PML Protein Expression in Hematopoietic and Acute Promyelocytic Leukemia Cells

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Acute promyelocytic leukemia (APL) is thought to be caused by the t(15,17) translocation that fuses the PML gene to that of the retinoic acid receptor α (RARα) and generates a PML/RARα fusion protein. Yet, paradoxically, APL cells are exquisitely sensitive to retinoic acid (RA), as they terminally differentiate upon RA exposure. In this report, we have examined the expression of PML and PML/RARα in normal and APL cells. By immunofluorescence or immunocytochemistry, we show that PML has a speckled nuclear pattern of expression that contrasts with that of PML/RARα (mostly a micropunctuated nuclear pattern or a cytoplasmic localization). The APL–derived cell line NB4 that expresses both the PML and PML/RARα genes also shows the fine micropunctuated nuclear pattern, suggesting a dominant effect of the fusion protein over the localization of wild-type PML. RA treatment of NB4 cells or clones expressing PML/RARα gradually leads to a PML pattern before apparent morphologic maturation. In 14 untreated APL patients, the PML-reactive proteins were cytoplasmic (by immunocytochemistry) or both cytoplasmic and nuclear with a micropunctuated pattern (by immunofluorescence). Strikingly, in 4 patients, after 1 to 2 weeks of RA therapy, the speckled nuclear PML pattern reappeared concomitant with the onset of differentiation. These results establish that fusion of PML to RARα results in an altered localization of PML that is reverted upon RA treatment. This observation, which highlights the importance of PML, is likely to be a key to unravelling the molecular mechanism of both leukemogenesis and RA-induced differentiation of APL.

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RETINOIC ACID (RA) administration leads to complete remissions in most patients with acute promyelocytic leukemia (APL) by inducing the differentiation of the leukemic clone.[1-5] RA therapy of APL represents one of the first relevant examples of differentiation therapy and, although APL is a rare disease, it constitutes an invaluable model system in cancer biology. APL is characterized by the presence of a specific t(15,17) translocation.[6] The retinoid sensitivity of APL and the chromosomal localization of the retinoic acid receptor α (RARα) gene cytogenetically close to the t(15,17) breakpoint lead to the identification of RAR rearrangements in APL.[7-10] The molecular cloning of the t(15,17) translocation showed that a new gene, PML, was fused to RARα.[11-13] The PML/RARα fusion gene product (for which several subtypes are found depending on the position of the breakpoints in PML) is a large, multifunctional protein of 100 Kd that possesses most of the presumed functional domains of both PML and RARα.[14-18] Consequently, the fusion protein might interfere with both transcriptional regulation pathways. Indeed, PML/RARα was shown to negatively interfere with RA response in cotransfection assays with RA-responsive promoters[14-17] as well as in RA-dependent differentiation of HL60 cells.[19] This finding strongly suggests that PML/RARα may contribute to leukemogenesis by inducing a differentiation arrest. RA efficacy in a disease presumably caused by a RAR alteration provides a unique example of an anticancer drug targeted at a specific genetic lesion in a human cancer. However, the molecular basis of the exquisite RA sensitivity of cells that are impaired in their RA transduction pathway is obscure and somehow paradoxical.[20]

Detailed functional analysis of transforming fusion proteins (in particular *bcr-abl*[21] and *myb-ets*[22]) has shown that in both cases either gene made important contributions to leukemogenesis. In APL, specific fusion of PML to RARα argues for a contribution of PML to transformation.[23] This gene contains several domains, including a C3HC4 (3 Cysteine, 1 Histidine, and 4 Cysteine) zinc finger motif, part of a larger cysteine-rich region, and a leucine-zipper, that could account for the dimerization of PML and PML/RARα. The C3HC4 motif that may represent a DNA or protein binding sequence is conserved among a group of proteins, the origins and functions of which vary from yeast to human and DNA repair to transcriptional activation.[16,24] The cysteine-rich region and leucine-zipper domain define a subfamily of 7 genes, 3 of which (PML, RFP, and T18) were discovered as fusion proteins associated with malignant transformation.[25] In all three cases, the fusion occurred immediately downstream of the leucine-zipper motif. This strongly suggests that C-terminal deletions of these proteins might be involved in oncogenesis.

