cells did not. Furthermore, whereas reintroduction of wild-type RARα or PML-RARα into the mutant HL-60 cells fully restored the ability of the cells to differentiate, as measured by the expression of leukocyte integrins, PLZF-RARα was only partially able to induce their expression. This information suggests that the differences in induction of endogenous genes by PLZF-RARα and PML-RARα are important for the ATRA-resistant clinical phenotype of t(11;17)(q23;q21)-associated APL.

More physiologically relevant data indicate that PLZF-RARα on its own may not fully account for the ATRA resistance of t(11;17)(q23;q21) APL. Marrow progenitor cells infected with a PLZF-RARα retrovirus are able to be serially passaged ex vivo and displayed a primitive hematopoietic phenotype. Upon treatment of these cells with 10⁻⁷ mol/L ATRA, proliferation ceased and differentiation ensued.

The role of the PLZF-RARα protein in leukemogenesis was further explored in a transgenic model using the cathepsin G promoter. These mice developed a chronic myeloid leukemia (CML)-like syndrome rather than APL. As in the case of the PML-RARα model, disease developed after a preleukemic phase, suggesting that secondary mutations are required for transformation. Acute leukemia developed more rapidly in these mice compared with the PML-RARα transgenics, suggesting that PLZF-RARα may be more oncogenic. Unlike PML-RARα transgenic mice, the PLZF-RARα mice did not achieve complete remission after ATRA treatment at a 10⁻⁶ mol/L dose, although they did show some evidence of myeloid differentiation. PLZF-RARα transgenic mouse leukemia cells treated with ATRA readily differentiated ex vivo, whereas in vivo, PLZF-RARα mice required a higher dose of ATRA than PML-RARα mice to induce short remissions. Thus, animals harboring PLZF-RARα were not absolutely insensitive to ATRA, suggesting that PLZF-RARα does not completely block ATRA-induced differentiation. The relative insensitivity of the disease in vivo correlates with the impaired ability of PLZF-RARα to transactivate in vitro, likely due to binding of corepressors even in the presence of ligand. This notion was further confirmed by the fact that the histone deacetylase inhibitor TSA in combination with ATRA synergistically inhibited growth and induced differentiation of PLZF-RARα harboring mouse leukemic cells. Therefore, the clinical phenotype of t(11;17)(q23;q21) APL might partly be due to the inability to achieve and sustain sufficiently high levels of ATRA required to stimulate the PLZF-RARα fusion product (Table 5). A combination of ATRA and sodium butyrate, the latter already in clinical trial, might alleviate this situation. These animal experiments are still imperfect models of APL. PLZF-RARα mice, unlike PML-RARα mice, did not accumulate promyelocytes, although transgenic mice expressing PML-RARα under the cathepsin G promoter did not develop true APL either. In addition, the animal model does not explain the poor response of these patients to chemotherapy and the resistance of fresh t(11;17)(q23;q21) APL cells to high doses of ATRA in vitro. This information implicates another oncogenic lesion, potentially the reciprocal RARα-PLZF protein in the aggressive nature of this form of APL. This notion is supported by recent studies with fresh APL blasts of a patient with t(11;17)(q23;q21). Neither ATRA nor arsenic treatment of these cells led to differentiation or apoptosis of the APL cells. Although arsenic can eliminate the microspleckled pattern of PML-RARα expression in APL associated with t(15;17), arsenic did not affect the punctate nuclear appearance of PLZF-RARα. ATRA treatment led to the degradation of PLZF-RARα, theoretically lifting the block to induction of RAR target genes, yet did not induce differentiation, suggesting that the reciprocal product offered a second oncogenic lesion.

