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A Chicco.

STATEMENT

The experimental work described is composed of two related parts.

The first part regards i) the biochemical purification of PML/RAR α containing nuclear complexes; ii) the demonstration that such complexes consist of oligomers, with mapping of the region involved in the formation of such structures; iii) the evaluation of the biological consequences of the existence of PML/RAR α oligomers. Most of the experiments described in the first part are part of a manuscript already published (Saverio Minucci, Marco Maccarana, Mario Ciocce et al: **Oligomerization of RAR α and AML1 Transcription Factors as a Novel Mechanism of Oncogenic Activation**: Molecular Cell 2000 5: 811) and have been personally performed by the candidate with some exceptions: the DNA-affinity based purification of PML/RAR α and AML1/ETO HMW complexes has been performed by Dr. Marco Maccarana; transient transfections of HeLa cells and reporter luciferase assays have been performed by Dr. Pasquale De Luca; the purification of hematopoietic precursors (Lin- cells) and the evaluation of the ability of the fusion proteins to block differentiation of such precursors have been performed by Dr. Sabrina Giavara.

All the mentioned authors are or were at the moment at the European Institute of Oncology, Milan, Italy.

The second part of the thesis regards i) the study of subcellular distribution of the fusion proteins ii) the identification of regions required for the subnuclear localization of PML, RAR α and PML/RAR α ; iii) the identification of relationships between the oligomerization state of PML, and nuclear matrix association of the protein. All of the experiments described in the second part have been personally performed by the candidate.

ABBREVIATIONS

AML: Acute Myeloid Leukemia

APL: Acute Promyelocytic Leukemia;

CoA: CoActivator;

CC: coiled coil

DMEM: Dulbecco's Modified Eagle Medium;

FPLC: Fast Pressure Liquid Chromatography;

GFP: Green Fluorescent Protein;

IP: ImmunoPrecipitation;

IVT: In Vitro Translated;

HMW: High Molecular Weight;

NPM: Nucleophosmin;

NuMA: Nuclear Mitotic Apparatus associated Protein;

PLZF: Promyelocytic Leukemia Zinc Finger Protein;

PML: Promyelocytic Leukemia Protein;

RA Retinoic Acid;

RAR: Retinoic Acid Receptor;

RIPA: RadioImmuno Precipitation Assay;

RXR: Retinoid X Receptor;

N-CoR: Nuclear Corepressor

SMRT: Silencing Mediator of Retinoic and Thyroid receptor;

ABSTRACT

Expression of the fusion protein PML/RAR α is an event pathogenetically linked to the development of APL.

Biochemically, the fusion protein has been demonstrated to be part of nuclear HMW complexes (Nervi et al, 1992). We provide evidence that such complexes are composed of PML/RAR α oligomers and exist in vivo: the coiled coil region of PML is a critical determinant for their assembly and is responsible for abnormal recruitment of NCoR-HDAC complexes, transcriptional repression, and impaired differentiation of primary hematopoietic precursors. Fusion of RAR α to an heterologous oligomerization domain recapitulated the properties of PML/RAR α , indicating that oligomerization *per se* is sufficient to achieve transforming potential. We extend these observations to other AML fusion proteins (PLZF/RAR α , NPM/RAR α , and AML1/ETO), suggesting that oligomerization of a transcription factor may represent a frequent mechanism of oncogenic conversion. In another aspect of this work, analysis of the subnuclear distribution and nuclear matrix association of PML/RAR α and other AML fusion proteins suggests that they all show a subnuclear distribution different from that of their physiological counterparts, suggesting that the delocalization may play a role in the leukemogenetic process. We identified a region in PML, lost in the fusion

protein, that promotes the nuclear matrix targeting of PML and correct assembly of nuclear matrix associated PML domains, via a modulation of the oligomerization state of the protein. PML/RAR α acts negatively on the oligomerization state of the protein. PML/RAR α acts negatively on the oligomerization state of PML, and this effect requires oligomerization and proper targeting of RAR α to PML-associated nuclear domains. We suggest that the altered regulation of oligomerization state of both PML and RAR α is likely to represent an unifying biochemical mechanism impinging on the complex process of the pathogenesis of APL.

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INTRODUCTION

Leukemia: a subversion of the physiological maturation of hematopoietic cells.

The mammalian hematopoietic system consists of a highly ordered cellular system “hierarchically” derived from precursor stem cells endowed with three characteristics: i) self-renewal, ii) proliferation, and iii) differentiative abilities. Hematopoietic Stem Cells (HSCs) are responsible for maintaining hematopoiesis throughout life. A complex combination of intrinsic and exogenous stimuli controls the stem cell life cycle.

Alterations in this tight mechanism of regulation, as a consequence of pathological molecular events, are responsible for the generation of a leukemic clone.

The word “Leukemia” (Λευκος + Εμω) indicates, macroscopically, the accumulation in the bone marrow and subsequently in the peripheral blood of immature hematopoietic cells that fail to differentiate in mature functional white cells: the blood, according to ancient descriptions of the syndrome, becomes typically “more viscous and less coloured”.

The subversion of the qualitative/quantitative composition of the hematopoietic compartment and the consequent invasion of the peripheral blood by the leukemic blasts are responsible for most of the clinical symptoms observed: haematological alterations, increased blood viscosity, hiper/hipo-coagulative disorders, hepato-splenomegaly, invasion of solid tissues, paraneoplastic syndromes, immunodeficiency.

Hallmark of AMLs is the relative abundancy of chromosomal translocations. Among the best characterized AML chromosomal translocations are those involving the Core Binding Factor family (CBF), the MLL and the Retinoic Acid Receptor α gene..

i) Abnormal cell proliferation, ii) anti apoptotic activity and iii) block of differentiation are events associated with neoplastic transformation. A grossly altered proliferation status (with fast growth rate) does not seem to be the principal feature of AML blasts. Indeed frequently leukemic blasts do not have cell cycle profiles different from that of their physiological counterparts.

Although it is likely that an increased anti-apoptotic activity is a common feature of AML blasts, it is not entirely clear so far how relevant this property is for the determination and maintenance of the leukemic phenotype. Defects in myeloid cell differentiation or maturation represent in any case critical events in the pathogenesis of

AML. Also the acquisition of the ability to invade tissues is an important element of leukemogenetic process.

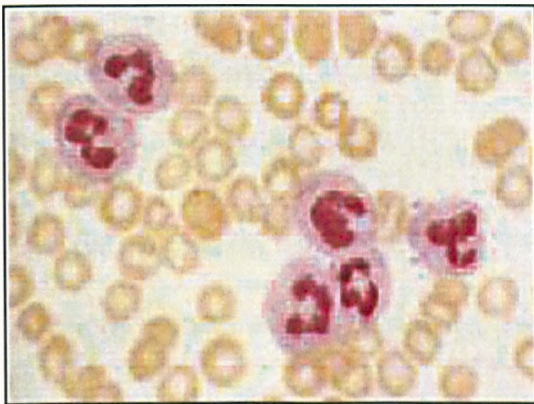
Blasts derived from AML patients show, *in vivo*, lack of expression of surface differentiation-related antigens and, *in vitro*, failure to differentiate upon treatment with appropriate differentiating stimuli. The block of differentiation could, from a pathogenetic point of view, alterate dramatically self-renewal abilities, with the consequent expansion of the neoplastic clone.

On the other hand, the block of differentiation could represent a necessary but not sufficient event in the pathogenesis of AML. At least two observations support this hypothesis: i) transduction of most AML fusion proteins is not sufficient to transform primary cells, ii) in murine models a relatively long latency time (months) is required to observe an overt leukemic phenotype. This suggest that additional, not yet identified, genetic lesions (such as the lack of telomerase activity) could be required for the AML development.

ACUTE PROMYELOCYTIC LEUKEMIA

Acute Promyelocytic Leukemia (APL) is among the best characterized forms of AML. It is observed in 5-10% of all AML cases. According to the cytomorphological French-American-British classification, it corresponds to the M3 subtype, in which leukemic blasts are arrested at a promyelocytic stage of differentiation. The great majority of cells in this leukemia are abnormal promyelocytes (fig.1), with very heavy granulations obscuring the basophilic cytoplasm. Some cells contain multiple Auer bodies resembling stacked firewood. In 1957, Hillstad first described the clinical entity of APL. He found 3 patients with the triad of a leukemic blood picture composed primarily of promyelocytes, hypofibrinogenemia and marked hemorrhage.

A



normal

B



leukemia

Fig.1:Peripheral blood from a normal and a APL patient.. Representative images of normal (A) and leukemic (B) blood samples. An increased number of big, immature, basophilic granulocytes is evident in the blood from leukemic patient.

APL is associated with at least four different chromosomal translocations, responsible for the generation of four fusion proteins: PML/RAR α t(15;17), observed in the vast majority of APL cases (95%); PLZF/RAR α t(11;17) (4% of APL cases) and the less common, sporadically observed, NPM/RAR α and NuMA/RAR α fusion proteins.

All fusion proteins share a “common” partner: the Retinoic Acid Receptor alpha (RAR α). This implicates a direct involvement of alterations of the retinoic acid signaling pathway in APL pathogenesis.

Aim of the experimental work here described is the biochemical and biological characterization of the t(15;17) derived PML/RAR α fusion protein. Before proceeding with a more detailed analysis of the PML/RAR α fusion protein, our current model of action of the natural RAR α and PML proteins will be shortly reviewed.

Retinoic acid receptors. RAR α (Retinoic Acid Receptor α) is a member of the nuclear hormone receptor superfamily. There are two families of RA receptors: RARs and rexinoid receptors (Botling et al., 1997; Chambon, 1996), RARs can bind both all-trans retinoic acid ATRA and 9-cis RA, whereas rexinoid receptors binds only 9-cis RA with high affinity. There are three members for each receptor family, encoded by different genes, namely RAR α , β and γ , and RXR α , β , and γ . Each member has a number of isoforms because of the alternative use of the promoters of the gene. Both RARs and RXRs share homology with other members of the nuclear hormone receptor superfamily, such as steroid hormone receptors, vitamin D receptors (VDRs), and thyroid hormone receptors (THRs). As other hormone-inducible transcription factors, these receptors bear six domains (A through F) (fig 2): A/B for ligand-independent transactivation, C with two zinc fingers for DNA binding, D which is a hinge region (involved also in binding to corepressors) and E responsible for dimerization with RXR, ligand binding, ligand-dependent transactivation and association with co-repressor (CoR) or co-activator (CoA) complexes. The function of the F domain is not clear.

A	Transcriptional activation (AF-1, ligand independent)
B	Transcriptional activation
C	DNA Binding Domain
D	Hinge region and corepressor binding
E	Heterodimerization Transcriptional activation (AF-2, ligand dependent) Ligand binding Coactivators binding Corepressors binding
F	Not characterized

Fig.2: RAR α structure.Schematic representation of RAR α domains with indication of putative functions.

Recently, new advances in understanding the molecular regulation of RA signaling has been made thanks to the discovery of corepressor (CoR) (Chen and Evans, 1995; Horlein et al., 1995; Zamir et al., 1996) and coactivator (CoA) complexes. Corepressor complexes are heterogeneous multiprotein complexes endowed with histone deacetylase activities. Histone deacetylases (HDACs) are enzymes able to deacetylate lysines in the histone aminoterminal tails. This modification is accompanied by a global increase of the positive charge of the molecule, which increases the affinity for the nucleosomal DNA and enforces nucleosome-nucleosome interactions. The latter events are responsible for the introduction of a “tighter”, more compact chromatin environment, leading to transcriptional repression (Tyler and Kadonaga, 1999; Wolffe, 1996). Transcriptional repression is probably due to a reduced accessibility of transcription activators to the DNA. Furthermore, deacetylation of histone tails can represent a “signal” for the recruitment of (multi)protein complexes, responsible for the maintenance (e.g. through methylation of DNA bases) of the chromatin in a repressed state. Retinoic acid receptors interact with at least two corepressor molecules: N-CoR and SMRT. The two proteins are part of HMW complexes of about 1.5-2 MDa (Guenther et al., 2000; Jones et al., 2001; Li et al., 2000; Underhill et al., 2000) and share a common

structural organization. Both proteins recruit HDAC activities either directly or indirectly through binding to the multiadaptor protein Sin3a/b. Binding of nuclear receptors to corepressors takes place through a motif, called CoR-box, which is conserved also in the thyroid receptor.(Cohen et al., 2001; Safer et al., 1998) The presence of this sequence has been correlated with the ability of the mentioned hormone receptors to mediate basal repression of target promoters, in absence of ligand.

Coactivator complexes are large multi-protein complex, containing, among the others, CREB binding protein (CBP)/adenoviral-E1A associated protein p300 (P300) (Arany et al., 1994). P300/CBP associated factor (P/CAF), P300/CBP interaction protein (P/CIP), also called activator of thyroid hormone and retinoid ACTR) and the nuclear receptors coactivator-1 (NcoA-1) or steroid hormone receptor coactivator-1 (SRC-1) or NcoA-2. Several CoA components possess histone acetyltransferase (HAT) activity (Bannister and Kouzarides, 1996; Goodman and Smolik, 2000; Ogryzko et al., 1996). Acetylation neutralizes the positively charged lysine residues of the histone tails decreasing their affinity for DNA, event leading to increased accessibility of transcriptional activators to the DNA target. Furthermore, since histone tails are involved in inter-nucleosomal contacts, acetylation may also alter the higher order chromatin structure.

It is also possible that acetylation of specific lysine residues inside the histone core influences directly the association of transcriptional activators to the promoter (Rhodes, 1997). In the absence of the ligand, RAR α /RXR is associated with corepressors, event which keeps the chromatin structure of the target genes in a repressed status through deacetylation of histones. Upon binding of RA, corepressors are dissociated from RAR α /RXR and coactivators will bind to the receptor heterodimer. Coactivators can not only induce ‘opening”of the chromatin structure by acetylating nucleosomes, but also recruit the basal transcription complex to the target genes thus leading to the activation of transcription.