In this context, PML expression was investigated in normal human cells and in APL blasts. By immunofluorescence and/or immunocytochemistry, we show that PML has a speckled nuclear localization. PML expression could be detected in most cell lines, whereas in human bone marrow it was restricted to the myeloid lineage. In the NB4-

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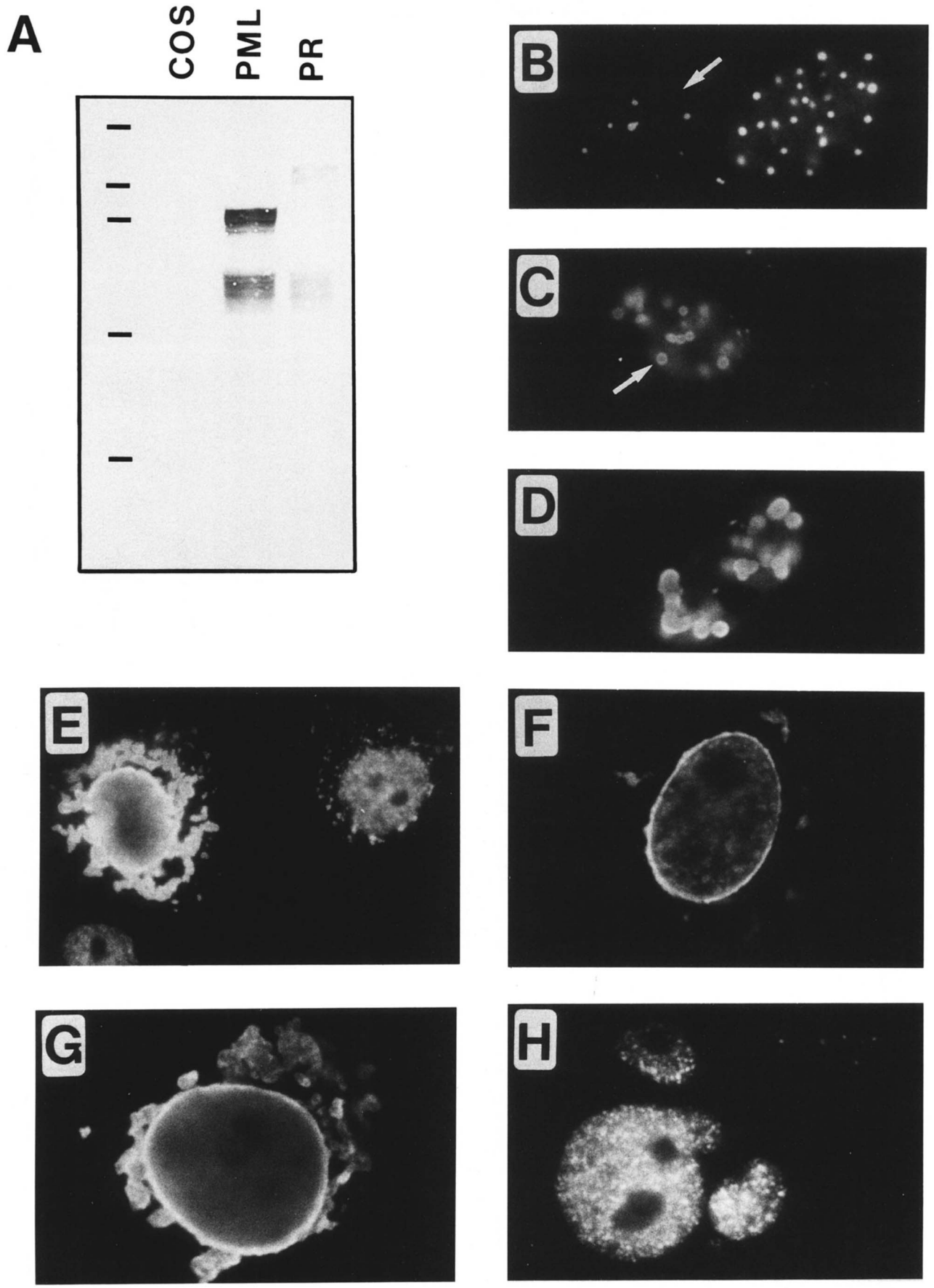
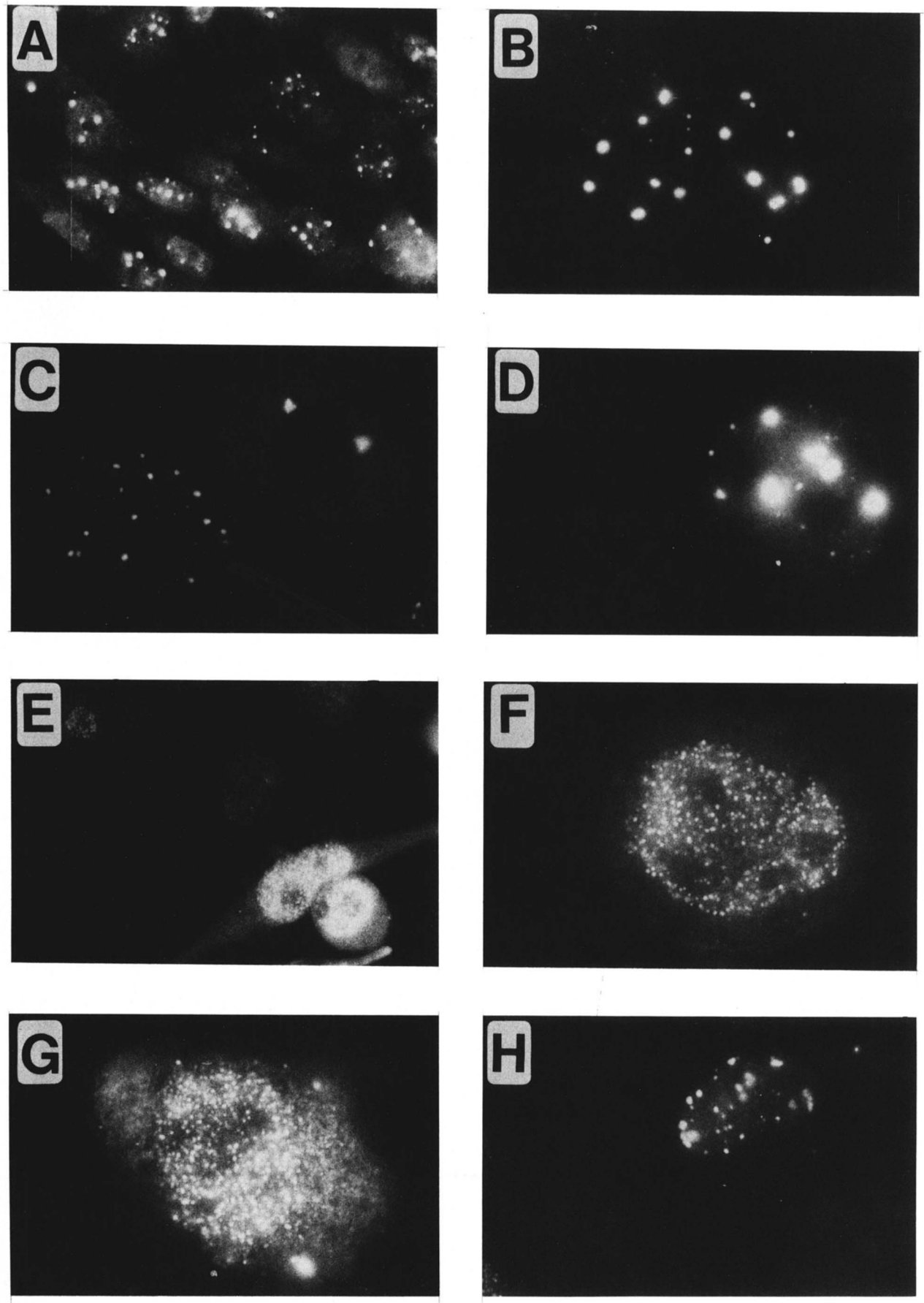


