Chromosomal Translocation t(15;17) in Human Acute Promyelocytic Leukemia Fuses $RAR\alpha$ with a Novel Putative Transcription Factor, PML

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Summary

A unique mRNA produced in leukemic cells from a t(15;17) acute promyelocytic leukemia (APL) patient encodes a fusion protein between the retinoic acid receptor α ($RAR\alpha$) and a myeloid gene product called PML. PML contains a cysteine-rich region present in a new family of apparent DNA-binding proteins that includes a regulator of the interleukin-2 receptor gene (Rpt-1) and the recombination-activating gene product (RAG-1). Accordingly, PML may represent a novel transcription factor or recombinase. The aberrant PML- RAR fusion product, while typically retinoic acid responsive, displays both cell type- and promoter-specific differences from the wild-type $RAR\alpha$. Because patients with APL can be induced into remission with high dose RA therapy, we propose that the nonliganded PML- RAR protein is a new class of dominant negative oncogene product. Treatment with RA would not only relieve this inhibition, but the activated PML- RAR protein may actually promote myelocyte differentiation.

Introduction

Karyotypic changes, including chromosomal translocation, are consistently associated with cancer (Mitelman, 1988). Specific changes, like those associated with *bcr-abl*, lead to the generation of fusion alleles and may be associated with initiation of cancer. In cases of acute leukemias, which represent clonal expansion of hematopoietic cells at certain developmental stages, genes located at the site of translocation may become activated to encode an oncogenic protein that represents the etiologic agent of the disease.

While chromosomal rearrangements are frequent hallmarks of malignant cells, the balanced t(15;17) (q22;q12-21) translocation is exclusively associated with acute promyelocytic leukemia (APL), and it is often the only visible karyotypic aberration present (Mitelman, 1988). This translocation is detected in as many as 90% of APL patients and has become the definitive marker for the disease (Rowley et al., 1977a, 1977b; Mitelman, 1988). The chro-

mosome 17 breakpoint in APL was recently mapped to the retinoic acid receptor α gene ($RAR\alpha$) (de Thé et al., 1990a; Borrow et al., 1990; Longo et al., 1990), and the translocation results in a fusion between $RAR\alpha$ and a region on chromosome 15 referred to as *myl* (de Thé et al., 1990a). *myl* has been renamed *PML* (for "promyelocytes"). RAR is a member of the steroid/thyroid hormone receptor superfamily and regulates gene expression by acting as a ligand-dependent transcription factor (Evans, 1988; Giguere et al., 1987; Petkovich et al., 1987). The ligand, retinoic acid (RA), triggers cellular differentiation in a wide variety of cultured cells and is a developmental regulator in vivo. RA promotes differentiation of the myeloid HL-60 cell line to postmitotic granulocytes (Breitman et al., 1980), and the all-trans RA induces complete remission in APL patients (Huang et al., 1988; Chomienne et al., 1990; Miller et al., 1990; Warrell et al., 1991). These observations suggest that the translocation activates or produces a fusion protein that is hormone responsive. The administration of the high doses of RA to APL patients must overcome the oncogenic defect to restore myelocytic differentiation.

We report here the isolation of *PML-RAR* fusion cDNAs from an APL patient as well as the corresponding wild-type *PML* and $RAR\alpha$ cDNAs. Structural analysis reveals that the PML protein is a member of a newly recognized protein family that includes a variety of putative transcription factors as well as the recombination-activating gene product (RAG-1) (Schatz et al., 1989). We show that the aberrant PML- RAR fusion product is RA responsive, although it displays both promoter- and cell-specific differences from the wild-type $RAR\alpha$. Our data support a model in which myeloid differentiation is blocked by the PML- RAR fusion product and this inhibition relieved by RA treatment.

Results

Identification of $RAR\alpha$ -Associated Reciprocal Transcripts in APL

To look for the presence of altered $RAR\alpha$ transcripts, RNA was isolated from bone marrow of an APL patient whose leukemic cells contained the t(15;17) translocation (Bennett et al., 1976). Figure 1a shows the results of a Northern blot analysis of such APL RNA probed with human $RAR\alpha$ cDNA, and for comparison we show RNA isolated from HeLa cells. Previous work (A. K. and R. M. E., unpublished data) has shown the presence of 2.8–3.6 kb $RAR\alpha$ transcripts from HeLa cells. Both RNA samples give rise to two common bands corresponding to normal $RAR\alpha$ transcripts (2.8 kb and 3.6 kb). In the APL patient sample, two additional bands (3.2 kb and 4.0 kb) are found that could be transcripts of the fusion between the $RAR\alpha$ gene and the translocation locus on chromosome 15. To examine the translocation products further, a cDNA library was constructed from the patient mRNA. After a series of sequential screenings (see Experimental Procedures), candidate cDNAs coding for $RAR\alpha$, PML- RAR , RAR -PML, and PML proteins were isolated. Restriction enzyme and sequenc-

Figure 1. A Novel RAR Fusion Product in a Patient with APL
(a) Five micrograms of total bone marrow RNA from an APL patient and HeLa cell RNA was analyzed by Northern blotting, using the human *RARα* cDNA as a probe. Two wild-type mRNAs of 2.8 and 3.6 kb (open arrows) as well as two additional bands of 3.2 and 4.0 kb (closed arrows) are seen on the patient RNA.
(b) Schematic structures of *RARα* and *PML-RAR* cDNA. The open reading frames are shown as open boxes, and untranslated regions as lines. The fusion portion (encoding 394 amino acids) from the *PML* gene is dotted. Regions encoding the DNA-binding domains derived from *RARα* are in black. The RA-binding domain is marked as RA. The segment used for Northern blotting is also shown.
(c) The nucleotide and predicted amino acid sequences around the fusion points are presented. The nucleotide sequence from *RARα* is bold and underlined.

ing analyses revealed that the fusion occurs in the N-terminal portion of the *RARα* coding sequence (Figure 1b). Figure 1c shows the nucleotide sequences and the predicted amino acid sequences surrounding these fusion points. Two reciprocal fusion cDNAs were formed along with the cDNAs derived from the normal alleles of the *RARα* and *PML* genes. In addition, two distinctive forms of both *RARα* and *PML-RAR* cDNAs were identified that differ in length in the 3' noncoding regions, reflecting the results from the Northern blot (Figure 1a). The clones with the longest 5' regions were selected for sequence analysis.