The physiological relevance of the RA pathway in vertebrate ontogenesis has been demonstrated in “knock-out” animal models. Mouse double mutants for RAR α and RAR β , RAR β and RAR γ or RAR α and RAR γ all died either in fetal life or shortly after birth, with a variety of congenital abnormalities similar to those reported in vitamin A-deficient mice (Lohnes et al., 1994; Mendelsohn et al., 1994).

RA regulatory pathways have been shown to play an important, modulatory role in the hematopoietic development. Vitamin A-deficient mice or animals treated with an RAR α antagonist accumulate more immature granulocytes in their bone marrow.

Recently, the function of RAR α during granulocyte development has been more precisely addressed. RAR α is important for the differentiative response of granulocyte precursors to RA. In absence of ligand, the same protein instead controls negatively differentiation, since granulocyte precursors derived from RAR α $-/-$ mice differentiate earlier than the cells derived from RAR α $+/+$ mice (Kastner et al., 2001).

PML and the Nuclear Bodies.

From a structural point of view, PML belongs to an expanding family of proteins characterized by the presence of the RBCC (RING-Bboxes-Coiled-coil) motif (fig3): this region is composed of three zinc finger-like domains, namely the RING finger and two B-boxes, and an extended coiled-coil domain. The RING finger and the two B-boxes are involved in protein-protein interactions. Recently it has been shown that several RING finger domains display an E3 ubiquitin ligase activity (Fang et al., 2000; Levenson et al., 2000; Tyers and Jorgensen, 2000) but until now there are no experimental observations about the existence of such activity in PML. The coiled-coil domain represents a hetero and homo-multimerization interface. Other domains of PML comprise a nuclear-localization-signal (NLS), a Ser/Pro rich region and a variable, isoform-specific COOH term region.

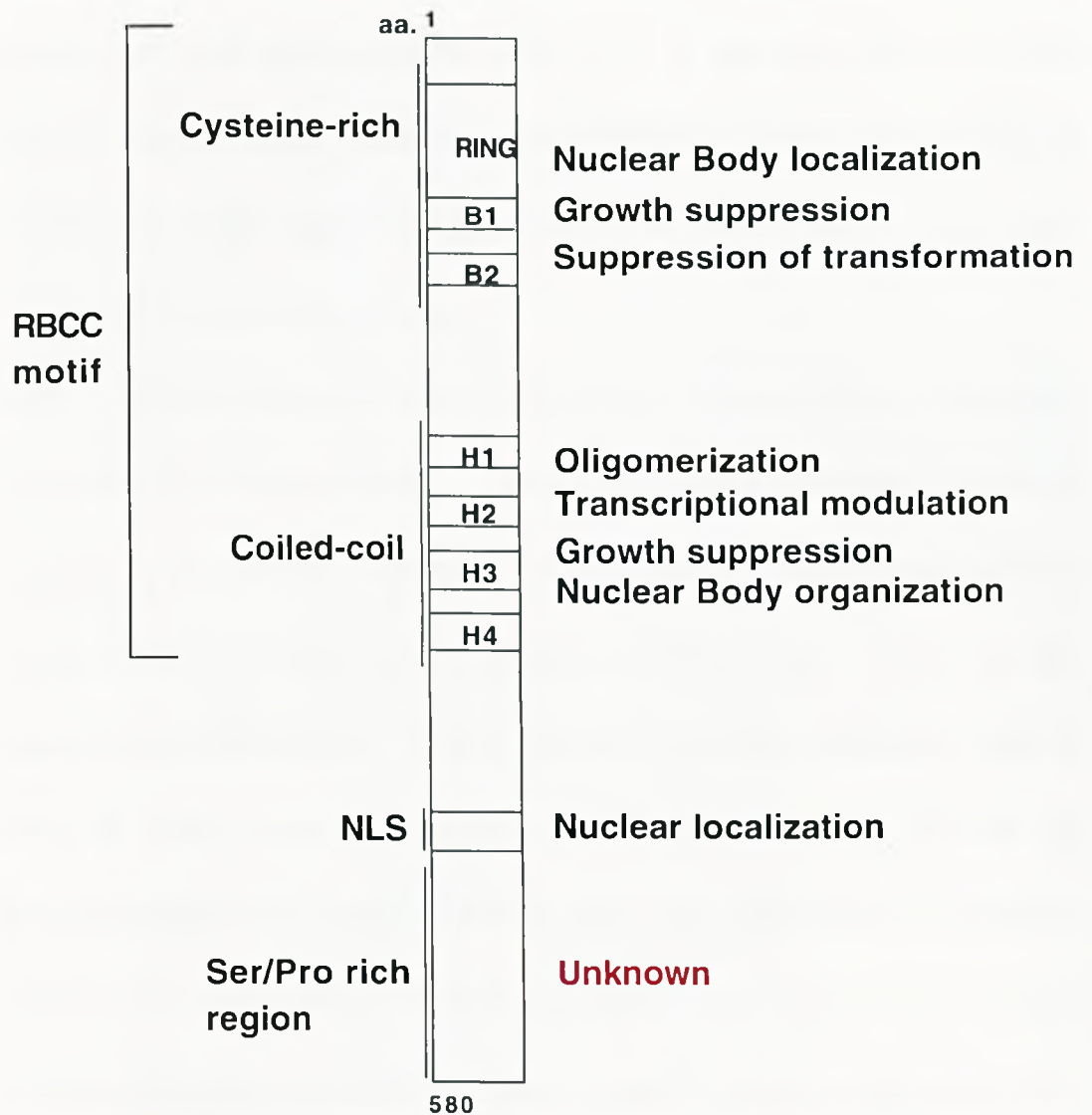


Fig.3: PML structure. Schematic representation of PML domains with indication of putative functions.

No specific functions up to now have been addressed to the last two domains. The Ser/Pro rich region is conserved in almost all known PML isoforms. This short region contains several serine residues. Some of them have been demonstrated to be sites of phosphorylation for the Protein kinase Casein Kinase II (unpublished observations from our group), but no biological role has been so far clearly addressed to these posttranslational modifications.

PML is found invariably, inside the nucleus, in specialized subnuclear domains: the Nuclear Bodies. These are complex structures, observed for the first time by screening of autoimmune sera from patients affected by primary biliary cirrhosis.(PBC), that reside in the interchromosomal space. Nuclear Bodies have been observed in almost all cell lines studied, varying in number between 10 and 20 bodies/nucleus (Dyck et al., 1994; Koken et al., 1994) (fig4A). Recently informations have been obtained regarding the ultrastructure of these nuclear organelles by electron spectroscopic imaging techniques (ESI) (Boisvert et al., 2000): a dense, nitrogen-rich, proteic central core is surrounded by branches of phosphorus-rich, compacted chromatin (fig4B).

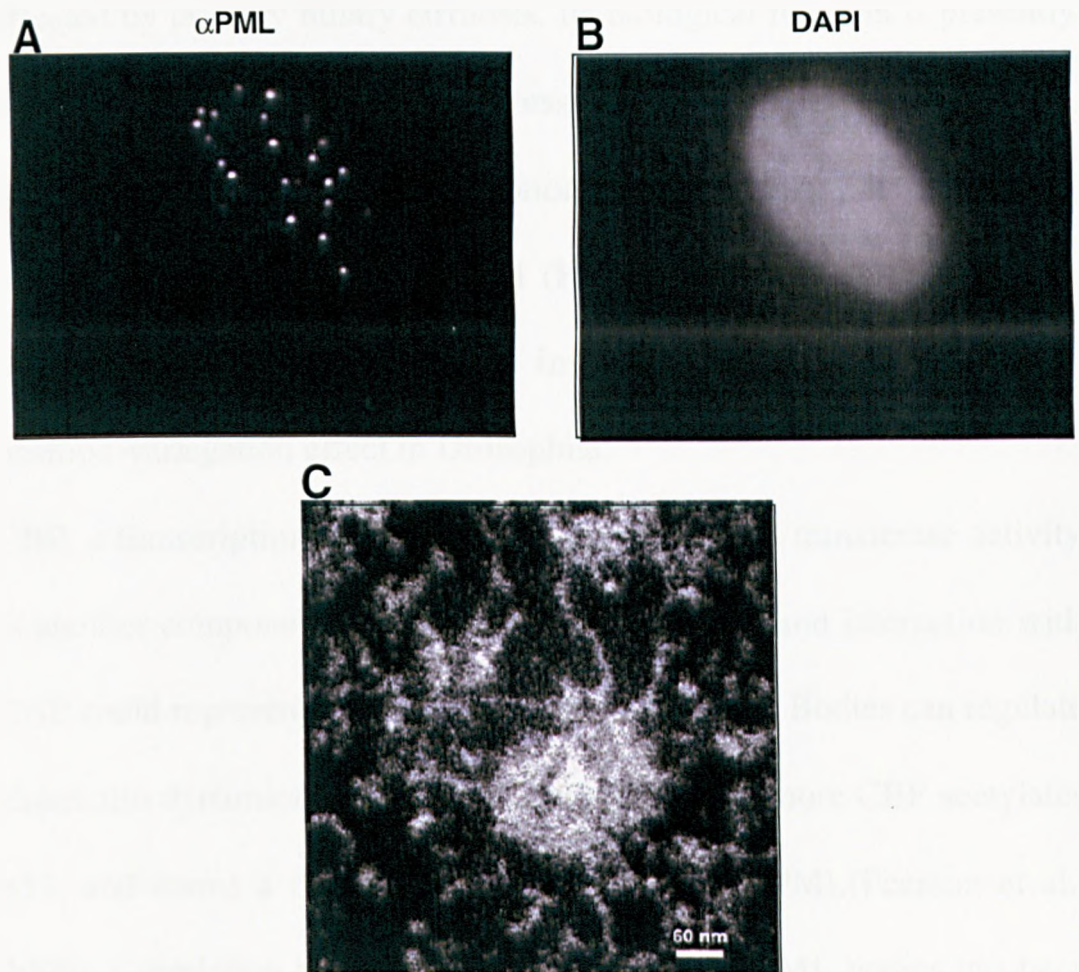


Fig.4: PML Nuclear Bodies. (A and B) Light microscopy: (A) PML containing bright nuclear foci are visualized with an anti-PML antibody (PGM3); (B) DAPI staining of the corresponding cell. (C) Electron microscopy: Thick section of a Nuclear Body, stained with uranyl acetate. (from: Boisvert et al, 2000)

In addition to PML, a high number of proteins involved in a variety of different cellular functions is represented in the Nuclear Bodies.

Sp100 is a 54 kDa protein recognized by auto-immune sera of patients affected by primary biliary cirrhosis. Its biological function is presently unknown. Like PML, SP100 expression is modulated by interferony. Sp100 might function in transcription, as suggested by its interaction with the heterochromatin protein-1 (HP1)(Seeler et al., 1998), another dynamic component of the NBs involved in the regulation of the position-variegation effect in *Drosophila*.

CBP, a transcriptional activator with histone-acetyl transferase activity is another component of these nuclear organelles, and interaction with CBP could represent a way through which the PML Bodies can regulate chromatin dynamics(Doucas et al., 1999). Furthermore CBP acetylates p53, and forms a stable complex with p53 and PML(Pearson et al., 2000). Correlation between p53 distribution and PML bodies has been observed in response to SV40 induced transformation. The viral Large T antigen redistributes p53 to nuclear domains adjacent to PML NBs (Jiang et al., 1996).

PML interacts with multiple corepressors such as c-SKI, N-CoR and mSin3a (Khan et al., 2001). Through these interactions, PML recruits histone deacetylase activities. Interestingly, PML can recruit at the same time, or at least in the same compartment, both histone deacetylase and

acetyltransferase activities. This finding suggests that Nuclear Bodies play a complex role in transcriptional regulation. Another possibility could be that acetylation/deacetylation of substrates different from histones could take place and play roles different from transcriptional modulation.

Other components of the PML-NBs are: Retinoblastoma protein (RB)(Alcalay et al., 1998) and p27, involved in cell-cycle regulation; DAXX, a regulator of FAS-induced apoptosis (Torii et al., 1999; Zhong et al., 2000); the P0, P1 and P2 ribosomal proteins, involved in the first steps of apoptosis and in the regulation of protein synthesis; the proteins int-6. (Desbois et al., 1996) and RFP (Cao et al., 1998) which have been suggested to modulate translational initiation processes.

In many cases, colocalization of the mentioned NBs components with PML is not complete, further underlying the dynamic nature of these nuclear subdomains.

Genetic evidences indicate that the structure of PML-NBs depends on the presence of PML (Ishov et al., 1999) since many other NB proteins are nuclear diffuse in cells derived from PML knock-out mice.

Up to now, it has been very difficult to address a specific function for the Nuclear Bodies compartment.

There are preliminary evidences which implicate NBs in the regulation of the anti-viral response, cellular senescence and transcription.