RARα-PLZF

In the case of the t(11;17)(q23;q21) APL variant, the reciprocal transcript encoding RARα-PLZF that yields a protein containing the last seven zinc fingers of PLZF fused to the A-domain of RARα (Fig 6) is consistently expressed. In contrast, the RARα-PML transcript of t(15;17) APL is absent in a significant number of cases. These seven zinc fingers can bind to the artificial PLZF binding site derived from PCR-based site selection as well as a site derived from selection from a human CpG island library. RARα-PLZF demonstrates properties that may be critical to the disruption of transcriptional and nuclear regulatory events. Whereas PLZF represses gene transcription through its cognate binding site, RARα-PLZF activates transcription through this site. Whereas PLZF is a growth suppressor and inhibits expression of the cyclin A2 gene, RARα-PLZF activates transcription, activates expression of cyclin A2 in an adhesion independent manner in 3T3 cells, and enhances cell growth. Hence, t(11;17)(q23;q21) may be an ATRA and chemotherapy-resistant disease due to the presence of two oncogenes working through different mechanisms. PLZF-RARα blocks retinoid-mediated activation of genes critical for myeloid differentiation. RARα-PLZF may activate cell cycle regulators such as cyclin A, accelerate cell growth, and block the antiproliferative effects of retinoid treatment. This notion is supported by the finding that mice harboring the RARα-PLZF protein develop a myeloproliferative syndrome. Whether these mice or the progeny of RARα-PLZF/PLZF-RARα crosses will develop leukemia is under study.

Finally, RARα-PLZF might also function through interference with RARα-mediated signaling. Inappropriate expression...
### Table 5. Comparison of N-Protein/RARα Fusion Products

<table>
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<th>Breakpoint variants</th>
<th>PML-RARα</th>
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<tr>
<td>Model</td>
<td>Multimerization, sequestration of RXR and other factors. Increased affinity for corepressors. Transcriptional effects on target genes. Interference with PLZF actions.</td>
<td>Multimerization, sequestration of RXR and other factors. Transcriptional effects on target genes. Reciprocal fusion may play a role.</td>
<td>Multimerization, sequestration of RXR and other factors. Interference with apoptosis program.</td>
<td></td>
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<tr>
<td>Reciprocal translocation</td>
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<td>Present in all cases tested. Activates PLZF target genes and induces cell proliferation1,289,394,505</td>
<td>Identified in the index case. Actions still unknown419</td>
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of this fusion protein, containing part of the AF1 activation domain of RARα, may act competitively with wild-type RARα for limiting cofactors. Similarly, a truncated form of RARγ containing the AF1 domain inhibited the ability of wild-type RARγ to activate transcription \(^{441}\) and transformation of keratinocytes, blocking their ability to differentiate. This supports the idea that a reciprocal RAR-X fusion protein could have an independent oncogenic effect.

**NUCLEOPHOSMIN (NPM-RARα)**

In t(5;17)-associated APL, RARα is translocated to a region on chromosome 5q35 encoding the ubiquitously expressed and evolutionarily conserved nucleophosmin gene (NPM). \(^{418-420}\) NPM, also known as B23, numatrin, and NO38, was initially isolated as a nucleolar phosphoprotein in hepatoma cells. \(^{421-423}\) The human NPM gene spans 25 kb, consists of 12 exons, \(^{424}\) and has a promoter region consistent with those of housekeeping genes. \(^{425,426}\) Alternative splicing yields two major isoforms: NPM\(^{B23.1}\) (294 amino acids) and NPM\(^{B23.2}\) (257 amino acids), differing in their C-terminal region. \(^{420,427-429}\) Major structural features of NPM include two Asp/Glu-rich acidic domains, which may serve as binding sites for basic regions of other proteins; a bipartite nuclear localization signal (NLS); a metal binding motif; an ATP binding site; phosphorylation sites for cdc2 kinase and casein kinase II; and a binding site for proteins that contain nucleolar localization signals. \(^{430,430-440}\) The C-terminal region of the 294 amino acid NPM\(^{B23.1}\) isoform is also involved in nucleic acid binding and stimulation of DNA polymerase activity. \(^{441-444}\) Both isoforms reversibly multimerize to a hexameric state via the N-terminal domain. \(^{445}\)

NPM is localized most prominently to areas of the nucleolus associated with ribonucleoprotein (RNP) processing. \(^{475,477-479}\) NPM binds to nucleic acids, altering their conformation, an effect that could facilitate binding of ribosomal proteins to rRNA. \(^{477}\) NPM also copurifies with proteins required for DNA replication. \(^{443,450-452}\) In addition, NPM, particularly at the N-terminal region, is highly homologous to the evolutionarily conserved protein nucleoplasm, a factor with chaperone activity that is involved in chromatin/nucleosome assembly. \(^{453,454}\) NPM also functions as part of a transport system used by ribosomal precursors to shuttle between the cytoplasm and nucleolus. \(^{436,440,455}\) NPM interacts with nonribosomal proteins via basic sequences (NoLS), assisting in transport to the nucleolus. \(^{436,440,456}\)