Structure of *PML*

Figure 2a shows the nucleotide sequence and predicted amino acid sequence of the *PML-1* cDNA. The *PML-1* cDNA (*pPML-1*) consists of 2155 nucleotides and has a long open reading frame with two potential translational initiation codons (ATG) in the 5' end of the cDNA. There is no in-frame upstream stop codon. The 5' ends of 22 cDNAs coding for PML and *PML-RAR* were sequenced to identify the longest extension, suggesting that the clone in Figure 2a codes for a nearly full-length cDNA. While the nucleotide sequences surrounding the second initiation codon more closely resemble the consensus initiation sequence (Kozak, 1987), more than 90% of translation in vertebrates starts from the first methionine (Kozak, 1987): we thus refer to the first ATG as the putative translational start site. The predicted *PML-1* protein consists of 560 amino acids, with a relative molecular weight of 62,006. Computer-assisted analysis of the *PML-1* protein sequence shows that *PML-1* contains four structurally interesting regions (Chou and Fasman, 1978), including a proline-rich N-terminus, a cysteine-rich portion, a potentially α -helical region, and a serine-rich C-terminus region (Figure 2b). There is no signal peptide sequence or a transmembrane region, suggesting that *PML-1* is an intracellular protein. Furthermore, there is no obvious nuclear localization signal or ATP-binding sequence.

The most striking sequence in *PML* is the cysteine-rich motif (amino acids 57–227), which can be divided into three clusters (Figure 3a). Data base searches that focused on this cysteine-rich region identified 11 protein sequences with almost perfect conservation of all cysteines in the first cluster (Doolittle, 1987; Freemont et al., 1991). In all 11 proteins, eight cysteines/histidines and one proline are conserved in the first cluster. This cluster is summarized as C-X-I-C-X₁₀₋₂₇-C-X-H-X-F-C-X₂-C-I-X₆₋₁₅-C-P-L-C, where the isoleucine (I), phenylalanine (F), and leucine (L) residues are highly conserved, but not invariant. Cysteine and histidine conservation is reminiscent of the zinc finger motif seen in several different classes of transcription factors (Miller et al., 1985; Evans and Hollenberg, 1988; Berg, 1990), although the domain here likely represents yet a new class. Of these 11 homologous proteins, several are putative DNA-binding proteins. Although the function of this specific cysteine-rich domain has yet to be clearly demonstrated, the ability of MeI-18 to bind to DNA-cellulose requires the presence of this motif (Tagawa et al., 1990). The LMCV-Z protein binds Zn²⁺ (Salvato and Shimomaye, 1989), supporting the possibility that the other members of this family may also chelate Zn²⁺. Among these 11 proteins, *PML*, *Rpt-1*, and *Rfp* share additional amino acid homology through a second cysteine cluster, suggesting that these three proteins may be more closely related. *Rpt-1* is a potential transcription factor for the interleukin-2 receptor gene (Patarca et al., 1988), and *Rfp* is a proposed regulator for spermatogenesis (Takahashi et al., 1988). In this second cysteine cluster, seven cysteines/histidines and one glutamic acid are conserved among these three proteins, while *PML* has a third cysteine cluster (located between amino acids 189 and 227). The spacing