Fig 1.



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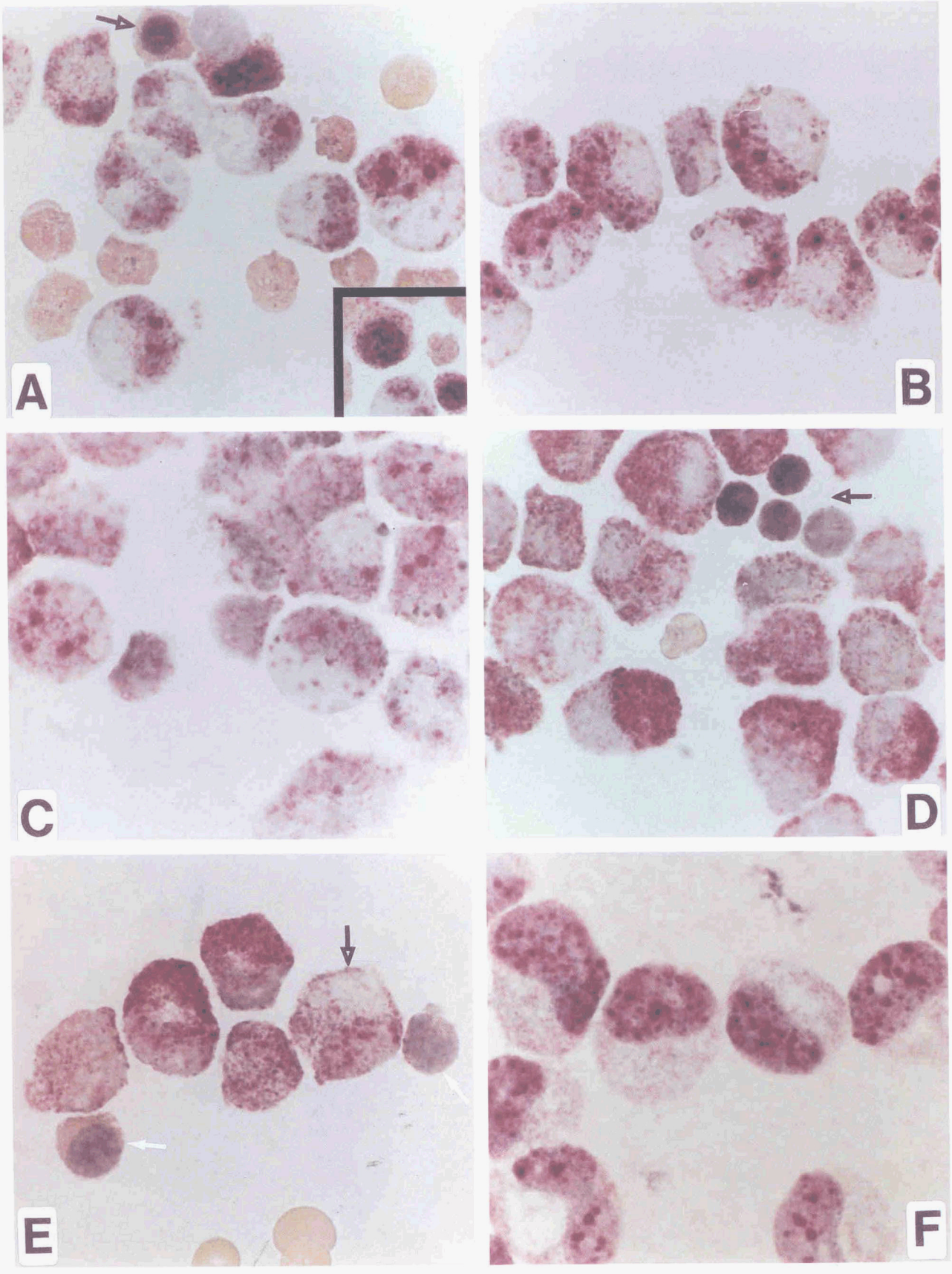