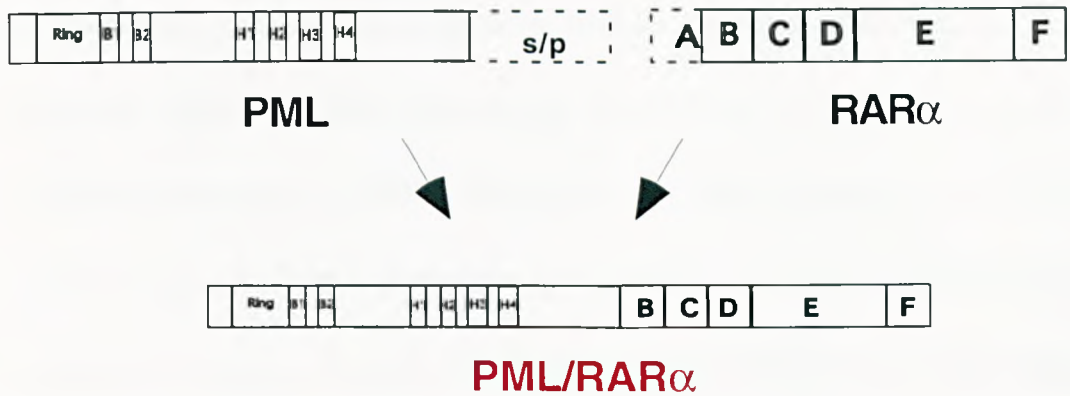
Many oncogenic viruses and viral proteins destroy the NBs and-or interact with PML during their replicative life cycle (Borden et al., 1998; Borden et al., 1998). It is possible to speculate that PML could act as an antiviral agent: the upregulation of PML induced by interferon, the IFN-induced accumulation of proteasome subunits inside the Nuclear Bodies (Fabunmi et al., 2001), the increased susceptibility of PML^{-/-} mice to viral infection and the recently demonstrated ability of the protein to upregulate the expression of MHC1 molecules (Zheng et al., 1998) strongly strengthen this hypothesis. In mouse primary fibroblasts, PML overexpression induces replicative senescence. Genetic evidences suggest that PML is part of the cellular machinery that triggers senescence, as shown by the fact that fibroblasts derived from PML knock out mice are resistant to Ras-induced senescence. Elucidation of the mechanisms through which PML regulates senescence brought to the discovery that PML regulates p53 acetylation and that integrity of the PML NBs is necessary for this effect. Upon Ras or PML overexpression, CBP and p53 are redistributed in the nucleus and recruited to PML-NBs. As a result, p53 transcriptional activity is regulated through the formation of a ternary complex including PML itself, p53 and CBP and leading to increased p53 acetylation. (Pearson et al., 2000).

It is still under debate whether and how PML and the Nuclear Bodies are directly involved in transcriptional modulation: despite the before mentioned interaction with nuclear corepressors and coactivators, there are contrasting reports regarding the presence of nascent RNA transcripts in these structures (Boisvert et al., 2000; LaMorte et al., 1998).

Properties of the fusion protein PML/RAR α .

Structurally, the translocation product retains most of the amino-terminal domains of the wt PML protein (The Pro-rich region, RING finger, B boxes and coiled-coil domains): PML/RAR α lacks the Ser/Pro rich region and the isoform-specific carboxyterminal region and retains most of the RAR α domains, except for the A region, localized at the extreme aminotermminus of the wt protein (fig 5A). Expression of PML/RAR α causes dramatic alterations in nuclear organization leading to the disruption of the PML-NBs. PML is recruited from its physiological sites to hundreds of new PML-RAR α containing sites called “microspeckles” (fig 5B).

A



B

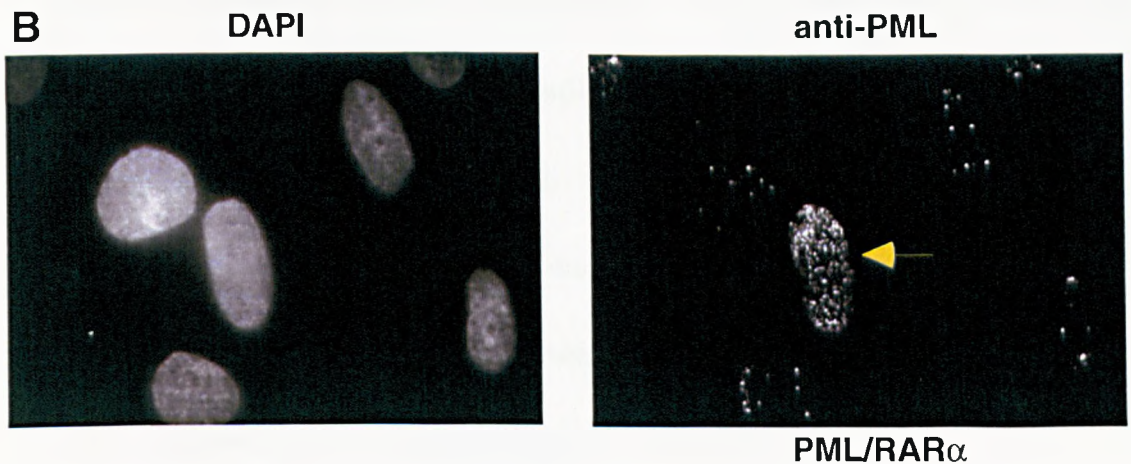


Fig.5: Nuclear Bodies are disrupted by PML/RAR in APL.

(A) Schematic representation of PML, RAR α and PML/RAR α domains. (B) HeLa cell microinjected with a PML/RAR α expression vector and subjected to indirect immunofluorescence with an α PML antibody (PGM3) shows the "typical" microspeckled pattern and fragmentation of Nuclear Bodies. Arrow indicates a representative microinjected cell.

Even if it does not appear to be a necessary requirement for the development of leukemia, fragmentation of Nuclear Bodies is always present in APL blasts, and RA-induced differentiation is accompanied by reappearance of these nuclear subdomains.

Expression of the fusion protein blocks terminal differentiation of isolated hematopoietic precursors and causes leukemia in animal models (Brown et al., 1997; Gelmetti et al., 1998; Grignani et al., 1996; Grisolano et al., 1997; Ruthardt et al., 1997). Unliganded PML/RAR α is able to recruit corepressor complexes (NcoR-HDACs) with higher affinity than its physiological counterparts and, unlike RAR α , pharmacological (10 μ M instead of 0.1-1 μ M) doses of RA are required to dissociate the complex and relieve the transcriptional repression (fig 6)(Grignani et al., 1998). Recruitment of HDACs, and insensitivity to physiological concentrations of RA, make the protein able to block differentiation of hematopoietic precursors. Treatment of APL patients with pharmacological concentrations of RA represents therefore the first example of differentiation therapy.

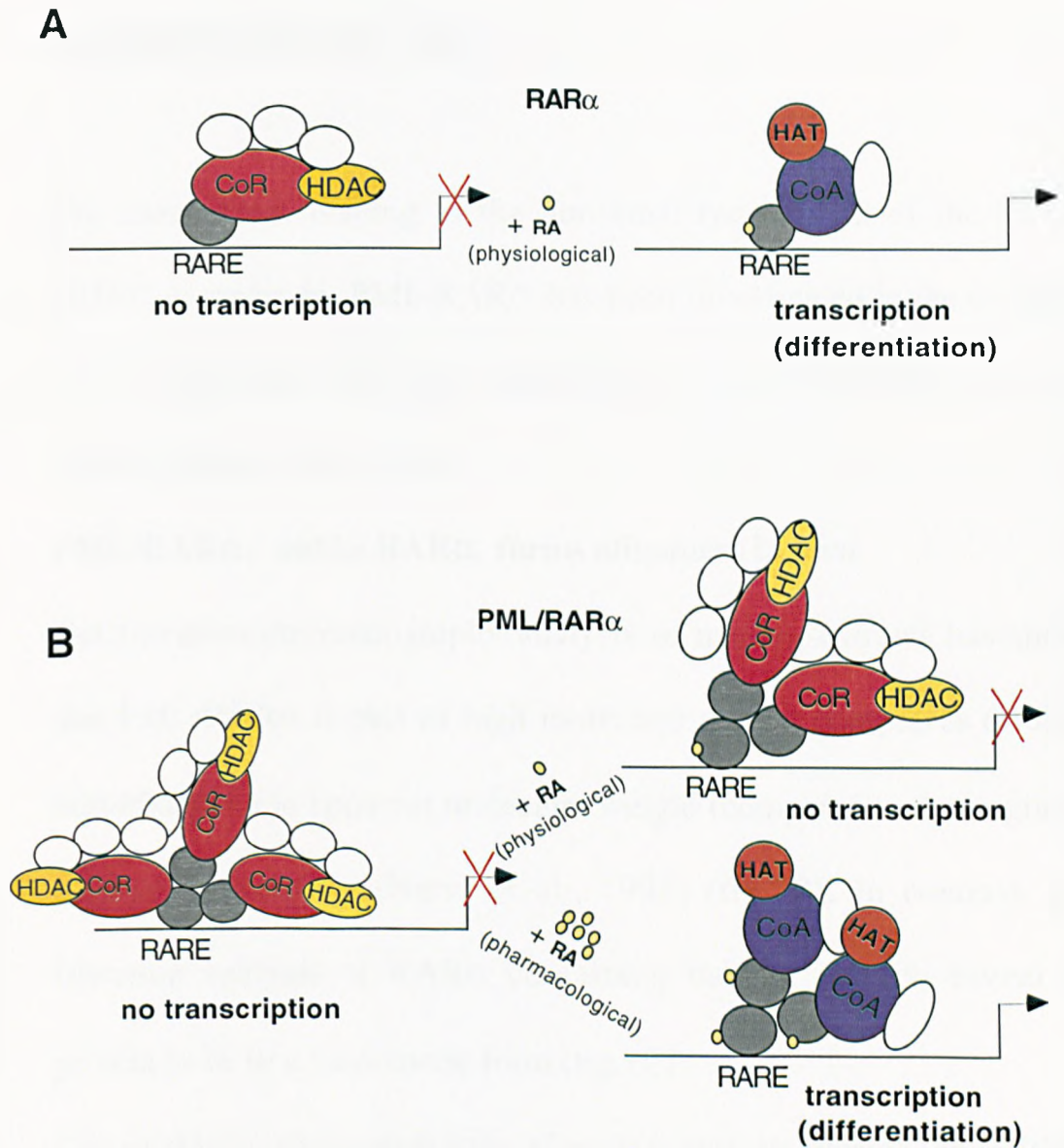


Fig.6: Schematic model for the pathogenesis of APL. (A) In absence of retinoic acid, RAR α binds corepressor complexes, represses transcription and limits differentiation. Physiological concentrations of ligand (RA,yellow) induce release of corepressors and recruitment of activators, with transcriptional activation and differentiation. (B) In the case of PML/RAR α , pharmacological concentrations of RA are required to release coactivator complexes, recruit coactivators and trigger differentiation of hematopoietic precursors.

RESULTS

ABERRANT OLIGOMERIZATION OF PML/RAR α IN THE PATHOGENESIS OF APL

The mechanism leading to the abnormal recruitment of the NCoR-HDAC complex by PML/RAR α has been investigated in the course of the experimental work here reported (Lin et al., 1998) (Minucci et al., 2000) (Minucci et al., 2001).

PML/RAR α , unlike RAR α , forms oligomers in vivo

Gel filtration chromatography analysis of nuclear extracts has shown that PML/RAR α is part of high molecular weight complexes of about 600-800 KD a in apparent molecular weight (compared to the migration of known proteins) (Nervi et al., 1992) (fig.7B). In contrast, gel-filtration analysis of RAR α containing nuclear extracts reveal the protein to be in a monomeric form (fig.7C).

Size exclusion chromatography of nuclear extracts obtained from PML3 expressing cells revealed that it is part of complexes (fig.7A) localized across the exclusion volume of the column, that are of larger MW than the PML/RAR α containing structures.

To identify which regions of PML and PML/RAR α could be responsible for the formation of the observed HMWCs, we have

evaluated the apparent molecular weight by SEC of PML/RAR α deletion constructs. From the structure/function analysis it emerges that the coiled coil region of PML represents a structural determinant for the formation of the high molecular weight complexes: a PML/RAR α mutant, which lacks the coiled-coil region, migrates in gel filtration at an apparent molecular weight compatible with dimeric and monomeric species (fig.7D). Furthermore, fusion of the isolated coiled-coil region of PML to RAR α is sufficient to drive the formation of high molecular weight complexes apparently identical to those of the PML/RAR α fusion protein (fig.7E).

Since the coiled-coil (CC) region of PML and PML/RAR α has been previously shown to mediate self-association (Grignani et al., 1996), we investigated whether the HMW complexes may represent oligomeric structures.

Bacterially expressed and purified recombinant PML/RAR α migrates, in gel-filtration experiments, at an apparent molecular weight of 800 kDa (data not shown). The fact that the preparation of the purified protein is devoid of eucaryotic cellular proteins led us to hypothesize that the purified PML/RAR α could have been able by itself to “organize” the 600-800 kDa structures.