a

1
M E P A

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CAACTGGCTCACGCCTCCCTTCAGCTTCTCTTCACGCACTCCAAGATCTAAACCGAGAATCGAAACTAAGCTGGGGTCCATGGAGCTCG 90
      10      20      30
P A R S P R P Q Q D P A R P Q E P T M P P P E T P S E G R Q
CACCCGCCGATCTCCGAGGCCCGCCAGCAGGACCCCGCCCGCCCGAGAGCCCAATGCCTCCCCCGAGACCCCTCTGAAGGCCGCC 180
      40      50      60
P S P S P S P T E R A P A S E E E F Q F L R C Q Q C Q A E A
AGCCCAGCCCCAGCCCCAGCCCTACAGAGCGAGCCCCCGCTTCGGAGGAGGAGTTCCAGTTTCTGCGCTGCCAGCAATGCCAGGCGGAAG 270
      70      80      90
K C P K L L P C L H T L C S G C L E A S G M Q C P I C Q A P
CCAAGTGCCCCAAGCTGCTGCTTGTCTGCACACGCTGTGCTCAGGATGCCTGGAGGCGTCGGGCATGCAGTGCCCCATCTGCCAGGCGC 360
      100     110     120
W P L G A D T P A L D N V F F E S L Q R R L S V Y R Q I V D
CCTGGCCCTAGGTGCAGACACCCGCCCTGGATAACGTCTTTTTCAGAGTCTGCAGCGCGCCTGTGCGGTGACCGCAGATTGTGG 450
      130     140     150
A Q A V C T R C K E S A D F W C F E C E Q L L C A K C F E A
ATCGCAGGCTGTGTGACCCGCTGCAAGAGTTCGGCCGACTTCTGGTGTCTTGTAGTGCGAGCAGCTCCTCTGCGCCAAAGTGCTTCGAGG 540
      160     170     180
H Q W F L K H E A R P L A E L R N Q S V R E F L D G T R K A T
CACACAGTGGTTCCTCAAGCACGAGGCCCGCCCTAGCAGAGCTGCGCAACAGTTCGGTGCGTGAGTTCCTGACCGCGCACCCGCAAG 630
      190     200     210
N N I F C S N P N H R T P T L T S I Y C R G C S K P L C C S
CCAACAACATCTTCTGCTCCAACCCCAACACCGCACCCCTACGCTGACAGCATCTACTGCCGAGGATGTTCCAAGCCGCTGTGCTGCT 720
      220     230     240
C A L L D S S H S E L K C D I S A E I Q Q R Q E E L D A M T
CGTGCGCGCTCCTTGACAGCAGCCACAGTGAGCTCAAGTGCGACATCAGCGCAGAGATCCAGCAGCGACAGGAGGAGCTGGACGCCATGA 810
      250     260     270
Q A L Q E Q D S A F G A V H A Q M H A A V G Q L G R A R A E
CGCAGGCGCTGCAGGAGCAGGATAGTGCTTTGGCGCGGTTTACGCGCAGATGCACGCGCGCTCGGCCAGCTGGGCCGCGCGCTGCCG 900
      280     290     300
T E E L I R E R V R Q V V A H V R A Q E R E L L E A V D A R
AGACCGAGGAGCTGATCCGCGAGCGCTGCGCCAGGTGGTAGCTCAGTGCGGGCTCAGGAGCGCGAGTGCTGGAGGCTGTGGACGCGC 990
      310     320     330
Y Q R D Y E E M A S R L G R L D A V L Q R I R T G S A L V Q
GGTACCAGCGGACTACGAGGAGATGGCCAGTGGCTGGGCGCGCTGGATGCTGTGCTGCAGCGCATCCGCACGGGCAGCGCGCTGGTGC 1080
      340     350     360
R M K C Y A S D Q E V L D M H G F L R Q A L C R L R Q E E P
AGAGGATGAAGTCTACGCTCGGACCAGGAGGTGCTGGACATGCACGGTTTCTGCGCCAGGCGCTCTGCCGCTGCCCCAGGAGGAGC 1170
      370     380     390
Q S L Q A A V R T D G F D E F K V R L Q D L S S C I T Q G K
CCCAGAGCCTGCAAGCTGCCGTGCGCACCGATGGCTTCGACGAGTTCAAGTGCGCCTGCAGGACCTCAGCTCTGCATCACCCAGGGGA 1260
      400     410     420
D A A V S K K A S P E A A S T P R D P I D V D L P E E A E R
AAGATGCAGCTGTATCCAAGAAAGCCAGCCAGAGGCTGCCAGCACTCCAGGGGACCTATTGACGTTGACCTGCCCGAGGAGGCAGAGA 1350
      430     440     450
V K A Q V Q A L G L A E A Q P M A V V Q S V P G A H P V P V
GAGTGAAGGCCAGGTTCAAGGCCCTGGGGCTGGCTGAAGCCAGCCTATGGCTGTGGTACAGTCAGTGCCCGGGGCACACCCCGTGCCAG 1440
      460     470     480
Y A F S I K G P S Y G E D V S N T T T A Q K R K C S Q T Q C
TGTACGCTTCTCCATCAAAGGCCCTTCTATGGAGAGGATGTCTCCAATACAACGACGCCAGAAGAGGAAGTGCAGCCAGACCCAGT 1530
      490     500     510
P R K V I K M E S E E G K E A R L A R S S P E Q P R P S T S
GCCCCAGGAAGTTCATCAAGATGGAGTCTGAGGAGGGAAGGAGGAAGGTTGGCTCGGAGCTCCCCGAGCAGCCAGGCCAGCCAGCACCT 1620
      520     530     540
K A V S P P H L D G P P S P R S P V I G S E V F L P N S N H
CCAAGGAGTCTCACACCCACCTGGATGGACCGCTAGCCCCAGGAGCCCGTCATAGGAAGTGAGGTCTTCTGCCCAACAGCAACC 1710
      550     560
V A S G A G E A G R E R N A L W *
ACGTGGCCAGTGGCGCCGGGGAGGCAGGTAGGGAGAGGAACGCGTTGTGGTGATCAGCAGCTCGGAAGACTCAGATGCCGAAAACCTCGTC 1800
CTCCCGAGAGCTGGATGACAGCAGCAGTGAGTCCAGTGACCTCCAGCTGGAAGGCCCGCAGCACCTCAGGGTCTGGACGAGAACCTTGC 1890
TGACCCCCAAGCAGAAGACAGACCTCTGGTTTCTTTGACCTCAAGATTGACAATGAAAGTGGGTTCTCTGGGGCTACCCCCACCCCTT 1980
TCTAATTAGTCTCTGAGTCCCAAAAAGAGTGCAGGCAGAGCCATCTGCCAGGCCAGGAGAGCTCTGAGCTCTGGCCAACTGTCAG 2070
CCAGGCTGGGCAGAGCACTCCGGCTCACCTGGGCTCCTGGCGTGTCAATTGCTGGCTTGAATAAGATGTCCGCTTAAAAAAA 2155
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b

Figure 2. Nucleotide and Amino Acid Sequence of *PML-1* cDNA Isolated from an APL Patient mRNA
(a) The triangle represents the fusion point seen in *PML-RAR*. A potential polyadenylation signal is underlined. Both ATGs are shown in bold type (nucleotides 81–83 and 147–149).
(b) Schematic structure of the *PML-1* protein. The encoded protein contains four regions of interesting structure. They are denoted as follows: Pro, proline-rich region; Cys, cysteine-rich region; α -Helices, α helix-rich region; Ser, C-terminus region where several serines are surrounded by prolines. The triangle shows the fusion point seen in *PML-RAR*. L-Z shows the position of a putative leucine zipper. The numbers above the boxes represent the amino acids at the junction of the assigned regions.