Fig 3.

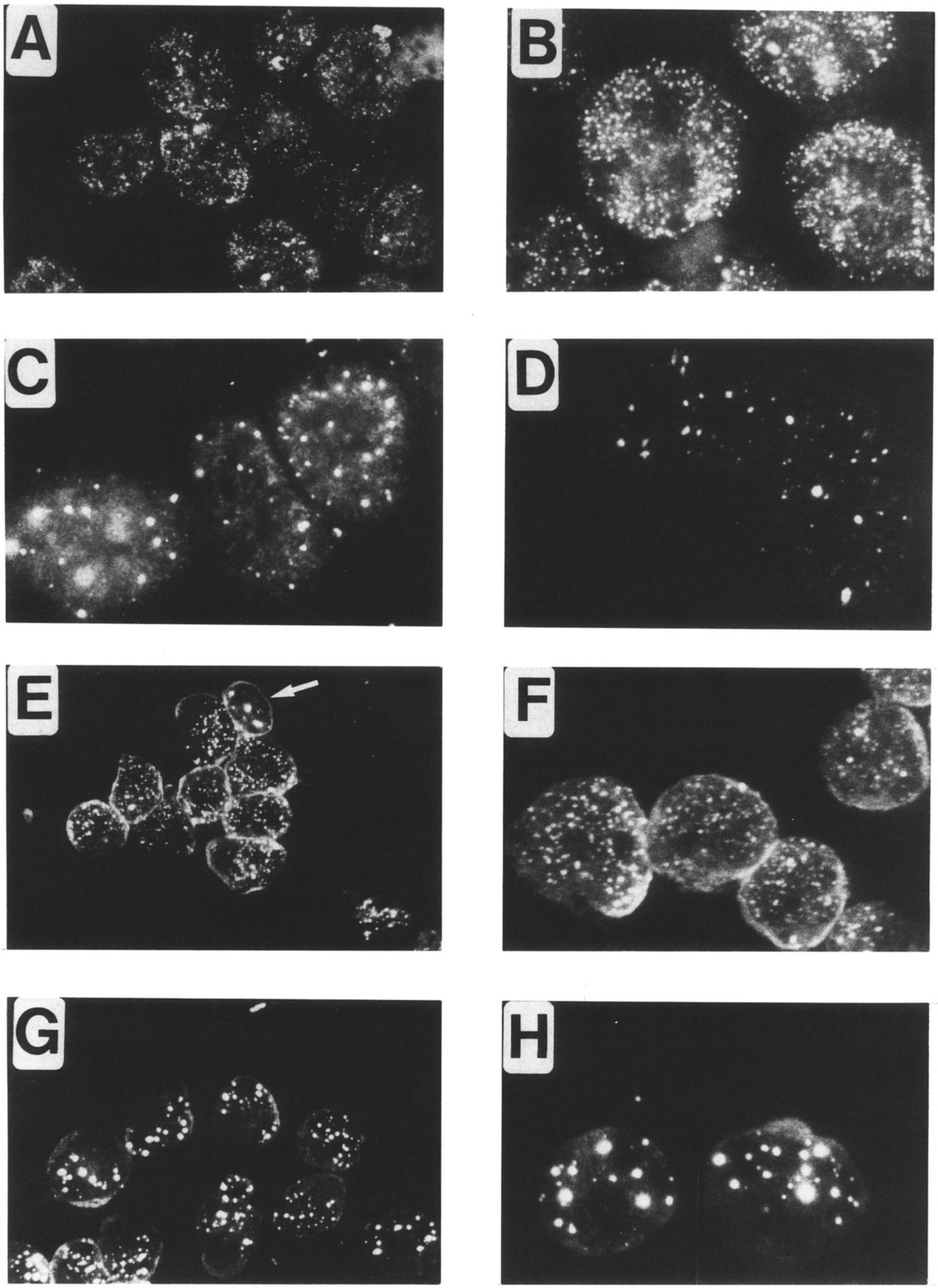

Fig 4.

APL cell line or in CHO clones stably expressing PML/RARα, a distinct micropunctuated nuclear pattern was detected. Analysis of fresh APL blasts shows a micropunctuated nuclear as well as cytoplasmic localization of PML and PML/RARα. In these patients (as well as in NB4 cells), RA therapy induces a nuclear transfer of the PML reactive proteins to PML sites that is coincident with the onset of differentiation. Thus, in APL, fusion of PML to RAR results in an altered localization of the PML proteins that is reversible upon RA exposure.

MATERIALS AND METHODS

Antibodies. PML was expressed as a Glutathion-S-Transferase (GST) fusion protein in *Escherichia coli* by inserting the complete PML coding region (*Nco*I to *Bam*HI, positions 80 to 2082 in de Thé et al[15]) in the plasmid BNAME (a derivative of pGEX3X; Pharmacia, Uppsala, Sweden; kind gift of T. Chouard). Protein expression was performed as described[26]; in short, *E coli* strain DH5aF' cells carrying the expression plasmid were induced by 0.8 mmol/L IPTG. The 90-Kd GST-PML fusion product was purified by affinity chromatography using glutathion-agarose (Sigma, St Louis, MO) and eluted with 5 mmol/L reduced glutathion (Sigma). Glutathion was removed from the eluate by centrifugation on a Centriprep 10 concentrator (Amicon, Beverly, MA). Rabbits and mice were immunized with 300 μg or 50 μg, respectively, of fusion protein in the presence of Freund's complete (and subsequently incomplete) adjuvant by multiple intradermal injections. Immune response was assessed by Western blot or immunofluorescence. Immune sera were purified by first selecting for IgGs using an ImmunoPure purification kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Subsequently, the IgGs were extensively adsorbed over a GST cross-linked Affigel column (BioRad, Richmond, CA). The eluant was applied to an Affigel column cross-linked to GST-PML. PML-specific antibodies were eluted according standard procedures.[27] The specificity of this reagent was assessed both by Western blot analysis and immunofluorescence. These affinity-purified antibodies were used at dilutions of 1/5 to 1/50. In the experiments reported here, several antisera gave identical results. Anti-RARα:Ab9α(F)' (kind gift of P. Chambon), a mouse monoclonal directed against the F region of RARα,[28] was used at a dilution of 1/100.