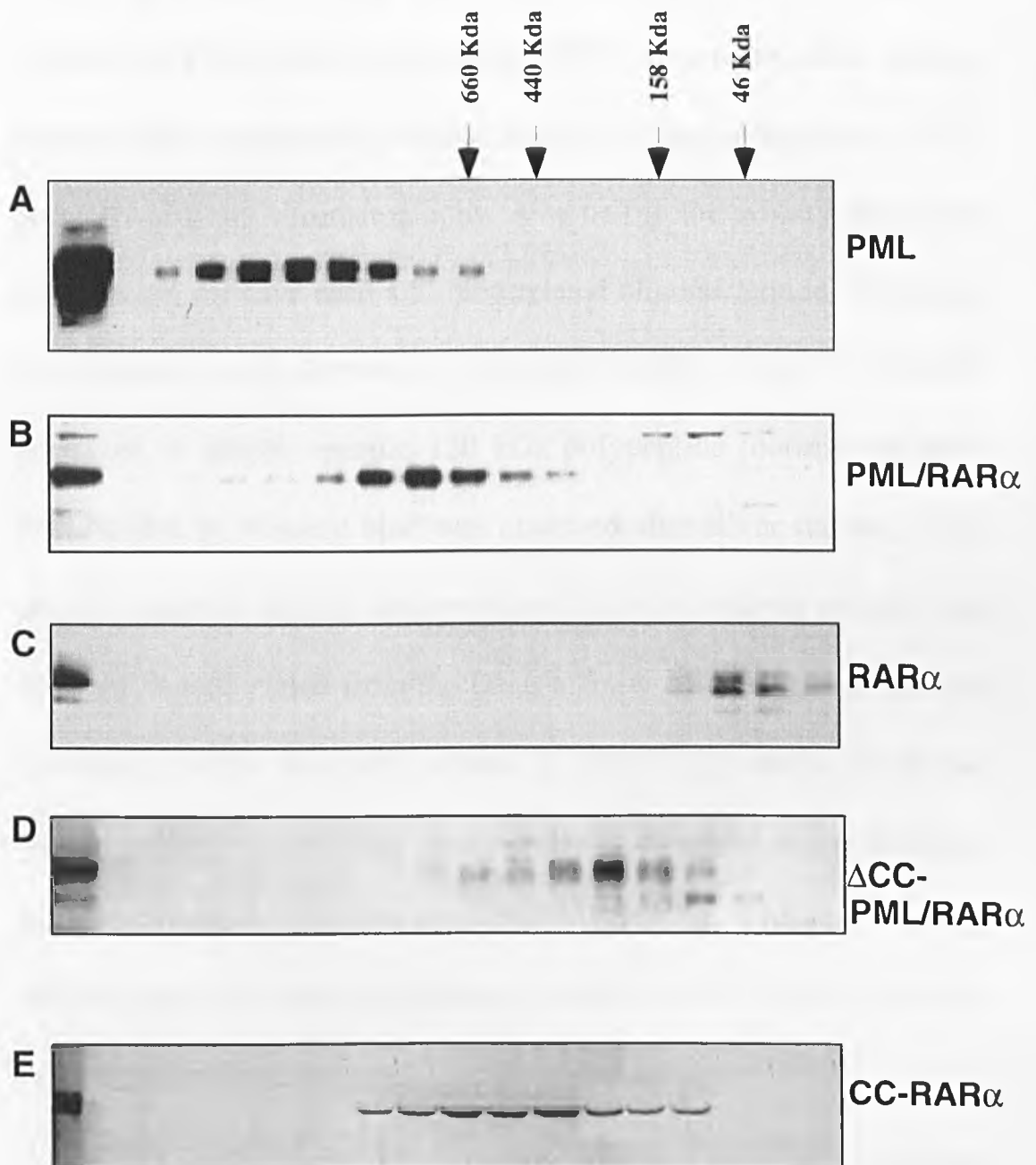


Fig.7: The coiled-coil region of PML mediates HMWC formation. Gel filtration analysis of nuclear extracts derived from cells stably expressing, respectively: **PML**, **RAR α** , **PML/RAR α** (A,B,C respectively); a PML/RAR α mutant (Δ CC-PML/RAR α) with a deletion in the coiled-coil region (D) and an *in vitro* translated labeled fusion protein containing the isolated coiled-coil region of PML fused to RAR α (CC-RAR α) (E). Elution profile of molecular weight markers is indicated by vertical arrows.

To test this idea, we have performed experiments to biochemically isolate the PML/RAR α containing HMW structures from nuclear extracts. Our purification scheme included Heparin-Sepharose, SEC, and DNA-affinity chromatography. As a bait in the affinity step of the purification, we have used a 5' biotinylated oligonucleotide, containing the Retinoic acid responsive element (RARE) from the RAR β 2 promoter. A single, specific 120 kDa polypeptide (corresponding to PML/RAR α by Western blot) was observed after silver staining of the purified material (fig.8A). Analytical gel filtration analysis revealed that the PML/RAR α eluted from the DNA affinity column with a high salt containing buffer was still present in HMW oligomeric complexes strictly (fig.8B) resembling the complexes observed in gel filtration analysis of the bacterially purified PML/RAR α . The last findings demonstrated that, in our experimental conditions, the HMW complexes of PML/RAR α are composed of oligomeric structures.

A possible criticism derives from the fact that the HMW oligomers could have been derived from *in vitro* "artefactual" rearrangements due to the conditions of extraction and that PML/RAR α may not exist in an oligomeric conformation *in vivo*.

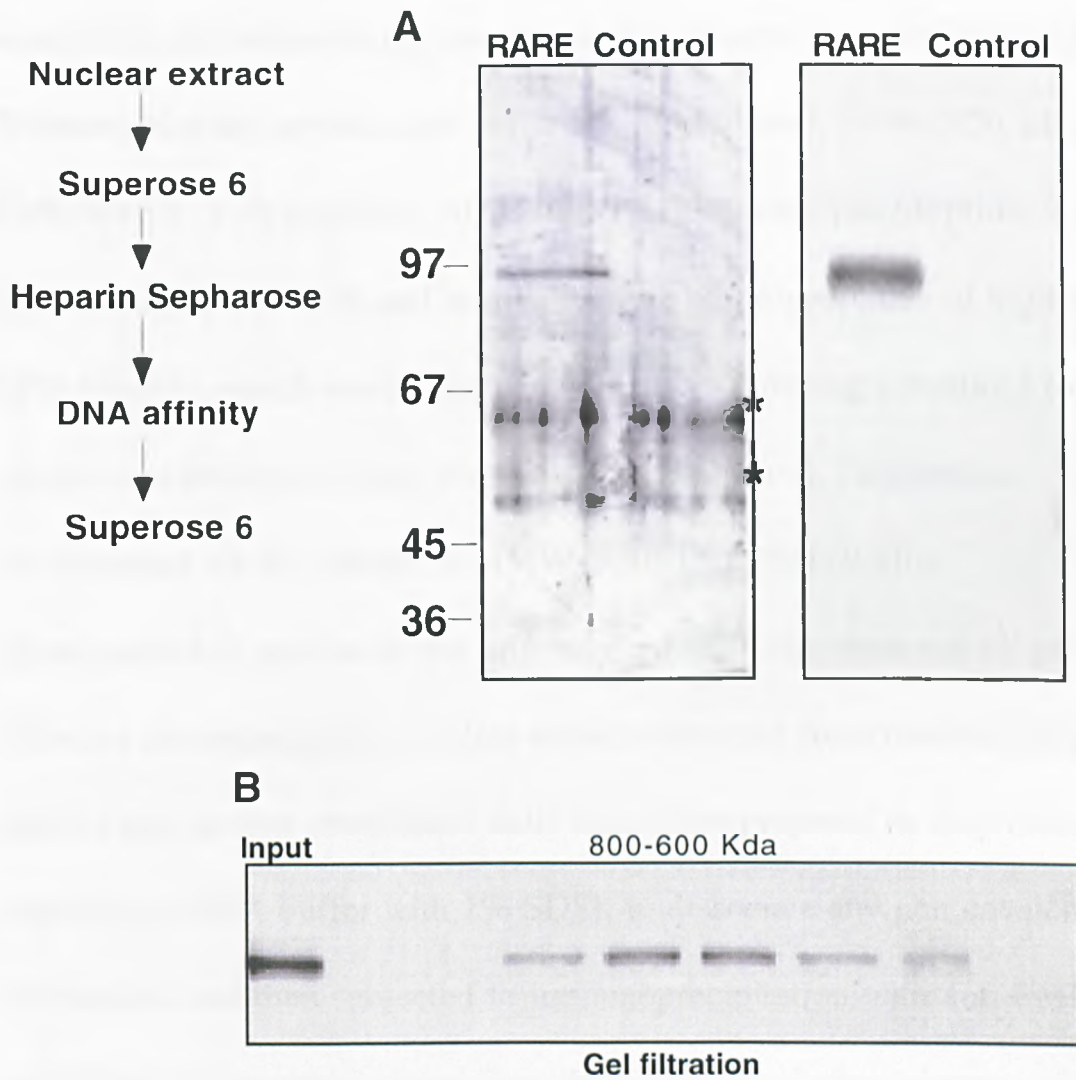


Fig. 8: Purification of PML/RAR α HMW complexes.

(A) Silver staining (left) and Western Blot analysis (right) of highly purified PML/RAR α after the final DNA-affinity chromatography. (B) Gel filtration analysis of the high-salt eluted PML/RAR α from the last DNA affinity step. Asterisks indicate aspecific bands.

In order to demonstrate the *in vivo* existence of HMW oligomers, we have performed *in vivo* crosslinking experiments using a bifunctional, thiol-sensitive, imidoesteric crosslinker (DTBP, Pierce). SDS-PAGE analysis under non-reducing conditions (to preserve cross-linking) and Western blotting reveals the existence, in addition to the 120 kDa PML/RAR α polypeptide, of a more abundant polypeptide of approximately 350 kDa and less well-resolved polypeptides of higher MW (fig 9A), which were absent in gels run in reducing conditions (to revert cross-linking) or from non-cross-linked material. To correlate the existence of the observed HMW (350kDa) PML/RAR α immunoreactive species to the oligomeric complexes observed by gel-filtration chromatography, nuclear extracts obtained from metabolically labeled and *in vivo* crosslinked cells have been prepared in very harsh conditions (RIPA buffer with 1% SDS), to dissociate any non covalent interaction, and then subjected to immunoprecipitation with anti-PML antibodies. The material eluted from the beads reveals the existence of a single, ³⁵S-labeled band of about 120 kDa, in reducing conditions, corresponding to the PML/RAR α protein (fig.9B). Crosslinked immunoprecipitated material detached from the beads by SDS treatment was then loaded on an analytical gel filtration column (fig 9C). The radioactive band corresponding to PML/RAR α migrates also in this case at an apparent molecular weight of 600-800 kDa.

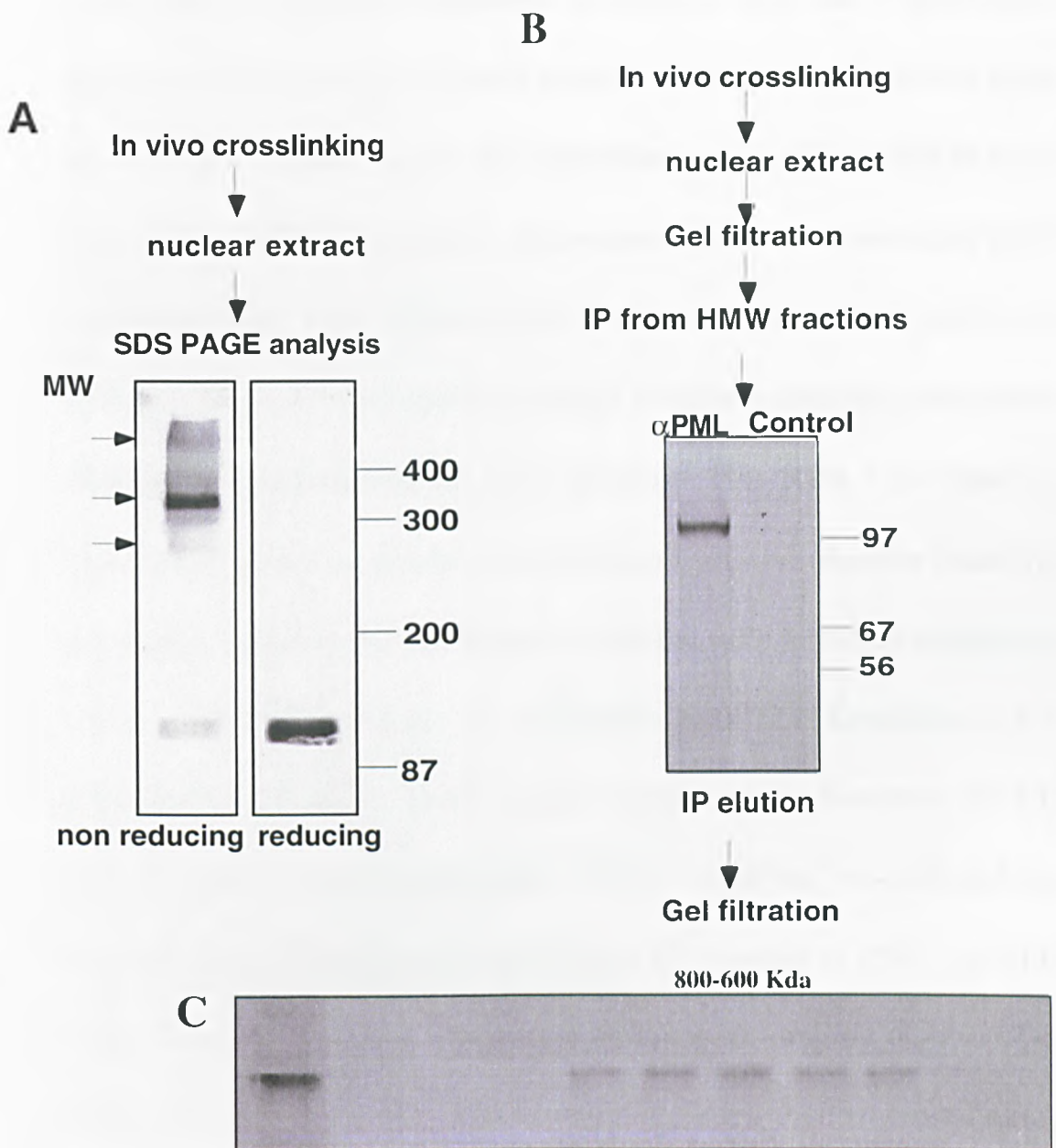


Fig.9: (A) PML/RAR α oligomers exist in vivo . (A)Immunoblot analysis of nuclear extracts from in vivo cross-linked PML/RAR α expressing cells after SDS PAGE in reducing or non-reducing conditions: arrows indicate the cross-linked species. (B)Autoradiography of immunoprecipitates obtained in not reducing conditions with an α PML antibody from HMW PML/RAR α containing fractions, derived from SEC analysis of nuclear extracts from in vivo cross-linked, metabolically labelled PML-RAR α expressing cells (C) Autoradiography of fractions obtained by SEC analysis of the crosslinked, immunoprecipitated material.