Figure 3. PML Shares Amino Acid Similarity to Proteins Containing Cysteine-Rich and Leucine Zipper Motifs

(a) Amino acid alignment of proteins with novel cysteine-rich motif. Three cysteine clusters are shown by arrows. Conserved amino acids, including cysteines/histidines, are denoted in bold type. Dashes represent gaps in amino acid alignment. The numbers represent the position of the first and the last amino acids in the respective protein sequences. The protein sequences are cited as follows: Rfp, Takahashi et al. (1988); Rpt-1, Patarca et al. (1988); Mel-18, Tagawa et al. (1990); RING1, Freemont et al. (1991); RAG-1, Schatz et al. (1989); RAD18, Jones et al. (1988); IE110, Perry et al. (1986); VZ61, Davison and Scott (1986); CG30, Thiem and Miller (1989); LCMV-Z, Salvato and Shimomaye (1989). Cysteines and histidines in the third cluster are denoted in shadowed lettering.

(b) Amino acid alignment of putative leucine zipper of PML and the leucine zippers of the Fos family. Bold type denotes the amino acids conserved in all four proteins. Underlining denotes the similar amino acids conserved in all four proteins. Dashes represent gaps in amino acid alignment. The numbers represent the position of the first and the last amino acids in their protein sequences. Asterisks mark the position of conserved leucines in proteins of the Fos leucine zipper family. The proteins from the Fos family are cited as follows: Fra-1, Cohen and Curran (1988); c-Fos, Van Beveren et al. (1983); FosB, Zerial et al. (1989).

of cysteine and histidine in this third cluster resembles a CCHC box (Berg, 1990) seen in retroviral zinc fingers. This structure analysis suggests that PML-1 may bind DNA through its cysteine-rich domain and is thus a candidate transcription factor.

It is noteworthy that this protein family includes the recombination-activating gene product (RAG-1) (Schatz et al., 1989) and RAD18, a yeast gene product that is involved in repair of UV-damaged DNA (Jones et al., 1988).

Following the cysteine-rich region is a large segment that could form multiple α helices. A segment in this α helix-rich domain shows homology to the leucine zipper region of the Fos family (Cohen and Curran, 1988; Van Beveren et al., 1983; Zerial et al., 1989) (Figure 3b). Because the leucine zipper mediates dimerization between Fos and Jun, the presence of this related sequence raises the possibility that PML or PML-RAR may be part of a homo- or heterodimer complex.

The C-terminal region following the α -helical region includes ten serines, five of which are surrounded by prolines. This type of motif is reminiscent of serine phosphorylation sites and suggests that PML may be a phos-

phoprotein and a substrate for serine/threonine protein kinases and/or phosphatases (Kemp and Pearson, 1990; R. Doolittle, personal communication). Interestingly, this region is deleted in PML-RAR (see below).

At the N-terminus 16 of the 46 residues are prolines. A proline-rich motif is part of the transcriptional activation domain of CTF (Mermod et al., 1989) and has also been noted in many other mammalian transcription factors (Mitchell and Tjian, 1989, and reference therein). In addition, six of these prolines are adjacent to serine residues and may also represent candidate phosphorylation sites (see above). We also identified multiple forms of PML cDNAs, presumably produced by alternative splicing, which give rise to different sized proteins as analyzed by in vitro translation analysis (data not shown).

The identification of the reciprocal fusion gene products strongly suggests that the PML gene is located on chromosome 15. This was confirmed by analyzing metaphase chromosome preparations by fluorescent in situ hybridization. Among 34 informative metaphase preparations, 46 fluorescent hybridization signals were observed and 22 (49%) were present on chromosome 15. Of the 22 hybrid-

Figure 4. The *PML* Gene Maps to Human Chromosome 15q22
Mapping of the *PML* gene to chromosome 15q22 region by in situ hybridization using a biotin-labeled *PML-1* cDNA as a probe. The ideogram of chromosome 15 depicts the distribution of the fluorescent signals relative to known banding patterns of chromosome 15.

ization events observed on chromosome 15, 11 were present at 15q22 (Figure 4) and correspond closely to the known site of translocation (Mitelman, 1988).

Structure of the *PML-RAR* Fusion cDNA

The *PML-RAR* cDNA (*pPML-RAR*) consists of 3036 nucleotides and has an open reading frame that continues in frame from *PML* to the *RAR* α terminus (Figure 5). The predicted protein consists of 797 amino acids, with a calculated relative molecular mass of 89,281, and gives rise to a 90 kd product when tested by the in vitro transcription-translation system (data not shown). Using an *RAR* α -specific antibody, the fusion protein can be detected in nuclear extracts of COS cells transfected with a *PML-RAR* cDNA expression vector (data not shown). The fusion point occurs between nucleotide 1249 and 1250 in the *PML-RAR* sequence (Figure 5) and produces a new codon for alanine at residue 395. The fusion *PML-RAR* protein substitutes 394 amino acids from *PML* in place of the first 59 amino acids from the N-terminus of *RAR* α .

If the *PML-RAR* fusion gene is a unique translocation product in all APL patients, the fusion mRNA should be diagnostic for the presence of leukemic cells. Accordingly, polymerase chain reaction (PCR) analysis with primers flanking the fusion point was used to characterize APL RNA described in Figure 1 and from an additional patient. Primers include a sense oligonucleotide from *PML* and an antisense oligonucleotide from *RAR* α (Figure 6a and Experimental Procedures). A 439 bp product is predicted for APL patients with *PML-RAR* α translocations. Figure 6b shows the ethidium bromide staining of an agarose gel for the PCR product from two patient RNA samples. Each patient yielded a band of about 440 bp (Figure 6b, open arrow), while HeLa RNA gave no signal. The identity of the PCR products was confirmed by restriction enzyme

analysis and verified the amplification (data not shown). Primers specific for *RAR* α cDNA (Figure 6a and Experimental Procedures) yielded identical PCR products in all three RNA samples (Figure 6b, bottom). These results confirm that the fusion transcript presented here is specific and common for these two APL patients. We have analyzed additional APL cases using these same oligonucleotide primers, all but one showing the same result. The one exception showed a longer amplification product, indicating that a translocation had occurred in the same genes but in a different position (W. H. M. and E. D., unpublished data).