Cell culture. Cos-7 and Chinese hamster ovary (CHO) cells were grown in Dulbecco's modified Eagle's medium (GIBCO, Paisley, UK) with 10% fetal calf serum (FCS; GIBCO). NB4 cells were propagated in RPMI 1640 with 20% FCS. Analysis was always performed under exponential culture conditions. RA was used at a concentration of 10^{-6} mol/L all-*trans* RA unless otherwise indicated.

Analysis of transfected cells. CHO cells (Pasteur strain) were stably transfected with retroviral expression vectors that drive the expression of PML and PML/RARα (the construction of these vectors will be described elsewhere). Three-day posttransfection cells were exposed to G418 (500 U/mL; GIBCO) or hygromycin (250 U/mL; Sigma). Individual antibiotic resistant clones, isolated by limiting dilution, were tested for PML or PML/RARα expression. Cos-7 cells were transiently transfected with PML or PML/RARα expression vectors derived from pSG5 by the calcium phosphate procedure.[27] Western blotting was performed by conventional methods using polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA) blocked in BLOTTO-Tween.[27] The primary affinity-purified antibody was used at 1/50 dilution at 4°C overnight. After extensive washes in phosphate-buffered saline (PBS) 0.5% Tween, an alkaline phosphatase-coupled antirabbit IgG conjugate was applied for 1 hour at room temperature and shown using nitro-blue-tetrazolium and 5-bromo-4-chloroindoxyl phosphate (Sigma) as a substrate.

APL patient samples. A total of 14 untreated APL patients were analyzed, and 4 of them were subsequently tested during RA therapy. Controls were performed on 5 aspirates, 3 patients with normal bone marrows, 1 patient with chronic granulocytic leukemia (CGL), and 1 patient with acute myeloid leukemia (AML; M2). Blood and bone marrow samples were obtained with the patients' informed consent. Mononuclear cells were further isolated by centrifugation on Ficoll-Hypaque (Pharmacia) gradients.