The results indicate that the PML/RAR α containing HMWC are constituted of discrete oligomeric structures, and that PML/RAR α oligomers exist *in vivo*. A further possible criticism would derive from the fact that estimation of the molecular mass of the PML/RAR α oligomer by SEC has intrinsic limitations, since the coiled-coil (CC) region of PML may influence the shape and the elution profile of PML/RAR α . By centrifugation through a sucrose gradient, unliganded PML/RAR α sedimented at approximately 700 KDa (not shown). Calculation of the molecular mass of the oligomeric complex based on the Stokes radius (from gel filtration) and the sedimentation coefficient (from sucrose gradients) is consistent with the formation of a PML/RAR α hexamer. There are no known cases, however, of CC domains forming hexamers (Lupas, 1996). Therefore, we analysed the self-associating properties of the isolated CC domain of PML. By SEC analysis, the 14 kDa CC eluted as a HMW peak ranging 60-150 kDa, confirming its capacity to oligomerize (Fig.10A). *In vitro* cross-linking with two different compounds (DTBP or BS³) resulted in the formation of higher MW bands, recognized by anti-CC antibodies and corresponding to di and trimeric species of the CC (Fig.10B). The CC domain can be therefore isolated from bacteria as a trimeric complex.

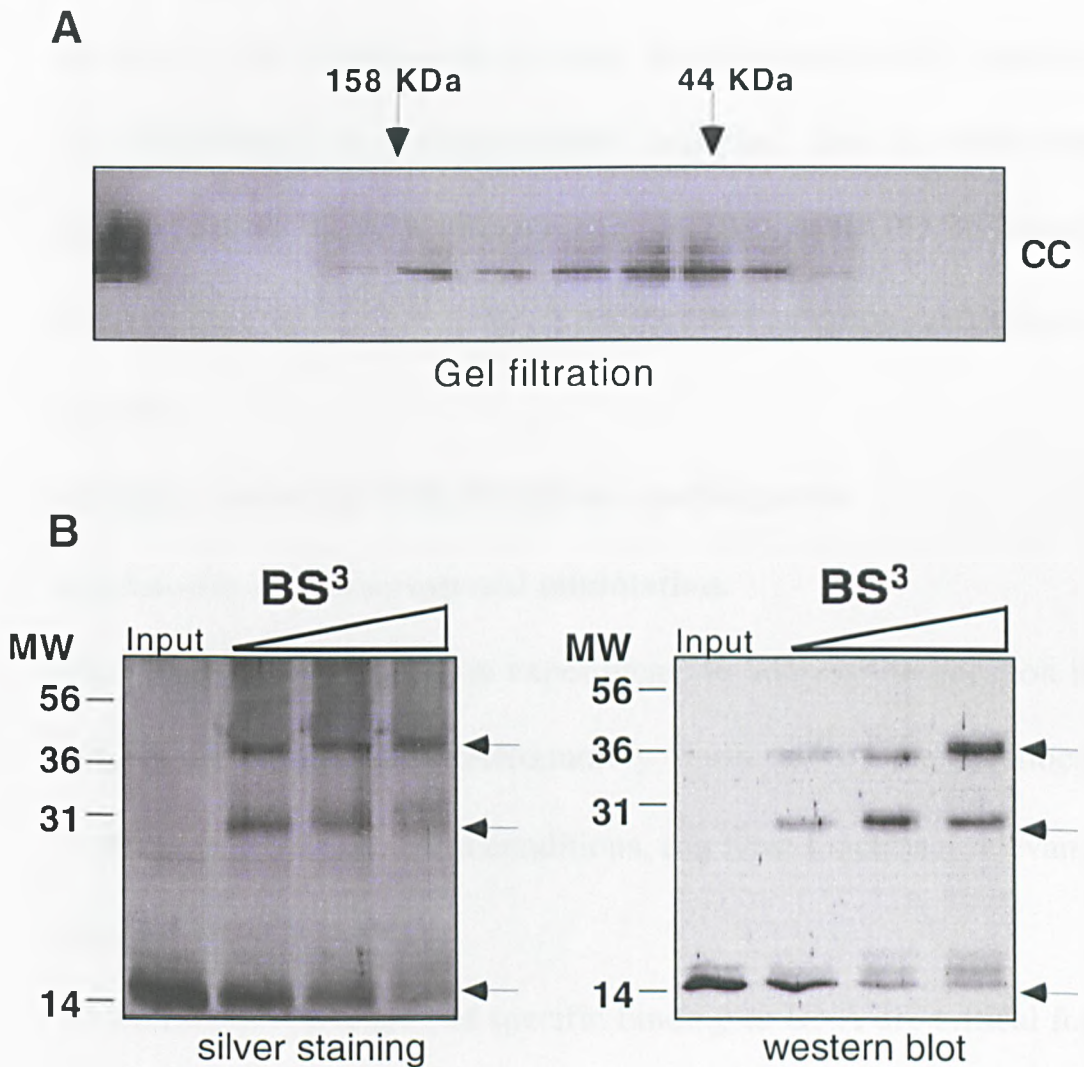


Fig.10: Biochemical properties of the isolated coiled-coil of PML. (A) SEC analysis of the bacterially expressed and purified coiled-coil (CC) region of PML. (B) SDS PAGE analysis and silver staining (left panel) or Western Blot of the in vitro crosslinked, purified coiled coil region of PML. Arrows indicate the monomeric (14 KDa) and the crosslinked, oligomeric species.

Based on these findings, two possible hypotheses can be formulated on the nature of the PML/RAR α complex:

- i) PML/RAR α is a trimeric complex with different migration properties with respect to the globular proteins used as MW markers;
- ii) PML/RAR α is a trimer-trimer complex, due to additional interactions mediated by other domains of PML or RAR α . In support of this latter model, Δ CC-PML/RAR α eluted as mono- and dimeric species.

Oligomerization of PML/RAR α as a pathogenetic mechanism of transcriptional modulation.

We then decided to perform experiments to address the question if the oligomerization of the RAR α moiety “induced” by the translocation and absent in physiological conditions, can have functional relevance in the pathogenesis of APL.

Recruitment of N-CoR and specific binding to DNA are critical for the oncogenicity of PML/RAR α (Minucci et al., 1999). N-CoR binds nuclear receptors through two interaction domains, ID1 and ID2 (Hu and Lazar, 1999). We have investigated if the oligomeric conformation can influence the binding of the PML/RAR α fusion protein to N-CoR. In GST pull-down assays, PML/RAR α shows the highest apparent affinity for GST N-CoR. The PML/RAR α mutant which lacks the coiled-coil region and is not able to oligomerize, behaves in a way very

similar to RAR α . On the other hand, fusion of the isolated coiled-coil region of PML to RAR α , event promoting the forced acquisition of an oligomerization state by RAR α , restores a binding to NcoR similar to that of PML/RAR α (fig.11).

To determine whether oligomerization is *per se* the critical function mediated by the CC domain of PML, we fused RAR α C-terminally to the tetramerization domain present in p53 (Friedman et al., 1993; Jeffrey et al., 1995). SEC analysis showed that p53-RAR α (unliganded or RA-bound) formed HMW complexes (Fig.12A), allowing us to analyze RAR α oligomers that do not contain PML sequences. Similarly to PML/RAR α and CC-RAR α , p53-RAR α bound even the lowest amounts of GST-NCoR tested (fig12B).

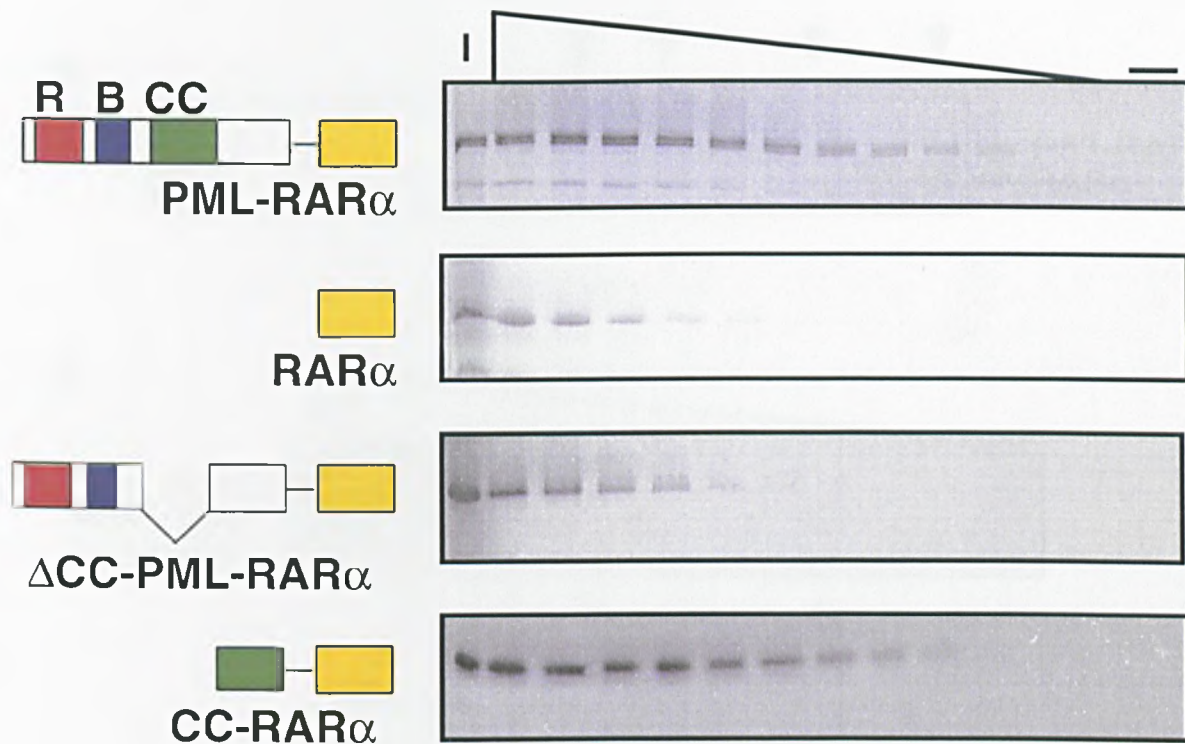


Fig.11:Oligomerization confers increased affinity for NCoR. Autoradiography:the indicated in vitro translated, ³⁵S labeled proteins were incubate with decreasing amounts of GST-NCoR (from 10 μ g to 150 ng), or GST as a control (10 μ g; lane "-") I = Input material.

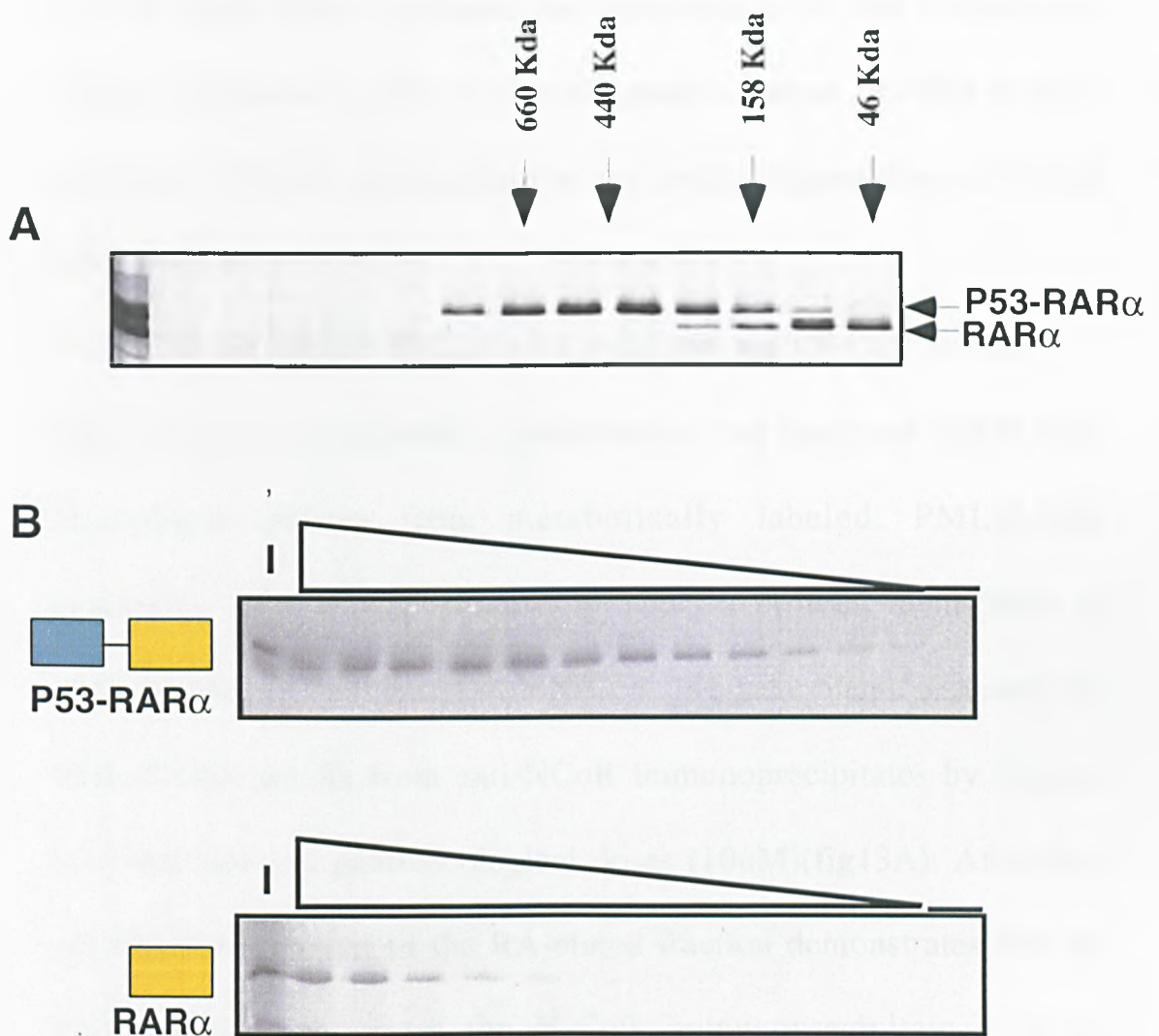


Fig.12: Fusion of a heterologous oligomerization domain to RAR α . mymics the properties of PML/RAR α .