We also identified multiple forms of *RAR-PML* fusion cDNAs resulting from the reciprocally translocated allele. The detailed structures of these products are being determined. Considering the overall structural feature of *PML-RAR* and *RAR-PML* fusion proteins and that *PML-RAR* contains the highly conserved domains from both gene products, *PML-RAR* represents the focus of the remainder of our analyses here.

PML-RAR Is a Transcription Activator

To facilitate functional analysis, wild-type *RAR* α and *PML-RAR* cDNAs were transferred to the pCMX expression vector (Umesono et al., 1991), creating pCMX *RAR* α and pCMX *PML-RAR*, respectively. A deletion mutant (pCMX Δ *PML-RAR*) that lacks the entire *PML* segment was also generated (see Experimental Procedures). The receptor expression vector was cotransfected with a set of CAT reporter plasmids based on a minimal SV40 promoter in which the enhancer was removed (Δ SV) and substituted with various RA response elements. The response elements included TREp, which is a palindromic response element activated by high levels of thyroid hormone and RA receptors (Umesono et al., 1988), and β RE, which is a natural element from the *RAR* β gene and is responsive to low levels of RA (Sucov et al., 1990; de Thé et al., 1990b). These reporter vectors allow comparison of the transcriptional activities of the *PML-RAR* fusion receptor with that of transfected *RAR* α and endogenous *RAR*.

PML-RAR fusion constructs could transactivate RA response elements when treated with RA (Figure 7). Unexpectedly, in CV-1 cells, the fusion receptor showed constitutive transactivation on both TREp and β RE (Figure 7a). This transactivation was further enhanced by adding RA exogenously, resulting in about 2-fold greater induction. In contrast, the deletion mutant (pCMX Δ *PML-RAR*) failed to display the constitutive phenotype and is nearly identical to the wild-type *RAR* α . This indicates that the constitutive activation property has resulted not from the truncation of the *RAR* α N-terminus, but from the fusion with the *PML* gene product.

To explore potential cell type variation, *PML-RAR* was expressed in a variety of cell lines. In Hep G2 cells (human hepatocellular carcinoma) and K562 cells (human myeloblast leukemia), *PML-RAR* responded similarly as in CV-1 cells (data not shown). The *PML-RAR* fusion protein resulted in a high constitutive activation that was further induced by RA. However, in the human acute my-

Figure 5. Nucleotide and Amino Acid Sequence of the *PML-RAR* cDNA Isolated from APL Patient mRNA
The triangle represents the fusion points seen in *PML-RAR*. A potential polyadenylation signal is underlined.

Figure 6. PCR Detection of *PML-RAR* Fusion Transcript

(a) Schematic showing the primers used for PCR detection. Arrows represent the positions of primers used in PCR (see Experimental Procedures). Numbers represent the predicted size of PCR products. (b) Ethidium bromide staining of the PCR products. (Top) The open arrow shows the products of the PCR, using *PML-RAR*-specific primers (see Experimental Procedures). The closed arrow shows unutilized primers. (Bottom) The products of the PCR with *RAR α* -specific primers are shown (see Experimental Procedures). The negative image of the photograph is printed.

eloid leukemia cell line HL-60, PML-RAR becomes fully ligand dependent (Figure 7b). Furthermore, in the absence of RA the fusion proteins actually suppress basal level transcription. Nonetheless, in this case, the RA-induced response is still 2-fold greater than that of wild-type RAR α (Figure 7b).

The transactivational function of PML-RAR was assessed in two other promoter contexts. In one case, using a thymidine kinase promoter containing a TREp (Damm et al., 1989), both RAR α and PML-RAR show comparable inductive profiles in CV-1 cells (Figure 7c). In another case the RA-responsive promoter from the *RAR β* gene (Sucov et al., 1990) was used to monitor PML-RAR activity (Figure 7d). In CV-1 cells with an RAR β element, a fully hormone-dependent response was observed with RA. While constitutive activity was not seen, it was clear that PML-RAR displays a more effective activation profile than the wild-type RAR. Again, when the PML extension is deleted, the superinduction is also lost (Figure 7d). These data indicate significant cell type variation in the activation profiles of PML-RAR. However, in each instance, PML-RAR is at

Figure 7. Comparison of the Transactivation Abilities of RAR α and PML-RAR

The transactivation abilities of RAR α and PML-RAR were monitored in the presence (hatched bars) or absence (solid bars) of 1 μ M RA. An expression plasmid coding for the firefly luciferase (Control), RAR α , PML-RAR, or a deletion mutant of PML-RAR (Δ PML-RAR) was tested in various combinations with five different CAT reporters (Δ SV, Δ SV-TREp, Δ SV- β RE, TK-TREp, RAR β (P)), in CV-1 cells (a, c, and d) or HL-60 cells (b). There is a significant increase in activation with PML-RAR compared with RAR α . This increase is dependent on the presence of the PML segment, since it is absent with Δ PML-RAR.

least as effective as wild-type RAR α and generally is a more effective activator than wild-type RAR α .