NPM levels are increased in proliferating cells \(^{457,458}\) and hypertrophic tissue to even higher levels in malignant cells, \(^{420,459,460}\) including leukemic blasts. \(^{461}\) The increase in NPM expression may just be the consequence of increased requirements for ribosomal precursors. However, engineered overexpression of NPM in 3T3 cells yielded a transformed phenotype. \(^{461}\) One explanation for this could be that NPM binds to the tumor suppressor IRF-1 and inhibits its ability to activate genes that mediate the antiproliferative effect of IFN. \(^{461}\) Thus, NPM could behave as an antitumor suppressor.

NPM binds to transcription factor YY1, which is involved in cell growth and differentiation, changing it from a transcriptional repressor to an activator. \(^{462,463}\) YY1 binds to the NPM enhancer, possibly constituting a feedback mechanism. \(^{428}\) ATRA-induced differentiation of HL60 cells, but not growth arrest by serum withdrawal, resulted in downregulation of NPM. \(^{464}\) When cellular NPM levels were decreased by an antisense oligonucleotide, there was potentiation of the ATRA-induced differentiation. \(^{464}\) Growth-suppressive IRF-1 is upregulated by ATRA during myeloid differentiation, in opposition to the effect of NPM. \(^{413}\) These results support a possible role for NPM in the control of cellular growth and differentiation and hint at involvement in retinoid and IFN pathway regulatory cross-talk.

NPM undergoes dynamic changes over the course of the cell cycle. Expression peaks at S or G2 phase and is minimal in cells at G1. \(^{460,465-467}\) This pattern of expression might be related to the fact that NPM\(^{B23.1}\) specifically stimulates the activity of DNA polymerase α. \(^{444}\) Alternatively, this may be a reflection of metabolic demands. In addition, NPM undergoes cdc2 threonine phosphorylation in G2/M. \(^{468,469}\) During M-phase progression, NPM associates with perichromosomal regions and nucleolar bodies, thus functionally linking the processes of nucleolar disassembly to mitotic chromosome condensation. \(^{469}\) The fact that NPM is intimately involved in events taking place at the G2/M regulatory point also underlines the strong relation between this protein and cellular proliferation.

NPM is preferentially regulated during apoptosis and cell damage. Apoptotic prostatic cells have hypophosphorylated NPM due to decreased CK II activity. A protease specific for unphosphorylated NPM then degrades the protein. \(^{470,471}\) NPM undergoes ADP-ribosylation after cells are exposed to x-rays, suggesting a role for NPM in DNA repair. \(^{472}\) NPM reversibly delocalizes from the nucleolus to the nucleoplasm \(^{35,473}\) when cells are exposed to conditions that discourage DNA or RNA synthesis or encourage terminal cell division, including stationary growth, serum starvation, hyperthermia, chemotherapeutic drugs, or ATRA (in HL-60 cells). \(^{473,474}\) This may reflect either decreased shuttling due to diminished metabolic requirements or a controlling role for NPM in regulating the cessation of cell growth. The dynamic response of NPM after various cell stresses resembles the reorganization of the PML-containing nuclear body under such conditions, indicating that both proteins may measure or control cell homeostatic processes.

NPM is fused to genes other than RARα in hematologic malignancies, as in the t(2;5)(p23;q35) translocation found in Ki-1\(^+\) anaplastic large-cell lymphoma (ALCL). \(^{425,484-495}\) In this situation, NPM is linked to ALK, a gene encoding a membrane spanning tyrosine kinase normally not expressed in lymphoid tissue. \(^{496}\) The resulting protein contains the N-terminal oligomerization domain of NPM intracytoplasmic to the tyrosine kinase domain of ALK. \(^{424,497}\) As a result, the ubiquitously expressed NPM gene drives the expression of an aberrant tyrosine kinase, which can multimerize, yielding constitutive kinase activity, and aberrant oncogenic signaling. \(^{490,495,500}\) In the t(3;5)(q25.1; q34) translocation, found in myelodysplasia and M6-AML, a larger 175 amino acid portion of the NPM gene is linked to the MLF1 gene encoding an abundant cytoplasmic protein of unknown function. \(^{424,503}\) The resulting NPM-MLF fusion aberrantly places the MLF moiety in the nucleus and nucleolus. This fusion protein might also block normal NPM function, disrupting normal cellular growth control mechanisms. A similar mechanism could be at play in the case of NPM-RARα.
NPM-RARα