(A) Gel filtration chromatography of in vitro translated P53 RAR α . Elution profile of the has been determined by comparison with standard molecular weight markers. (B) GST-N-CoR pull down. In vitro labeled P53 RAR α or RAR α were incubated with decreasing amounts of GST-NCoR (from 10 μ g to 150 ng), or GST as a control (10 μ g; lane "-").

The increased stability *in vitro* of the interaction of PML/RAR α with N-CoR could likely represent the consequence of two intrinsically related mechanisms, due to the oligomeric nature of PML/RAR α : avidity for N-CoR, and increase of the local concentration of N-CoR binding sites.

To investigate whether PML/RAR α associates with N-CoR *in vivo* when it is in an oligomeric conformation, we analyzed anti-N-CoR immunoprecipitates from metabolically labeled, PML/RAR α expressing cells. An approximately 120kDa protein, identifiable as PML/RAR α , coprecipitated with N-CoR. We have then dissociated the PML/RAR α protein from anti-N-CoR immunoprecipitates by retinoic acid treatment, at pharmacological doses (10 μ M)(fig13A). Analytical gel filtration analysis of the RA-eluted fraction demonstrates that the PML/RAR α eluted from the N-CoR immunoprecipitate is in an oligomeric conformation. The absence, even after very long exposure of the gel, of any other PML/RAR α specific signal in fractions different from the HMW indirectly suggests that, when complexed to N-CoR, PML/RAR α is an oligomer (fig.13B).

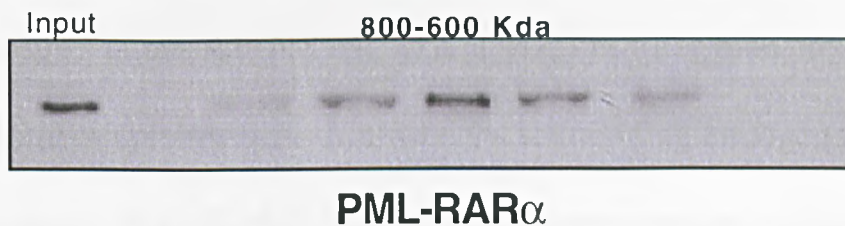
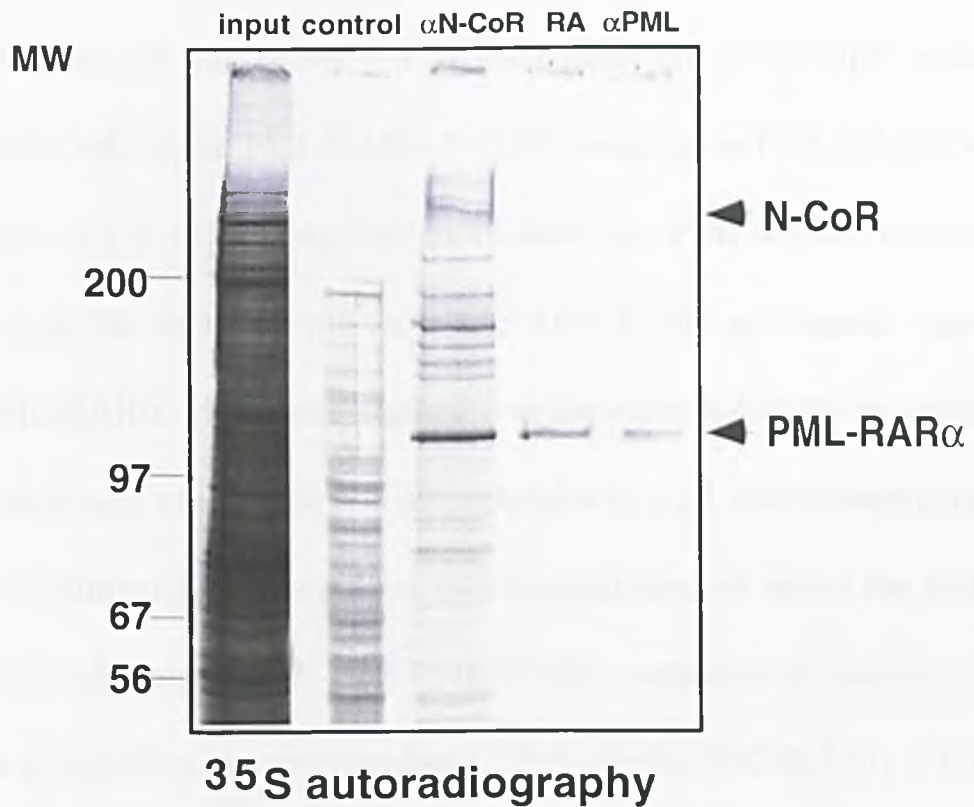


Fig.13: PML-RAR α binds N-CoR in an oligomeric conformation.

Metabolically labelled cells were immunoprecipitated with anti-NCoR (aN) or control (PI) antibodies. The anti-NCoR immunoprecipitates were incubated in the presence of RA (10 μ M) for 2 hours at 4 $^{\circ}$ C (RA lane). Control anti-PML immunoprecipitates (aPML) are shown in the last lane. (B) The RA-eluted material was then analyzed by SEC, followed by SDS PAGE and autoradiography

In order to better clarify the nature of the higher avidity/affinity of PML/RAR α for N-CoR (respect to RAR α), it has been interesting, at this point, to address the question whether in the PML/RAR α -N-CoR complex, the oligomeric PML/RAR α binds one or multiple molecules of N-CoR. In the PML/RAR α -N-CoR complex the PML/RAR α /N-CoR ratio is >1 , suggesting that more than one PML/RAR α molecule is bound for each N-CoR molecule. Due to the multimeric nature of PML/RAR α , we cannot exclude, on the other hand, the possibility of recruitment of multiple N-CoR molecules in a 1:1 stoichiometric ratio.

To distinguish between these two possibilities, we tested the ability of immunoprecipitated N-CoR-PML/RAR α complexes to mediate further recruitment of in vitro translated, 35 S-labeled N-CoR (ID1 1 ID2, the two previously characterized PML/RAR α and RAR α N-CoR interacting domains). N-CoR (ID1 1 ID2) did not bind to anti-N-CoR immunoprecipitates from control cells (containing endogenous RAR α). In contrast, N-CoR-PML/RAR α immunoprecipitates mediated further N-CoR (ID1 1 ID2) recruitment (fig14), suggesting a potential for PML/RAR α oligomers to recruit multiple N-CoR molecules.

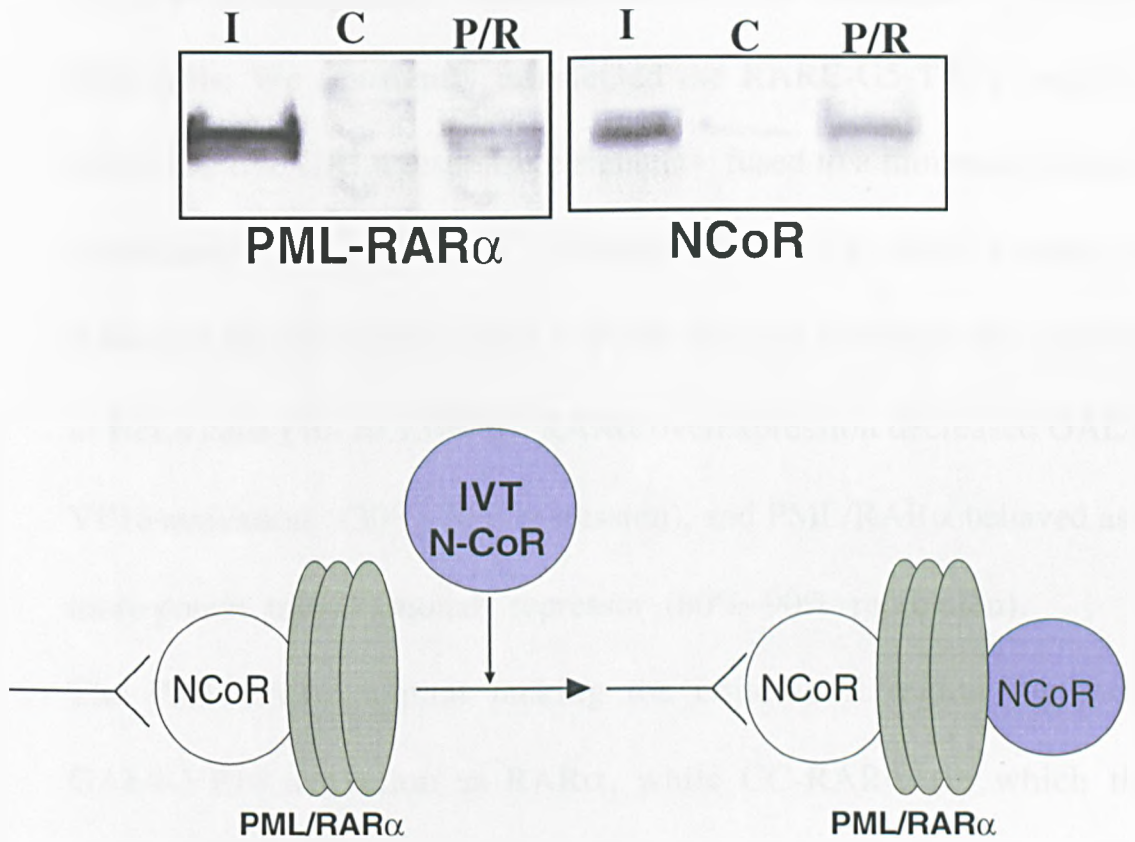


Fig.14: PML/RAR α recruits multiple NCoR molecules. Anti-NCoR immunoprecipitates from PML/RAR α expressing cells or control cells were incubated with an in vitro translated, 35 S-NCoR (C-terminal region), and then analyzed by SDS-PAGE followed by Western blot with anti-RAR α antibodies (left panel) or autoradiography (right panel).

As a functional “read-out” of the increased ability of the PML/RAR α oligomers to recruit multiple N-CoR molecules, we have evaluated the ability of PML/RAR α versus RAR α , and the coiled-coil deleted mutant to act as transcriptional repressors in transient transfection assays in HeLa cells. We transiently transfected the RARE-G5-TATA reporter which has five GAL4 responsive elements fused to a minimal promoter containing a RA responsive element (RARE) to allow binding of RAR α or fusion proteins. GAL4-VP16 strongly activated this reporter in HeLa cells (10- to 13-fold). RAR α overexpression decreased GAL4-VP16 activation (30%–40% repression), and PML/RAR α behaved as a more potent transcriptional repressor (80%–90% repression).

The PML/RAR α mutant lacking the coiled-coil region repressed GAL4-VP16 activation as RAR α , while CC-RAR α (in which the coiled-coil region of PML has been fused to RAR α) was comparable to PML/RAR α (fig.15).

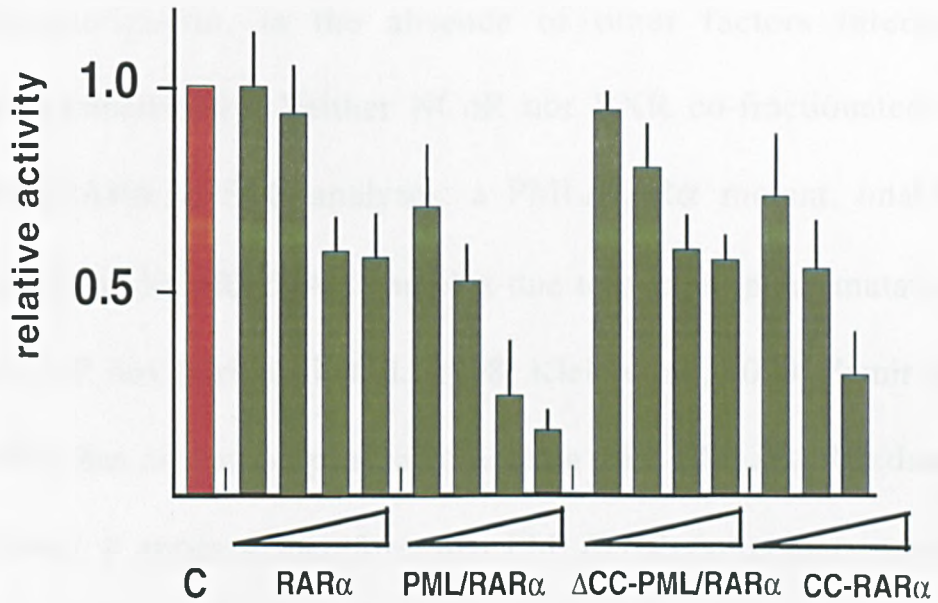


Fig.15: Transcriptional repression. Luciferase activity of extracts from HeLa cells cotransfected with the RARE-G5-TATA reporter in the absence (C=control) or presence of increasing amounts (50, 100, 250, 1000 ng) of the indicated expression vectors.