Discussion

Evidence is presented that the t(15;17) translocation in APL involves reciprocal fusions between the *RAR α* gene on chromosome 17 (Mattei et al., 1988) and a gene (*PML*) encoding a novel putative DNA-binding protein. The translocation is proposed to create an oncogene whose product, PML-RAR, is suggested to be a chimeric transcription factor.

The creation of a chimeric transcription factor is reminiscent of the translocation of t(1;19) in pre-B cell acute lymphocytic leukemia, which fuses a homeobox protein (Prl) with a helix-loop-helix protein (E2A) that is a known regulator of the κ -immunoglobulin gene enhancer (Nourse et al., 1990; Kamps et al., 1990). PML is part of a newly

recognized class of regulatory proteins that includes candidate transcription factors such as Rpt-1 (Patarca et al., 1988) (a regulator of the interleukin-2 receptor gene) and two potential oncoproteins, Rfp (Takahashi et al., 1988) and Mel-18 (Tagawa et al., 1990). This family also includes the products of the recombination-activating gene (*RAG-1*), which controls immunoglobulin gene rearrangement (Schatz et al., 1989), and *RAD18*, a yeast gene for postreplication repair of UV-damaged DNA (Jones et al., 1988). While perhaps serving different functions, these gene products are thought to bind to DNA, suggesting that the conserved cysteine cluster may encode this property. The total of eight cysteine/histidine residues suggests that this region could coordinate two zinc ions, which would thus represent a novel class of DNA-binding finger (Berg, 1990).

The second cysteine cluster in PML contains seven cysteine/histidine residues and is similar to those of the Rfp and Rpt-1 proteins. The functional significance of this cluster is less clear. Finally, a third cluster (amino acids 189–227) containing cysteine/histidine residues is currently unique to PML and represents yet a third potential region for zinc coordination and DNA binding.

Within the potentially α -helical region in the center of PML is a segment (amino acids 280–327) with homology to the leucine zippers from c-Fos, FosB, and Fos-related antigen (Fra-1) (Van Beveren et al., 1983; Zerial et al., 1989; Cohen and Curran, 1988). If analogous in function this would present a potential dimerization interface for PML. Regardless, the three cysteine clusters, as well as the candidate leucine zipper, are all present in the PML-RAR chimeric product and their properties are potentially important in transforming potential. In addition to the DNA-binding motifs, the N-terminus of PML contains 16 prolines in the first 46 amino acids. Proline-rich sequences have previously been associated with transcription activation domains in the CTF family of transcription factors (Mermod et al., 1989) and have also been found in the N-terminal portion of the E12 transcription factor (Murre et al., 1989). Together, these results support the proposal that PML is a candidate transcription factor.

The PML-RAR fusion protein lacks the N-terminal 59 amino acids of RAR α but retains intact the DNA-binding and ligand-binding domains. The deleted N-terminal 59 amino acids of RAR α appear to have little function in transactivation, as pCMX Δ PML-RAR has properties similar to those of RAR α (Figures 7a and 7d). The PML-RAR fusion protein creates a potentially bifunctional protein with multiple DNA-binding and transcriptional regulatory properties. Consistent with this suggestion, PML-RAR has altered transactivation properties relative to wild-type RAR α (Figure 7). Two conspicuous properties are its superinducible activity by RA and its suppression of basal activity in the absence of RA (Figure 7).

In the accompanying paper an additional isoform of PML-RAR has been isolated from an APL patient. This additional isoform (PML-RAR(L)) includes 552 rather than 394 amino acids from the PML protein (de Thé et al., 1991). In cotransfection using reporters and cells shown in Figure 7, no significant differences were observed between these isoforms (data not shown). However, both isoforms showed unique transcriptional activities on a TK promoter carrying three tandem copies of the β RE (TK-(β RE) $_3$). Each fusion protein showed very weak transactivation properties compared with that of transfected wild-type RAR α in Hep G2 cells (Figure 8a). In this assay transactivation levels of both isoforms did not rise above the endogenous level, and in the case of PML-RAR(L), it displayed a mild suppressive phenotype. However, when tested on the RAR β (5K) promoter both isoforms showed a net positive induction relative to endogenous activity in Hep G2 cells. It is noteworthy that in CV-1 cells, PML-RAR is a better activator than RAR α on the RAR β promoter (Figure 7d). Finally in Figure 8c, reducing the number of response elements from three to one (TK- β RE) reverts the neutral or suppressive phenotype of PML-RAR into a positive activator. These lines of evidence clearly indicate that both isoforms show comparable altered transactivation phenotypes, which include cell-specific and promoter-specific variation. These patterns of variation are associated with the fused PML segment, suggesting that PML-RAR interacts with transcription factors in a fashion distinct from RAR α alone.

Figure 8. Comparison of the Transactivation Abilities of PML-RAR Isoforms on Different Promoters

An expression plasmid coding for RAR α , PML-RAR, PML-RAR(L), or Δ PML-RAR was tested on three different LUC reporters (TK-(β RE) $_3$, RAR β (5K), or TK- β RE) in Hep G2 cells. The pCMX vector alone was used in control transfections. PML-RAR(L) is an isoform of PML-RAR and has 552 rather than 394 amino acids from the PML protein (see Figure 2a and text). The percent LUC activity is calculated relative to that of transfected RAR α as 100%. Hatched bars and solid bars are as in the legend to Figure 7.

How might PML-RAR contribute to APL? While there are several possibilities, each must account for two clinical observations: greater than 90% of APL patients have t(15;17), suggesting that the mutant *RAR* allele is a new oncogene; and patients with APL can be induced into remission by high dose RA treatment (Huang et al., 1988; Chomienne et al., 1990; Miller et al., 1990; Warrell et al., 1991). The most parsimonious explanation is that PML-RAR acts to block or interfere with myelocyte differentiation. This idea has precedence with the v-ErbA oncoprotein that is thought to cause erythroleukemia by blocking the functions of its endogenous cellular counterpart, the thyroid hormone receptor (Damm et al., 1989; Zenke et al., 1990). According to this model, v-*erbA* and the *PML-RAR* fusion gene could be thought of as dominant negative oncogenes.