The t(5;17)(q35;q21) translocation was first described in a 2-year-old girl with APL who achieved cytogenetic remission after treatment with ATRA and chemotherapy. Blasts from this index patient, when thawed and treated with ATRA, differentiated into mature granulocytes. Subsequently, at least two other cases were described, one of whom has had a prolonged survival after ATRA and bone marrow transplantation. Like the t(11;17)(q23;q21) APL syndrome, t(5;17) patients present with atypical morphological features, but unlike PLZF-associated APL, these patients are sensitive to ATRA therapy. This gene rearrangement joins the NPM gene 5’ to exon 3 of RARα in a manner similar to the other forms of APL. From a cDNA library derived from the marrow of the index patient, two fusion cDNAs were isolated. In the NPM2-RARα (520 aa), NPM contributes its oligomerization, metal binding, and nucleoplasmin homology domains as well as its highly active promoter. NPM2-RARα (563aa) harbors an additional sequence of unclear origin, possibly from the noncoding region of NPM (Fig 9).

Like PML-RARα and PLZF-RARα, the NPM-RARα fusion acts as a ligand-dependent transcriptional activator when coexpressed with reporter genes containing RAREs, although it is not yet known whether NPM-RARα can act as a dominant negative inhibitor of wild-type RARα. This is likely, because the NPM moiety of the fusion, like the PML-and PLZF proteins, contains a multimodification domain and hence could sequester RXR and other cofactors from wild-type RARα. NPM-RARα has biological effects in cell culture similar to PML-RARα and PLZF-RARα. Its engineered expression in U937 cells blocked monocytoid differentiation in response to vitamin D3 and TGF-α.

This furthers the notion that all of the RARα fusion proteins elicit the APL phenotype through disruption of nuclear receptor signaling. This was supported by the finding that PML-RARα, PLZF-RARα, and NPM-RARα could enhance the proliferation of primitive marrow progenitor cells after retrovirus-mediated gene transfer. Treatment of these cells with ATRA induced differentiation and inhibited cell growth. A transgenic model of t(5;17)-APL was recently created using the cathepsin G promoter. These mice developed an APL-like syndrome after a latent period, and blasts derived from these animals were ATRA sensitive. The response of this disease to ATRA may be mediated in part by downregulation of translation of the NPM-RARα fusion, akin to the decline of NPM levels in differentiating HL60 cells. This would relieve the putative dominant negative effect of the fusion protein.

NPM-RARα is expressed in a microspeckled pattern throughout the nucleus, similar to PML-RARα and PLZF-RARα. In this regard, NPM-RARα probably does not affect PML function, because PML was expressed in the wild-type nuclear body configuration in HeLa cells engineered to overexpress NPM-RARα, whereas NPM-RARα was expressed in a diffuse nuclear pattern and APL blasts from a t(5;17) patient exhibited a normal NB configuration. Furthermore, NPM did not interact with PML in vitro. Interestingly, these experiments supply additional evidence that disruption of at least the PML component of the nuclear body is not required for the pathogenesis of APL. However, PLZF was delocalized in t(5;17) APL cells in a microspeckled pattern distinct from its wild-type distribution in normal marrow progenitor cells. This finding further supports a broad role for the PLZF protein in the pathogenesis of APL.

A reciprocal RARα-NPM mRNA was identified in the index t(5;17) patient, leading to fusion of the A domain of RARα to the acidic domains, NLS, and the rest of the C-terminus of NPM. This molecule could associate with wild-type NPM through C-terminal sequences; however, it is unknown whether it interferes with any NPM function such as nucleolar-cyttoplasmatic shuttling or apoptosis. RARα-NPM may bring the RARα activation domain in contact with NPM transcriptional targets such as YY1 and IRF-1, altering NPM-dependent transcriptional modulation. Lastly, similar to the other reciprocal fusion proteins, the ectopic expression of the RARα activation domain could interfere with wild-type RARα function by competition for AFI cofactors.