Thus, PML/RAR α oligomers bind DNA (alone or in association with RXR) and are able to recruit NCoR to DNA. The association of PML/RAR α oligomers with NCoR (and RXR) does not contradict our finding that the HMW complexes originate from PML/RAR α oligomerization, in the absence of other factors interacting stoichiometrically. Neither NCoR nor RXR co-fractionated with PML/RAR α in SEC analyses; a PML/RAR α mutant, unable to recruit the NCoR/HDAC complex due to a triple point mutation in the CoR box (Grignani et al., 1998; Klein et al., 2000; Zamir et al., 1996), has an elution profile coinciding with PML/RAR α (data not shown). It appears, therefore, that PML/RAR α oligomers represent the "core" complex responsible for the interactions (at lower affinity and/or stoichiometry) with other co-factors.

Effects of RAR α oligomerization on differentiation of hematopoietic precursors. In order to better define the consequences of the altered oligomerization of RAR α , we measured the ability of PML/RAR α , CC-RAR α and p53-RAR α to block differentiation of primary murine hematopoietic precursors. Briefly, murine hematopoietic progenitors were purified from the bone marrow of BALB-C mice on the basis of the absence of lineage differentiation markers (lin⁻). Purified cells were transduced with

retroviral constructs encoding for the proteins of interest and GFP as a marker. GFP-positive cells were sorted and seeded in methylcellulose plates, to allow terminal myeloid differentiation. Pooled colonies were analysed for the expression of differentiation markers (Mac-1 and GR-1)(Fleming et al., 1993; Hayashi et al., 1996) (fig 16). Cells that undergo terminal myeloid differentiation express early differentiation markers (MAC-1 and GR-1). PML/RAR α , CC-RAR α and p53-RAR α caused a strong differentiation block. Cells expressing Δ CC-PML/RAR α showed much higher levels of protein compared to PML/RAR α . High levels of RAR α lead to a differentiation block, likely by sequestering of RXR (Grignani and Pelicci, 1996). We sorted the Δ CC-PML/RAR α GFP+ cells in two populations, according to their mean fluorescence levels: GFP^{low} cells expressed lower levels of Δ CC-PML/RAR α than the GFP^{high} cells. Low levels of Δ CC-PML/RAR α had no effects, whereas at higher levels it induced a consistent differentiation block (>30%). These results show that the capacity of PML/RAR α to block myeloid differentiation depend on the CC domain of PML, and that addition of an oligomerization domain to RAR α is sufficient to obtain a fusion protein with full transforming potential.

Finally, we compared the capacity of PML/RAR α , CC-RAR α , and p53-RAR α to respond to RA. The differentiation block by

PML/RAR α and CC-RAR α was relieved exclusively at high concentrations of RA (1 μ M) In contrast, p53-RAR α expressing cells were insensitive to RA treatment (fig. 16, *ibidem*). Accordingly, RA did not relieve transcriptional repression by p53-RAR α . It appears, therefore, that RAR α -fusion protein oligomers exert differential responses to RA on the basis of the identity of the oligomerization domain fused to RAR α .

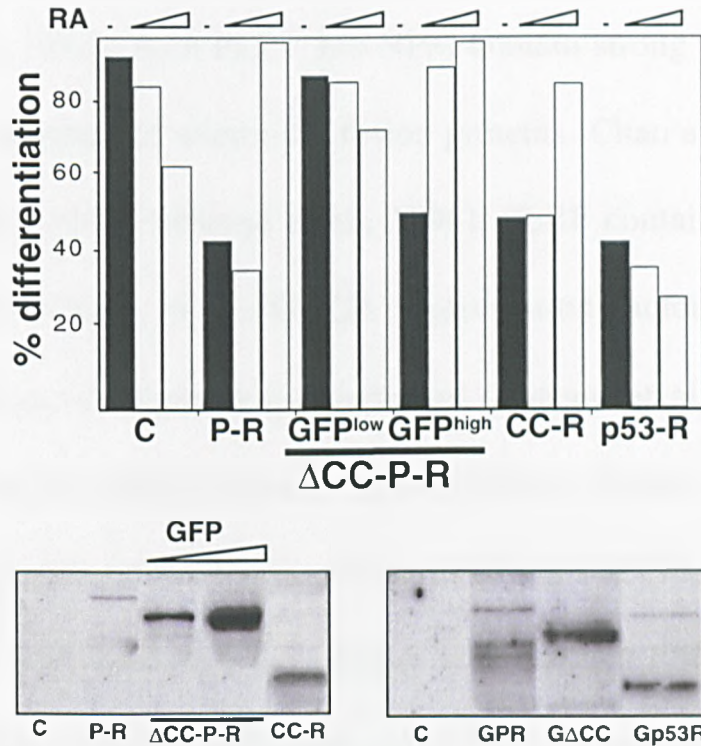


Fig.16: Differentiation of murine hematopoietic progenitors.

Lin⁻ cells were transduced with the indicated retroviral vectors (C control; P-R: PML/RAR α ; Δ CC-P-R: Δ CC-PML/RAR α ; CC-R: CC-RAR α ; GPR: GFP-PML/RAR α ; G Δ CC: GFP- Δ CC-PML/RAR α ; Gp53R: GFP-p53-RAR α), and GFP⁺ cells were sorted by FACS. Δ CC-PML/RAR α , GFP⁺ cells were sorted in GFP high or GFP low expressors. After sorting, cells were either plated in differentiation medium in the absence or in the presence of RA (3nM or 1 μ M) (upper panel), or analysed by Western blot (lower panels). Cells that undergo terminal myeloid differentiation express high levels of MAC-1 (upper panel) and GR-1 (not shown) early differentiation markers.

PML, PLZF and NPM form HMW complexes. Other RAR α fusion proteins (such as PLZF/RAR α and NPM/RAR α) are infrequently associated with APL (Zelent, 1994) (Pandolfi, 1996) (Zelent, 1998). Both PLZF and NPM contain strong self-association domains, retained within the fusion proteins (Chan and Chan, 1995; Li et al., 1997) (Ahmad et al., 1998). PLZF contains a BTB-POZ domain, which, in the GAGA transcription factor, mediates the formation of oligomeric complexes (Katsani et al., 1999); NPM contains an amino-terminal oligomerization domain, that mediates the formation of hexameric structures (Chan and Chan, 1995).

As previously noted, SEC analysis revealed that PML is distributed in HMW complexes migrating across the void volume of the column. Furthermore, purification of a recombinant MBP fusion protein and SEC analysis showed that also PML, like PML/RAR α , is able to form very HMW oligomeric structures, similar in MW to the structures observed in nuclear extracts from eucaryotic cells (data not shown).

PLZF and NPM were also found in HMW complexes: PLZF peaked at an apparent MW >440 kDa, whereas NPM was found as a monomer (30% of total NPM), or in HMW complexes (from 200 to >400 kDa) consistent with a hexameric state, as previously described (fig.17A).

NPM/RAR α and PLZF/RAR α also formed HMW complexes. NPM-RAR α peaked with a lower apparent MW (400 kDa), consistent with the lower MW of NPM/RAR α (60kDa;)(fig.17B). Therefore, all RAR α fusion proteins form HMW complexes through their corresponding PML, PLZF or NPM moieties. A detailed analysis, as in the case of PML/RAR α , has not been performed to investigate the nature of the PLZF/RAR α and NPM/RAR α HMW complexes. By analogy with PML/RAR α , however, it is reasonable to assume that the HMW structures represent oligomers. Thus, oligomerization of RAR α seems to represent a leitmotiv in other APL subtypes.

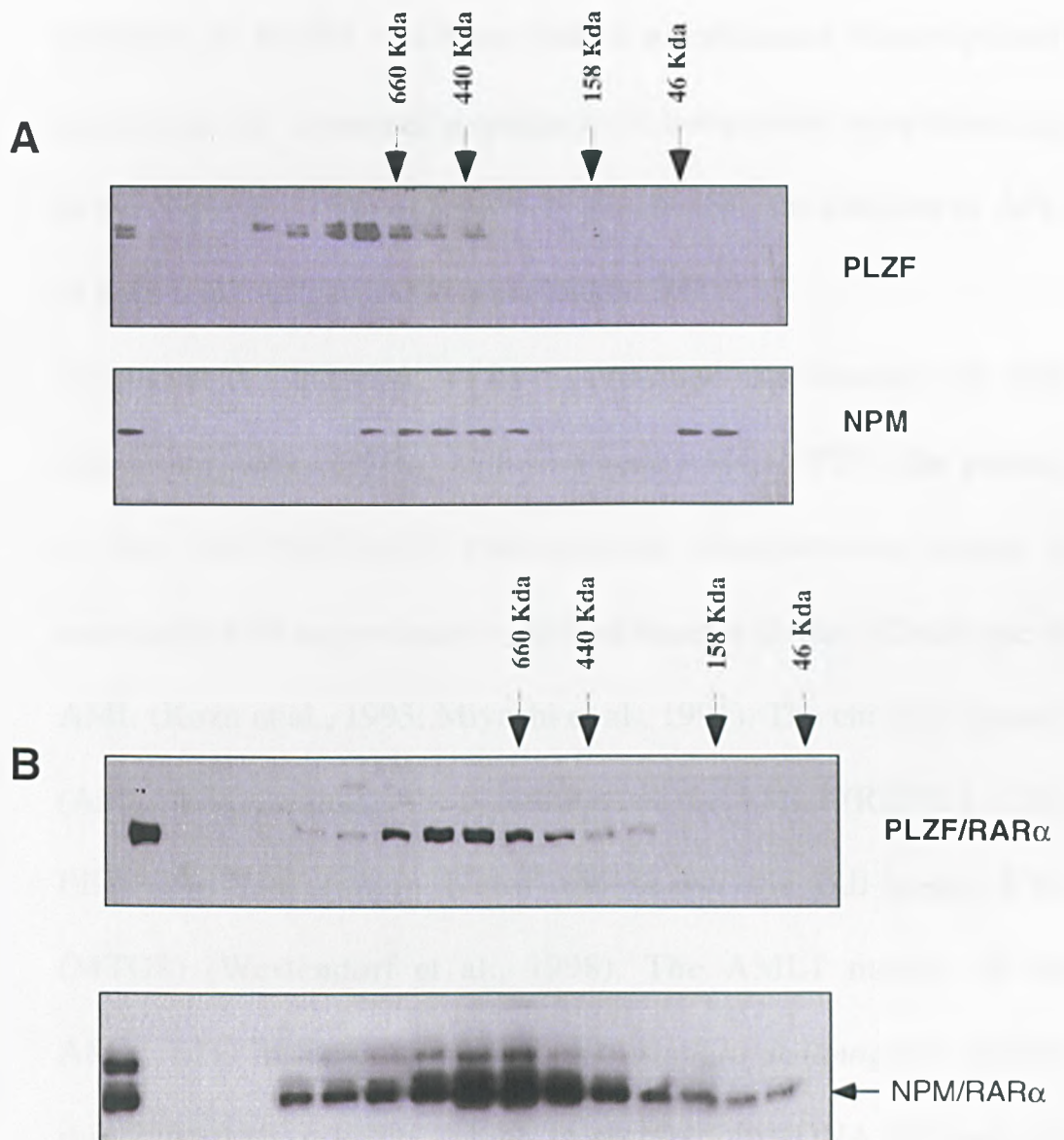


Fig.17: PLZF, NPM , PLZF/RAR α and NPM/RAR α form HMWC. Gel filtration analysis (Superose 6) of nuclear extracts derived from U937 cells stably expressing: PLZF, NPM, and PLZF/RAR α , NPM/RAR α (A and B, respectively). Elution profile of molecular weight markers is indicated by vertical arrows.

Oligomerization of the fusion protein AML1/ETO.

In the course of the experimental work here described, leukemogenic potential of RAR α has been linked to enhanced transcriptional repression, via abnormal recruitment of corepressor complexes, due to the oligomerization of RAR α . Is this mechanism a feature of APL, or does it take place also in other leukemias?

To answer this question, we have performed experiments with cells expressing AML1/ETO. The fusion gene AML1/ETO is the product of the t(8;21)(q22;q22) chromosomal translocation, which is associated with approximately 40% of cases with the M2 subtype of AML (Kozu et al., 1993; Miyoshi et al., 1991). The chimeric protein (AML1/ETO) contains the N terminus of the AML1 (RUNX1, CBF, PEBP2B) transcription factor and nearly the full-length ETO (MTG8) (Westendorf et al., 1998). The AML1 moiety of the AML1/ETO fusion protein has the *Drosophila melanogaster* protein runt homology domain, which is required for DNA binding and interaction with its heterodimerization partner, CBF, but lacks the transcriptional activation domain of AML1 (Meyers et al., 1993). Analysis of *AML1* knockout mice demonstrates that AML1 is a crucial factor for definitive hematopoiesis (Odaka et al., 2000). The ETO moiety of AML1/ETO is able to recruit the nuclear receptor corepressor (N-CoR)-mammalian Sin3 (mSin3)-histone deacetylase-

1 (HDAC1) complex (Lutterbach et al., 1998)(Gelmetti et al.,1998) (Wang et al., 1998). ETO recruitment of the N-CoR-mSin3-HDAC1 complex leads to lower levels of histone acetylation and less-accessible chromatin. ETO forms HMW complexes (Lutterbach et al., 1998) and has two putative protein-protein interaction domains, retained in AML1/ETO: a coiled-coil region (P1, residues 444-492) and an amphipatic α -helix (P2, residues 352-378). SEC analysis revealed that AML1/ETO forms HMW complexes, while deletion of P1 and P2 shifted the fusion protein to lower molecular weight forms (fig18A). AML1a, which is an isoform of AML1 corresponding essentially to the portion of AML1 retained in the fusion protein, was found by SEC as a monomeric species. This finding confirms the requirement for ETO in the formation of HMW complexes. Bacterially expressed AML1/ETO formed HMW complexes, indicating its oligomeric state (not shown). We then asked if the loss of the capacity to form HMW complexes correlated with changes in the ability of AML1/ETO to recruit NCoR and to repress transcription. The P1-P2 motifs do not interact with NCoR, but their deletion led to a strong decrease in the amount of fusion protein bound to GST-NCoR, despite the fact that Δ P-AML1/ETO retains the NCoR binding site (fig.18B).