In one model, PML-RAR would function as an RAR antagonist and be insensitive to RA induction. Indeed, on the TK-(β RE)₃ reporter PML-RAR is a poor transactivator and may suppress endogenous RAR activity. Furthermore, PML-RAR is frequently able to suppress basal activity of noninduced promoters (Figures 7b, 8a, 8b, and 8c) relative to RAR α , which generally enhances these effects. However, support for this model is reduced by the observation that the PML-RAR fusion protein contains an intact RA ligand-binding domain and, in general, shows hormone responsiveness in most cell lines and promoters tested (Figures 7, 8b, and 8c).

An alternative, but not exclusive, model is that PML-RAR functions as a dominant negative inhibitor of the endogenous PML activation pathway. It has previously been shown that heterologous transactivators fused to the ligand-binding domain of a steroid receptor become hormone dependent. Thus, E1A or GAL4 fused to the glucocorticoid receptor show dexamethasone-dependent activity (Picard et al., 1988; Hollenberg and Evans, 1988). Similarly, the transforming activity of the Myc protein becomes estrogen dependent when fused to the estrogen receptor (Eilers et al., 1989), and the transcription factor C/EBP, which induces hepatocyte differentiation, becomes hormone dependent in this process when fused to the glucocorticoid receptor or estrogen receptor (Umex et al., 1991). For PML-RAR we propose that the t(15;17) translocation places the PML product under the control of the RAR ligand-binding domain. Similar to v-ErbA, the fusion protein might block function of wild-type PML by forming nonproductive heterodimers or by blocking target DNA sites. Addition of RA would reverse this inhibition by transforming the receptor to an activated state. This model provides a simple explanation as to why APL patients are amenable to RA treatment. A conclusive test for either model will require identifying PML-RAR target genes crucial for leukemogenesis.

It is hoped that these studies will help to bring a sharper focus on the molecular defect in APL as well as provide some insights into both its diagnosis and treatment. First, as indicated in Figure 6b, it should be possible to use PCR as a definitive form of diagnosis and a means to monitor the progress of APL patients. Second, because the fusion occurs uniquely with RAR α , it should be possible to identify

therapeutic RA analogs that would be selective for this receptor subtype. Finally, this work should lead to a better understanding of the role of retinoids in normal myelocyte differentiation and the role of nuclear receptors in human disease.

Experimental Procedures

RNA Preparation and Northern Blotting

Bone marrow cells from an APL patient were aspirated, and mononuclear fractions were collected by Ficoll-Paque sedimentation (Pharmacia-LKB) (Miller et al., 1990). Total RNA was prepared from patient mononuclear fractions and HeLa cells by the guanidium thiocyanate method (Chirgwin et al., 1979). Northern blotting analysis was performed as described previously (Kakizuka et al., 1990; Mangelsdorf et al., 1990). The PstI fragment covering nucleotides 538-1156 from human RAR α (Giguere et al., 1987) was used as hybridization probe.

Patient cDNA Library Construction and Screening for PML-RAR, RAR-PML, PML, and RAR α

One microgram of poly(A)⁺ RNA was purified from 100 μ g of total RNA from the mononuclear bone marrow cells of one APL patient, using oligo(dT)-Latex (Kuribayashi et al., 1988). Double-stranded cDNA was synthesized by the method of Gubler and Hoffman (1983) with minor modifications. After attachment of an EcoRI-NotI adaptor (Pharmacia-LKB), double-stranded cDNA was size fractionated by electrophoresis on a 0.8% agarose gel, and the cDNAs longer than 2 kb were ligated to a λ ZAP II vector (Stratagene). Approximately 5×10^5 plaques were screened with the probe described above for the Northern blotting. The first screen gave more than 100 positives, of which 40 random clones were purified. The 40 clones could be classified into two major groups. One major group consisted of RAR α and the other corresponded to PML-RAR. Each major group had a unique KpnI site in the 5' part of the cDNA. EcoRI-KpnI fragments of about 700 bp and 1000 bp were isolated from clone 2 (RAR α) and clone 66 (PML-RAR), respectively. Half of the membrane filters used for initial screen were rehybridized with each of the EcoRI-KpnI fragments. The probe from RAR α and PML-RAR gave rise to 9 and 19 nonoverlapping clones, respectively. These clones identified cDNAs coding for RAR-PML and wild-type PML. Wild-type RAR α (clone 2), PML-RAR (clone 66), and PML-1 (clone 124) were sequenced entirely. The amino acid sequence of RAR α (clone 2) was identical to that published (Giguere et al., 1987).

Chromosomal Assignment and Mapping of PML by In Situ Hybridization

PML-1 (clone 124) was labeled with biotin 11-dUTP (Enzo Biochemicals) using Escherichia coli DNA polymerase I (Maniatis et al., 1982). Metaphase chromosome preparations from phytohemagglutinin-stimulated and 5-bromodeoxyuridine-synchronized cultures of normal male lymphocytes (Zabel et al., 1983) were utilized as previously described (Neel et al., 1982; Jhanwar et al., 1983). The hybridization signal was detected by immunofluorescence using avidin-conjugated fluorescein isothiocyanate (FITC-avidin) and biotinylated anti-avidin using the in situ hybridization kit (ONCOR) as described (Pinkel et al., 1986; Fan et al., 1990).