NUCLEAR MATRIX-MITOTIC APPARATUS PROTEIN (NuMA)

The gene encoding the NuMA protein is the newest reported fusion partner of RARα in APL and the first mitotic apparatus protein found to be genetically rearranged in a human malignancy. NuMA is a large, highly abundant, conserved, and ubiquitously expressed protein that is intimately involved in the completion of mitosis and reformation of nuclei in the postmitotic daughter cells. NuMA is also a structural unit of the interphase nucleus. NuMA was first identified as an insoluble nonhistone chromatin-associated protein distributed diffusely throughout the interphase nucleus and displays a remarkable crescent-shaped mitotic staining pattern.

The NuMA gene, located on chromosome 11q13, encodes a highly conserved protein of 2,115 amino acids with a molecular weight of approximately 230 kD and by alternative splicing yields 1776aa and 1763aa proteins. The NuMA protein is divided into two globular domains at either end of the protein, with a central coiled region of 1485 aa. The coiled motifs likely mediate protein homoaossociation and heteroaossociation. Little is known about the N-terminal globular region, although its absence impairs nuclear reformation after mitosis. The C-terminal region contains basic sequences, motifs for phosphorylation by cdc2 and other kinases, and sequences that confer localization to the nucleus (NLS) and mitotic spindle.

There is experimental evidence to support a role for NuMA in mitosis, apoptosis, and interphase nuclear matrix functions. It can participate in these diverse processes due to complex regulatory posttranslational modifications across the cell cycle. For example, NuMA is phosphorylated by the cdc2/cyclin B-regulatory kinase at the initiation of mitosis and associates with the spindle microtubules. A number of experiments demonstrated that NuMA is essential in forming convergent mitotic spindles and organizing and stabilizing parallel arrays of microtubules. NuMA may be required for the proper organization of newly forming daughter nuclei as cell division ends. NuMA undergoes proteolytic cleavage and dephosphorylation forming a pathway for proteins to transit to daughter nuclei. Towards the end of mitosis, NuMA reverts to an insoluble form, yielding a
Fig 10. Functional domains of the NuMA protein and structure of the NuMA-RARα protein generated in t(11q13;17)-APL. As in the other fusion proteins of APL, an oligomerization domain contributed in this case from NuMA is linked to RARα. It is uncertain if a reciprocal protein is generated.

**Axis 1: N-RARα ⇒ RARα**

N-RARα fusions are dominant negative RARs, multimerize and sequester RXR and other RARα cofactors. Repress targets by aberrant high affinity for co-repressor. Result is inhibition of key myeloid genes, in most cases reversible by ATRA treatment.

**Axis 2: N-RARα ⇒ N**

N-RARα fusions may associate with wild type N protein delocalize the N protein and or inactivate the N protein in a dominant negative manner. Growth suppressive activity of the N protein may be abrogated.

**Axis 3: RARα-N ⇒ N**

RARα-N may behave as a second dominant negative protein blocking the growth suppressive properties of N or alternatively, as in PLZF, may represent a gain of function contributing an independent oncogenic stimulus.

Fig 11. Three axes to be investigated in understanding the pathogenesis of APL.
fibrous network that may play a structural role during interphase. NuMA, like NPM, is an important target for regulation at the G2/M boundary as well as during postmitotic reorganization.

In interphase cells, immunofluorescence staining with anti-NuMA antibodies shows diffuse and/or speckled nuclear patterns,511,517,530,537 NuMA associates with a subset of nuclear core filaments around which proteins and nucleic acids coalesce to form the nuclear matrix.538 PML, NPM, and probably PLZF associate with the nuclear matrix.191,197,216,460,467 Other roles for NuMA were proposed based on coprecipitation with snRNPs and association with splicing complexes.539 In addition, NuMA specifically attaches to DNA matrix attachment regions (MAR), which are important for chromatin compaction and isolation of transcriptionally active loops of DNA.540,541 NuMA may thus provide structural support for RNA processing pathways, organize chromatin condensation, and participate in the regulation of transcriptional units.