Fig 1. Expression of PML and PML/RARα in transfected cells. (A) Western blot analysis of transfected Cos-7 cells. Cos, untransfected control; PML, PML expression vector; PR, PML/RARα expression vector. The horizontal bars represent molecular weight markers; from top to bottom, 200, 97, 69, 46, and 30 Kd. The group of bands at 50 Kd likely represents degradation products. (B, C, and D) Immunofluorescence analysis of PML localization in Cos-7 cells transfected with a PML expression vector. Various typical aspects are shown. The left part of (B), white arrow, is an untransfected cell. (C) Note the "doughnut-shaped" image (arrow) suggesting that PML is coating a sphere. (E through H) Immunofluorescence analysis of Cos-7 cells transfected with a PML/RARα expression vector; various typical aspects are shown. The aspect was identical with the anti-RARα antibody in double labeling experiments (data not shown).

Fig 2. Expression pattern of PML and PML/RARα in stable CHO clones. (A) Low power field view of a CHO clone expressing PML. (B, C, and D) High-power field views of various cells in a pool of stable PML transfectants. The human PML protein is specifically bound to the nuclear dots (untransfected CHO cells are negative for PML staining). (E through H) Immunofluorescence analysis of CHO cells expressing PML/RARα. Identical images were obtained with the anti-RARα for cells with a high level of PML/RARα expression (data not shown). Note the clear cut difference between the localization of PML and PML/RARα. (H) CHO cell expressing PML/RARα after a 3-day treatment with RA. Aggregation of the nuclear dots is observed, suggesting a retargeting of PML/RARα to PML sites.

Fig 3. Immunostaining of normal and leukemic bone marrow cells. (A) Normal bone marrow immunostained with the anti-PML antibody. The speckled nuclear staining is clearly visible. Note the strong positivity in the granulocytic lineage. Erythroblasts may occasionally be positive as in (A), black arrow and boxed insert, but are generally negative as in (E), white arrow, left. Exceptionally, megakaryocytes may be positive data (not shown). Lymphoid cells were constantly negative (see D, black arrow, and E, white arrow right). (B) CGL. (C) AML M2. The labeling pattern is identical to that of normal bone marrow. (D and E) APL before RA therapy. The PML staining is intense in the cytoplasm of some blasts. Note the presence of a maturing cell with a speckled nuclear pattern (black arrow). (F) Blood sample containing 95% blasts from the patient of (D) after 10 days of RA therapy. A speckled nuclear profile is found.

Fig 4. PML immunofluorescence in APL cells. (A and B) Untreated NB4 cells at low and high magnifications. Note that this pattern is similar to the one of CHO PML/RARα clones. (C) NB4 cells after a 24-hour RA exposure. (D) NB4 cells after 48 hours of RA exposure. RA induces a reappearance of the PML pattern. Insufficient sensitivity of the antibodies precluded the detection of PML/RARα (or RARα) in NB4 or APL cells. (E and F) Immunofluorescence analysis of APL blasts (from the marrows of two different patients) before treatment. (E) A cell with a normal PML pattern is indicated by an arrow. (G and H) Blood (G) and marrow (H) samples with at least 90% blasts from the same patients (E is G, F is H) after 10 days of RA therapy. Note the modifications in both cytoplasmic labeling and in the size of the dots.

Immunochemical and immunofluorescence studies. Transfected or untransfected cells were grown in Lab-Tech micro well plates and fixed in 4% paraformaldehyde in PBS at 4°C for 20 minutes followed or not by methanol for 5 minutes at 4°C. Cytospins of nonadherent cells were performed with 40,000 cells at 400 rpm for 10 minutes followed by air drying and fixation as previously described. The primary antibody was incubated on the cells for 1 to 3 hours at 37°C at a dilution of 1/50 in PBS. After an extensive washing in PBS, a second fluorescien-coupled antirabbit (or mouse) antibody was added for 1 hour at 37°C. Immunostaining was performed using the APAAP immuno alkaline phosphatase technique according to Cordell et al.[29] Double labeling was performed using a rhodamine-coupled antimouse conjugate (Nordic Immunology, Tilburg, The Netherlands).