Reverse Transcription/PCR Detection of the PML-RAR Fusion Gene

One microgram of total RNA was converted to cDNA with 40 ng of random hexanucleotide (pd(N)₆, Pharmacia-LKB) and 200 U of RNAase H⁻ Super Script reverse transcriptase (Bethesda Research Laboratories) in 20 μ l of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, and 500 μ M of each dNTP at 37°C for 1 hr. Ten to 20 ng of cDNA was used for PCR amplification in 25 μ l of 67 mM Tris-HCl (pH 8.8), 16.6 mM (NH₄)₂SO₄, 10 mM β -mercaptoethanol, 10% dimethylsulfoxide, 2 mM MgCl₂, 250 μ M of each dNTP, 1 μ M of each primer, and 1.5 U of Taq polymerase (Cetus). Amplification was carried out for 35 cycles in an Eppendorf MicroCycler (1 min denaturation at 92°C, 2 min annealing at 55°C, 3 min elongation at 72°C). One pair of primers (5'-GCG GTA CCA GCG CGA CTA CGA GGA GAT-3' and 5'-GCG GCG GAA GAA GCC CTT GCA GCC CTC ACA GG-3') was used for PML-RAR, and another pair (5'-ATG GCC

AGC AAC AGC AGC TCC TGC CCG ACA-3' and 5'-GCG GCG GAA GAA GCC CTT GCA GCC CTC ACA GG-3' for *RAR α* .

Plasmid Constructions

The EcoRI fragment from clone 2 (*RAR α*) and clone 66 (*PML-RAR*), both of which cover the entire cDNA, was transferred to the pCMX expression vector (Umesono et al., 1991), which contains the cytomegalovirus promoter. These plasmids were named pCMX *RAR α* and pCMX *PML-RAR*, respectively. To delete the *PML* region from pCMX *PML-RAR*, we first introduced a NcoI site changing T at nucleotide 1254 (Figure 5) to G and changed Ile to Met. The transactivation capacity of pCMX *PML-RAR* and this mutant (pCMX *PML-RAR NcoI*) was identical in CV-1 cells (data not shown). Because the translation start site of *PML-RAR* is a NcoI site (Figure 5), the fragment between the two NcoI restriction sites was removed from pCMX *PML-RAR NcoI* (pCMX Δ *PML-RAR*). pCMX *PML-RAR(L)* was constructed by replacing the entire *PML* coding segment (BglII-BstEII fragment) from pCMX *PML-RAR* with that of pSG5 *PML-RAR(L)*, a gift from Drs. Anne Dejean and Hugh de Thé. pCMX LUC (Umesono et al., 1991), which contains the coding sequence of luciferase, was used as a control.

The reporter plasmid Δ SV-CAT was constructed by replacing the TK promoter in TK-CAT (Damm et al., 1989) with the SphI-HindIII fragment of the SV40 early promoter. Oligonucleotides coding for TREp (Umesono et al., 1991) and β RE (β RE2) (Sucov et al., 1990) were reported previously. Each oligonucleotide was inserted at the unique HindIII site upstream of the SV40 promoter (Δ SV-TREp-CAT and Δ SV- β RE-CAT), and the identity of the inserted oligonucleotide was confirmed by sequencing. Only single response elements were used to avoid potential artificial responses following multimerization of the binding sites. *RAR β (P)-CAT* (a gift from Dr. Henry Sucov) contains about 600 bp of the promoter region of the mouse *RAR β* gene (Sucov et al., 1990). TK-(β RE)₃-LUC and *RAR β (5K)-LUC* are gifts from Drs. Anne Dejean and Hugh de Thé (1990b).

Cell Culture and Transfection

CV-1 cells were maintained in DMEM supplemented with 10% charcoal-resin double-stripped calf serum. Hep G2 cells were grown in DMEM with 10% fetal bovine serum. K562 cells and HL-60 cells were grown in RPMI 1640 medium with 10% and 20% fetal bovine serum, respectively. CV-1 and Hep G2 cells were transfected by the calcium phosphate method (Graham and van der Eb, 1973; Kakizuka et al., 1990) with 0.5 μ g of pCMX receptor plasmid, 1 μ g of reporter CAT or LUC plasmid, and 5 μ g of pRAS- β GAL (Umesono and Evans, 1989) or pCMX- β GAL as an internal control. The cells were transfected for 8 hr, and after the DNA precipitates were washed out, they were incubated for an additional 36 hr with or without 1 μ M RA (Sigma). K562 and HL-60 cells were transfected using the Gene Pulser electroporation apparatus (Bio-Rad). Cells were collected by centrifugation at 600 $\times$ g for 5 min, washed twice with ice-cold opt-MEM (GIBCO), and suspended in opt-MEM. Samples (0.2 ml) of cell suspension ($5-10 \times 10^6$ cells/ml) were kept on ice and electroporated in a 0.4 cm cuvette, using 960 μ F at 250 V with 1 μ g of a pCMX receptor plasmid and 2 μ g of a reporter CAT plasmid and 6 μ g of pCMX- β GAL as an internal control. The samples were kept at room temperature for 20–40 min and then transferred to 10 ml of medium and incubated at 37°C. After 3 hr, cells were split into two samples in 10 ml of medium and further incubated for 36 hr with or without 1 μ M RA. Cell extracts were prepared for β -galactosidase, LUC, and CAT assays as described (Umesono and Evans, 1989).

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GenBank Accession Numbers

The accession numbers are M73778 for *PML-1* and M73779 for *PML-RAR*.

Note Added in Proof

A new member of the *PML* family, called *bmi-1*, has recently been identified (van Lohuizen et al., 1991, *Cell* 65, 737–752; Haupt et al., 1991, *Cell* 65, 753–763) that functions as a putative oncogene in transgenic mice. *bmi-1* is apparently activated by retroviral insertion and cooperates with *myc* in triggering B cell lymphoma. These results support the idea that perturbation of the *PML* transcriptional pathways by *PML-RAR* may contribute to leukemogenesis.