NuMA may play a role in apoptosis and is an early target for proteolysis by caspase-3 and caspase-6,542 yielding an approximately 180-kD NuMA proteolytic product,526,543,544 lacking the C-terminal globular effector domain after several different apoptotic stimuli.544-547 This proteolytic product could act as a dominant negative, disrupting normal nuclear structure. Alternatively, release of NuMA from the DNA matrix attachment regions may facilitate the DNA fragmentation characteristic of apoptosis.548 However, in some of these experimental models, there was no correlation between nuclear fragmentation and NuMA proteolysis,544 suggesting that NuMA may be specifically targeted to prevent dying cells from proceeding through M-phase. In summary, NuMA would appear to be a structural component of the cell that responds to cell cycle signals on cue rather than a controlling factor in cell proliferation. It is unclear whether inhibition of NuMA function might contribute to oncogenesis. In fact, many NuMA mutants seriously disrupt mitosis and would not be expected to be compatible with normal cell growth and proliferation.

**NuMA-RARα**

The NuMA-RARα fusion protein was first described in a 6-month-old boy with morphologically diagnosed APL harboring a translocation t(11;17)(q13;q21).509 The patient had a complete remission after ATRA therapy and was disease free at 24 months after a bone marrow transplant.509 The t(11;17)(q13;q21) results in a 2286 aa protein predicted to consist of 1883 amino acids of NuMA, including the N-terminal globular and coiled-coil domains of NuMA fused to RARα domains B through F, as in all the other RARα fusion proteins548 (Fig 10). NuMA-RARα localized to sheetlike nuclear aggregates in the patient’s leukemic cells, but NB structure and PML staining were unperturbed. Introduction of C-terminal deletion mutants of NuMA into cells completely disrupts mitosis,522,523 and it is surprising that the existence of NuMA-RARα is even compatible with cell division. Perhaps the RARα moiety mislocalizes the mutant NuMA from the daughter nuclei reforming after mitosis, allowing the remaining wild-type NuMA to perform its function. In the nucleus, the fusion may, in a dominant negative manner, interfere with regulatory events during interphase or G2. NuMA-RARα might also compete with wild-type NuMA for caspasases, interfering with apoptosis.

The most likely mechanism of action of the NuMA-RARα is interference with nuclear receptor function. Although no functional data for the NuMA-RARα fusion are yet available, it is probable that, like the other RARα fusion proteins, NuMA-RARα is a dominant negative retinoid receptor with diminished intrinsic transcriptional activity. A fundamental common denominator among APL fusion proteins is their ability to form higher order complexes. Like PML, PLZF, and NPM, NuMA can multimerize.521,524 NuMA-RARα might thus sequester RARα partner proteins or have aberrant affinity for nuclear receptor coactivators or corepressors. Like PML, NuMA is a component of the nuclear matrix and NuMA-RARα might further inhibit RARα function by confinement of RARα cofactors in a subnuclear compartment (such as the MAR) apart from wild-type RARα. Another common theme among RARα fusion proteins is their downregulation when treated with ATRA. Similarly, NuMA-RARα might decrease as t(11;17)(q13;q21) APL cells are induced to terminally differentiate by ATRA. This is consistent with the fact that normal neutrophils do not express NuMA.547 Finally, it is unknown whether a reciprocal RARα-NuMA transcript is expressed in this disease. In fact, there might be selection against this protein as it could represent a detriment to mitosis.

**COMMON THEMES, SPECULATIONS, AND DEPARTURES FOR FUTURE INVESTIGATION**

There are three axes to be investigated in understanding the pathogenesis of APL (Fig 11).

First, it is clear that certain RARα target genes must be activated (or repressed) for myeloid development to proceed. In all APL patients, one allele of RARα is disrupted and fused to the partner gene, N. All of the N genes encode a protein able to multimerize. The multimerization of the N-RARα fusion protein may be a key factor in interfering with normal ATRA-mediated signaling and sequestering cofactors. Recent evidence shows that PML-RARα and PLZF-RARα have abnormally high affinities for corepressors. Because PML alone does not seem to bind these factors, it appears that fusion of RARα to the PML moiety effects an allosteric change in the entire molecule, causing the fusion protein to retain the corepressors at physiological concentrations of ATRA. The structural basis of this change is unknown. NPM and NuMA might have the same effect on RARα, and this should be investigated. In contrast, PLZF binds the corepressors even in the presence of ATRA. The net result is that, at physiological levels of ATRA, the fusion receptors do not activate the critical genes for myeloid differentiation and only can do so with pharmacological doses. In the case of PLZF-RARα, even the usual pharmacologic dose of ATRA is insufficient for full activation of the RARα axis. The use of 10⁻⁵ mol/L ATRA and/or addition of HDAC inhibitors are required to allow retinoic acid signaling to proceed.