RESULTS

Expression of PML and PML/RARα in transfected cells. In Western blots of transfected Cos cells, the PML or PML/RARα proteins appear as a group of bands of 70 and 100 Kd, respectively, in accordance with their calculated molecular weights[15] (Fig 1A). The existence of several molecular species suggests the presence of posttranslational modifications of the protein. The endogenous PML proteins could not be detected under the conditions used, which suggests that they are not abundant enough. However, using immunofluorescence in untransfected Cos cells, we could detect the speckled nuclear pattern of the endogenous PML protein (arrow in Fig 1B). In PML-transfected cells, PML was overexpressed as a few large nuclear dots of up to 2 μm of diameter (Fig 1B through D). In the case of PML/RARα-transfected cells, PML antisera detected a massive cytoplasmic and nuclear protein accumulation (Fig 1E, left part, and G), whereas in some other cells, PML was apparently stuck to the nuclear membrane (Fig 1F). Finally, a third common pattern consisted of hundreds of very small intranuclear dots, the nucleolus being prominently seen by exclusion (Fig 1E, right part, and H). Identical images were obtained with an anti-RARα antibody (not shown). As previously described, RA treatment lead to a nuclear transfer of the PML/RARα protein[17] (not shown). This crude approach suggested that fusion of PML to RARα has altered the localization of both of these proteins, corroborating previous studies.[17]

To circumvent potential artefacts due to transient expression, we generated stable clones expressing either PML or PML/RARα in CHO cells. Although untransfected CHO cells showed no reactivity to the anti-PML sera, in clones expressing PML, the profile was similar to the one previously observed in PML-transfected or untransfected-Cos cells (Fig 2A through D). PML was exclusively speckled and nuclear, without any labeling of other cellular structures. Therefore, the targeting of PML is a process conserved from hamster to human. For PML/RARα, hundreds of very small nuclear dots could be detected with both PML or RARα antibodies, as in some transfected Cos cells (Fig 2E through G; compare with Fig 1E and H). In contrast to transfected Cos cells, no massive cytoplasmic accumulation was observed. In most cells, RA administration lead to a progressive change in PML/RARα localization characterized by an aggregation of the dots after 3 days (Fig 2H). Thus, it appears that RA retargets PML/RARα to PML sites.

Expression of PML in bone marrow and APL cells and effect of RA therapy. Because PML antisera allow the detection of endogenous PML proteins, we explored 3 normal human bone marrow samples. In all 3, PML expression was primarily restricted to myeloid cells. A speckled nuclear labeling was evident and constant in the granulocytic lineage at all stages of maturation (Fig 3A). Occasionally, PML staining may be found in erythroblasts (Fig 3A, black arrow and the two cells in the boxed insert for labeled erythroblasts, and Fig 3E, white arrow on the left for an unstained erythroblast). PML labeling may be exceptionally observed in megacaryocytes (data not shown). Lymphoid cells were negative in all marrow aspirates examined (including a case of chronic lymphocytic leukemia (CLL), data not shown; see Fig 3D, black arrow, and E, white arrow on the right). When we examined CGL as well as M2 AML, an identical pattern was found (Fig 3B and C, respectively), suggesting that, except in APL, the PML pattern is unaltered in leukemic cells. This observation contrasts with previous reports based on mRNA detection that claimed that PML expression was ubiquitous.[14-17,30,31] Such demonstration of a myeloid-specific expression with constant and intense positivity of the granulocytic lineage may be important with regard to the pathogenesis of APL.

The APL-derived cell line NB4[32] is a valuable system with which to study PML expression, because both the normal and rearranged PML gene are present. The anti-PML antisera showed discrete micropunctations, seemingly located in the nucleus, identical to what we observed in stable PML/RARα CHO clones (Fig 4A and B). This argues that the profile in NB4 cells corresponds to a PML/RARα pattern and that PML/RARα colocalizes with the wild-type PML, as previously shown in transfected Cos cells.[17] In these cells (as in APL blasts) immunofluorescence analysis with the anti-RARα antibody was repeatedly negative. Most likely, this is the consequence of the low sensitivity of the anti-RARα antibodies and of the much lower level of PML/RARα protein expression in APL cells compared with PML/RARα-transfected Cos or CHO cells. When NB4 cells were induced to differentiate in the presence of RA, the PML/RARα pattern was gradually lost and substituted with a typical PML profile as early as 2 days after RA. Again, as in PML/RARα CHO cells, the first detectable modification was a perinuclear coalescence of the PML containing structures (day 1; Fig 4C), followed by appearance of a normal PML pattern (day 2, Fig 4D).

When cytospins from bone marrow aspirates or peripheral blood samples of 14 untreated APL patients were tested for PML expression by immunocytochemistry, the label was cytoplasmic for at least 90% of the blasts in all patients (Fig 3D and E). It should be noted that the intensity of the labeling of the blasts is variable. By immunofluorescence, nuclear dots reminiscent of the PML/RARα pattern were clearly detected in the two patients analyzed (Fig 4E and F), although some of the PML protein was cytoplasmic. Blood or marrow samples from 4 of the previously studied patients containing at least 90% blasts were analyzed during RA ther-

apy. After 10 days, PML was found to be mainly nuclear with a speckled aspect by both immunocytochemistry and immunofluorescence (Fig 3F, Fig 4G and H, same patients as Fig 4E and F, respectively). These maturating leukemic cells appear to have an intermediate pattern as they show more and smaller nuclear dots than in the normal granulocytic lineage (compare Fig 3F with 3A and B). Interestingly, in untreated patients, a few cells that could be spontaneously maturing blasts (eg, Fig 3E, black arrow) have the same profile. PML sites labeled distinctly as early as 7 days of treatment, coincident with the onset of leukemic maturation.