Second, the N gene is disrupted with only one normal allele remaining. Loss of N gene dose and/or function might play a role in the pathogenesis of APL. The N-RARα fusion could sequester the normal N product, altering its role in growth control. PML-RARα delocalizes both PML and PLZF from nuclear bodies, potentially altering their function. Whether
NPM-RARα and NuMA-RARα affect the function of the wild-type proteins is unknown.

Third, the reciprocal RARα-N fusion gene could play a role in the pathogenesis or clinical phenotype of the APL syndrome. The N-RARα fusion, in addition to altering RARα function, could act as a second dominant negative protein to inhibit the function of the N protein. In the cases of t(11;17)(q23;q21)-associated APL, the RARα-PLZF gene product could both interfere with normal PLZF function and have novel gains of function. Hence, many questions remain, including the following:

1. What are the critical RARα target genes required for myeloid differentiation that are inhibited in their expression by the N-RARα protein?

2. What are the molecular details of the corepressor complex bound to N-RARα that inhibit the activity of the fusion protein? Are all the corepressors directly bound or are a chain of protein-protein interactions required? Are coactivators also sequestered by the N-RARα fusions? Will manipulation of coactivators (histone acetylases) as well as corepressor histone deacetylase factors play a role in restoration of normal myeloid differentiation and the treatment of APL?

3. Is the NB an active organelle or an intranuclear storage site? What is significance of the PML and PLZF association with the NB? Is there a role for NB disruption in the pathogenesis of APL?

4. What is the mechanism of action of the PML protein in transcription, apoptosis, and growth control?

5. What is the role of PLZF in hematopoiesis and what are its target genes?

6. How do RARα fusion proteins transform cells? Do the fusion proteins alter expression of regulators of apoptosis? Are the RARα fusion proteins dominant negative inhibitors of PML or PLZF? Will APL develop both in PML or PLZF null animals?

7. How does arsenic cause the apoptosis of APL cells? Is arsenic response dependent on the presence of N-RARα fusion proteins or is it reflective of the particular stage of differentiation of the APL cell?

8. Do RARα-N reciprocal fusion proteins cooperate with N-RARα proteins to induce leukemia?

9. Can other molecular pathways, such as caspases or IFN mediators, be exploited as avenues for future therapies of APL?

10. Are there molecular interactions between PML and NPM or NuMA?

The study of the molecular pathogenesis of APL is at the forefront of the application of molecular biology to clinical medicine, because this disease is the paradigm for successful differentiation therapy. The spectacular response of these patients to ATRA has underlined the importance of continued efforts to understand the basic biology of leukemia. We now understand that the RARα fusion genes of APL are the key to the cause and cure of this disease. Work to date has elucidated how the RARα fusion proteins may block differentiation and how ATRA can reverse this block and promote differentiation and death of the malignant clone. Translational studies using clinical samples have highlighted how retinoic acid resistance can occur in patients who develop secondary mutations in the PML-RARα fusion gene. New work has led to a greater understanding of how arsenic, a reactivated agent of the cancer armamentarium, may promote death of neoplastic cells. The occurrence of naturally resistant forms of APL such as that associated with rearrangement of the PLZF gene indicates that molecular heterogeneity can occur in APL and that a definitive diagnosis of t(15;17)-APL must be made before ATRA can be used with confidence. The study of the resistant and sensitive forms of APL, characterized by rearrangement of the PML and PLZF genes, respectively, yielded an appreciation of the importance of transcriptional repression by histone deacetylation in the development of the disease. This led to the recent use of the deacetylase inhibitor sodium butyrate as a form of targeted transcription therapy in a patient with resistant APL.549 With the development of animal and cell models of APL of sensitive and resistant forms of APL and the advent of more powerful technologies for gene discovery and cell biology, the next 5 years should offer continued insights leading to the development of more effective therapies for this fascinating disease as well as other forms of leukemia.

ACKNOWLEDGMENT

The authors thank S. Waxman, J. Gabrilove, G. Atweh, M. McConnell, and A. Zelent for review of the manuscript and C. Brechok for editorial assistance.

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