DISCUSSION

The results presented in this article shed light on several important aspects of APL. They show that, in contrast to what was previously assumed, PML is specifically expressed in at least some cell types in the hematopoietic system. The fusion of PML to RARα alters the intracellular distribution of PML. These alterations are reversible upon RA administration, which may relate to the induction of differentiation in these patients.

As expected from the presence of zinc-finger and leucine-zipper motifs, the localization of the normal PML protein is nuclear. A speckled PML protein pattern could be detected in all human and simian cell lines analyzed, although the level of expression was highly variable. No cross-reactivity was found in rodent cells, suggesting that the PML proteins are not antigenically conserved. Expression in only a subset of hematopoietic cells may suggest a role in myeloid maturation, but the detection of PML in many other cells types in vivo (skin and breast; data not shown) does not favor an exclusive role in the hematopoietic system.

The speckled appearance of PML is an important feature and may be a clue to unravelling its function. In transiently or stably transfected cells, the size of the dots reflects the absolute level of PML expression (compare Fig 2B with D). The clear enhancement of fluorescence at the border of the dot-like structures (see arrow in Figs 1C and D) suggests that PML is coating a sphere and contributes to the determination of its size. Electron and confocal microscopy studies are in progress to identify the nature of the PML-associated structures. These may correspond to intranuclear organelles known as nuclear bodies,[33] which are known sites of such key events as mRNA splicing or repetitive DNA replication.[34,35] Several proteins show a dotted pattern similar to the one discovered here such as RO-52K,[36] a protein part of the spliceosome complex that belongs to the same gene family as PML; and methyltransferase[37] and PCNA,[38] proteins associated with DNA replication. The high specificity of PML binding to this structure could be taken advantage of to characterize it.

We have not been able to precisely assign to PML/RARα a specific cellular compartment. In APL blasts, immunofluorescence and immunochemical analyses were not fully concordant. We note that the same discordance is found in NB4 cells as, by immunochemistry, PML staining was cytoplasmic in untreated and speckled nuclear in RA-treated cells (data not shown). We cannot, at present, account for the differences between the results obtained with these two techniques. Our data may suggest the existence of a soluble and speckled form of PML/RARα with a favored detection of the soluble form of the protein by immunochemistry and of the speckled one by immunofluorescence. That PML/RARα might be found exclusively cytoplasmic is puzzling, because both PML and RAR possess nuclear transfer signal sequences and are normally found in the nucleus.[17] The pattern of PML/RARα is clearly distinct from the one of PML. PML/RARα is not associated with the nuclear dots but with much smaller structures. This implies that the loss of the C-terminal region of PML and/or the fusion to RARα induce a nuclear retargeting and/or a cytoplasmic sequestration of the fusion protein. Whether the loss of binding of PML to the nuclear dots results in modifications of their structure or functions is not known.

In all the models that we have studied, RA administration lead to a progressive transfer of PML/RARα to PML sites. The experiments in CHO cells establish that the PML/RARα fusion is retargeted and not degraded. However, by immunofluorescence, we cannot precisely quantify PML/RARα during RA treatment. The molecular basis of PML/RARα nuclear transfer in the presence of RA, as well as the precise intracellular route, remains to be elucidated. It had previously been suggested that fusion of PML to RARα might lead to an RA dependence of PML function.[14] Our observations lend weight to that hypothesis. In patients, the reappearance of the PML profile during RA therapy is particularly striking. From a clinical point of view, it is often critical to predict response in RA-treated APL patients.[39] Detecting modifications in the PML profile might be worthwhile in predicting the time to the onset of differentiation.

These observations likely relate to the pathogenesis of APL and may provide major clues as to the molecular mechanism of RA action. In APL cells, the altered PML pattern implies that PML/RARα acts dominantly over the wild-type PML most likely through dimerization, as previously suggested in transfected cells.[17] PML sequestration at sites other than its nuclear binding sites most likely precludes a normal role of PML and may participate in the leukemic phenotype. We and others have already presented evidence for impairment of RAR function.[14-17] These alterations might be the consequence of the abnormal PML/RARα intracellular localization, because the chimeric protein (that in APL is considerably more abundant than RARα) may titrate out and delocalize the RXR cofactors, as initially suggested by Leid.[40] Via its abnormal localization and through dimerization, PML/RARα may dominantly interfere with both PML and RA signal transduction pathways. Thus, the RA-induced nuclear shift of the fusion protein may be at least one of the bases of RA action in APL.

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