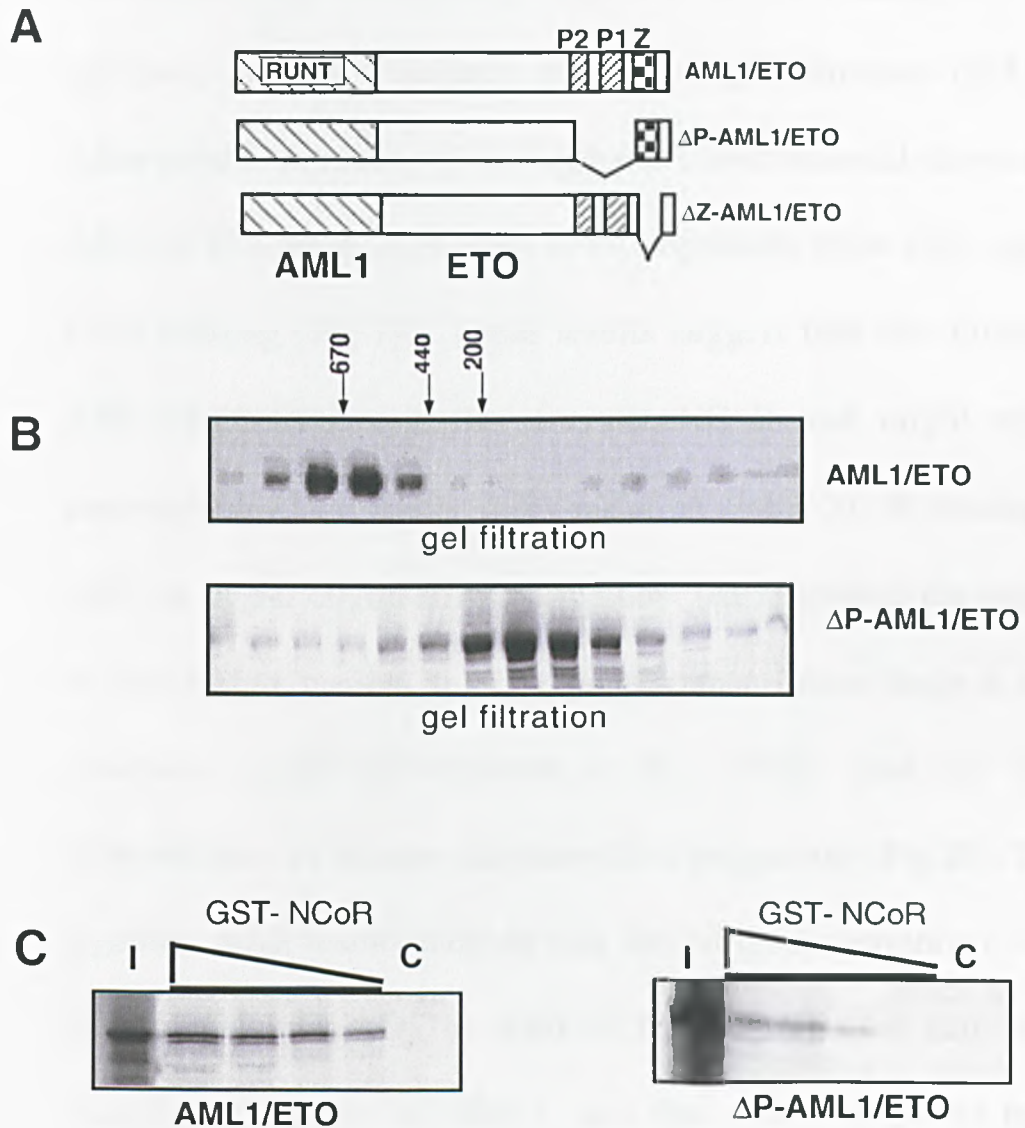


Fig.18: AML1/ETO forms HMW complexes. (A) scheme of AML1/ETO and the deletions used : Z, zinc fingers (NCoR interaction domain).(B) AML1/ETO (A-E) and Δ P-AML1/ETO were *in vitro* translated, fractionated by SEC, and analysed by SDS-PAGE followed by autoradiography (lower panel). (C) **Interaction of AML1/ETO with NCoR.** Pull-down assays of *in vitro* translated AML1/ETO and Δ P-AML1/ETO incubated with GST-NCoR (RDIII), or GST beads (control) as control.

We analyzed whether HMW AML1/ETO complexes are able to bind DNA. AML1/ETO was partially purified by DNA affinity, with biotinylated oligos containing a specific AML1 binding site or an unrelated sequence (panel). Analytical gel filtration (SMART, Amersham Pharmacia) of the high-salt eluted material showed that AML1/ETO can be recovered in its oligomeric form after specific DNA binding (Fig.19). These results suggest that the oligomeric AML1/ETO/DNA complex recruits NCoR and might repress transcription. Consistently, deletions of either the NCoR binding site (ΔZ), or of the oligomerization regions (ΔP) impaired the capacity of the fusion protein to: i) repress transcription from a target promoter: MDR-1(Lutterbach et al., 1998); and ii) block differentiation of primary hematopoietic progenitors (Fig.20). Taken together, these results indicate that the efficient recruitment of the NCoR-HDAC complex by AML1/ETO is required to activate the oncogenic potential of AML1, and that this is achieved by the formation of AML1/ETO oligomeric complexes. This is the demonstration that aberrant oligomerization of a transcription factor may represent a common theme in myeloid leukemias and, may be represents a mechanism of oncogenic conversion in other tumors.

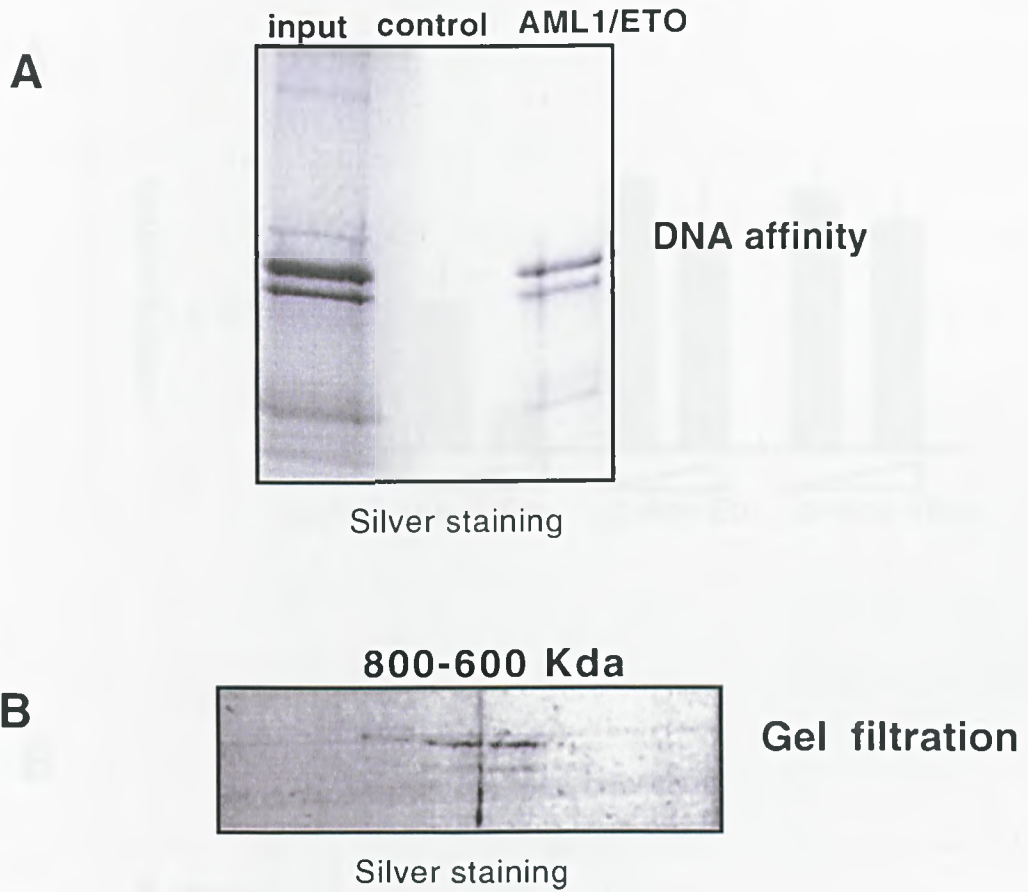
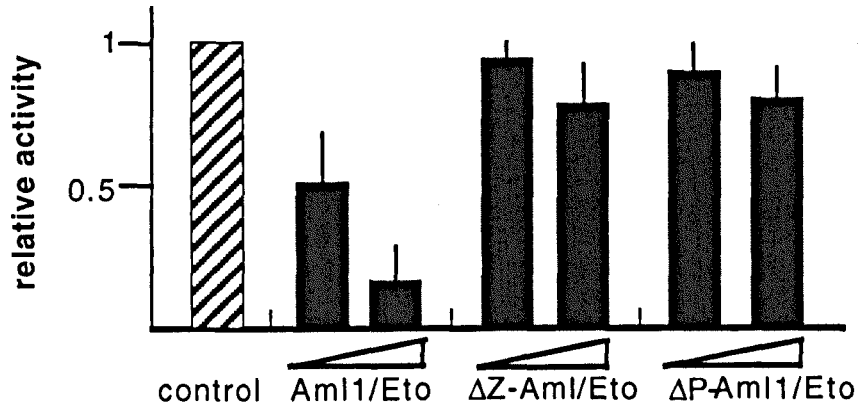


Fig.19: Purification of AML1/ETO HMW complexes.
 (A) Silver staining of the high salt eluted material from a DNA affinity chromatography of nuclear extracts from AML1/ETO expressing cells performed with biotinylated oligos containing a specific AML1 binding site or a unrelated sequence(control)(B) SEC analysis of the high salt eluted material.

A



B

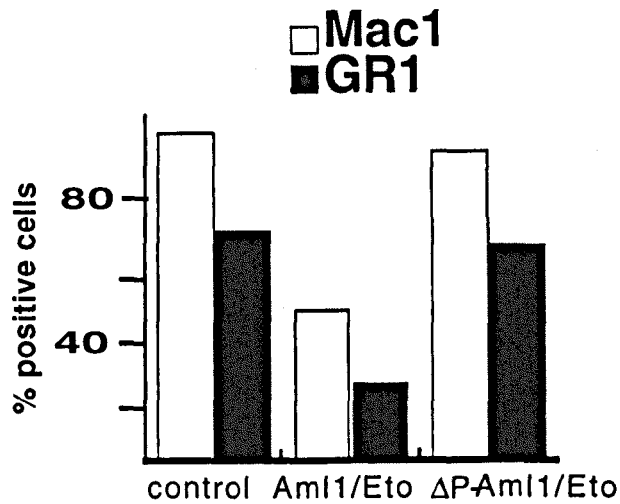


Fig.20: (A) Oligomerization of AML1 is required for transcriptional repression and block of differentiation. C33A cells were transiently transfected with the MDR1-luc reporter and the indicated expression vectors. **(B) AML1/ETO blocks differentiation of hematopoietic precursors.** Lin⁻ cells transduced with the indicated retroviral vectors were sorted and then treated as described.

SUBCELLULAR DISTRIBUTION OF PML, RAR α AND PML/RAR α .

In the course of the experiments aimed at the biochemical and functional dissection of the PML/RAR α high molecular weight nuclear complexes, some observations came to our attention: after high salt extraction of nuclei isolated from PML and PML/RAR α expressing cells, analysis by western Blot of the soluble, high salt nuclear extract and of the “post nuclear extraction” pellet revealed that, after extraction, a conspicuous fraction of PML wt was still entrapped in the pellet. Differently, PML/RAR α was more represented in the soluble, high salt extracted fractions, and less in the pellet. The “post-nuclear “pellet is composed, in our experimental conditions, of nuclear membranes, nuclear laminae, nucleoli and of the so called “nuclear matrix”.

The finding that the PML/RAR α fusion protein behaves differently from wtPML, led us to a more detailed investigation of the subcellular distribution of PML/RAR α (and its physiological counterparts), and of the cause of the relative resistance to the high salt extraction. We have therefore performed experiments in order to investigate if there is a relationship between the oligomerization state, leukemogenic potential, and the nuclear matrix targeting of the proteins investigated.

Nuclear organization and nuclear matrix.

The eucaryotic nucleus is a complex , three-dimensional structure.

A growing number of evidences, based mainly on the visualization of incorporated DNA and RNA labeled precursors and immunolocalization experiments, suggest that key cellular processes, such as DNA replication, transcription as well as RNA processing, take place in specialized nuclear compartments, or "nuclear factories". The "local" concentration of factors allows a better spatial and temporal organization of the nuclear activities, compared to a modulation based only on the free diffusion of molecules, and contributes to the high degree of functional specialization of these dynamic structures.

In this way, the spatial distribution of nuclear proteins is intimately linked to their functional role. The "nuclear matrix" (NM) plays an important role in this scenario (Berezney et al., 1995; Pederson, 1998).

The term nuclear matrix refers to an electron-dense, fibrogranular network observable after that nuclei are digested with DNAses and extracted to remove the chromatin and the vast majority of histone and non-histone soluble proteins (Belgrader et al., 1991; Berezney and Coffey, 1974; Berezney and Coffey, 1976) (fig.21A). Two distinct types of structures constitute the electron-dense "matrix": perichromatin fibrils and interchromatin granule clusters (ICGs)(Fey et al., 1991; He et al., 1990; Nickerson, 2001; Nickerson et al., 1997).

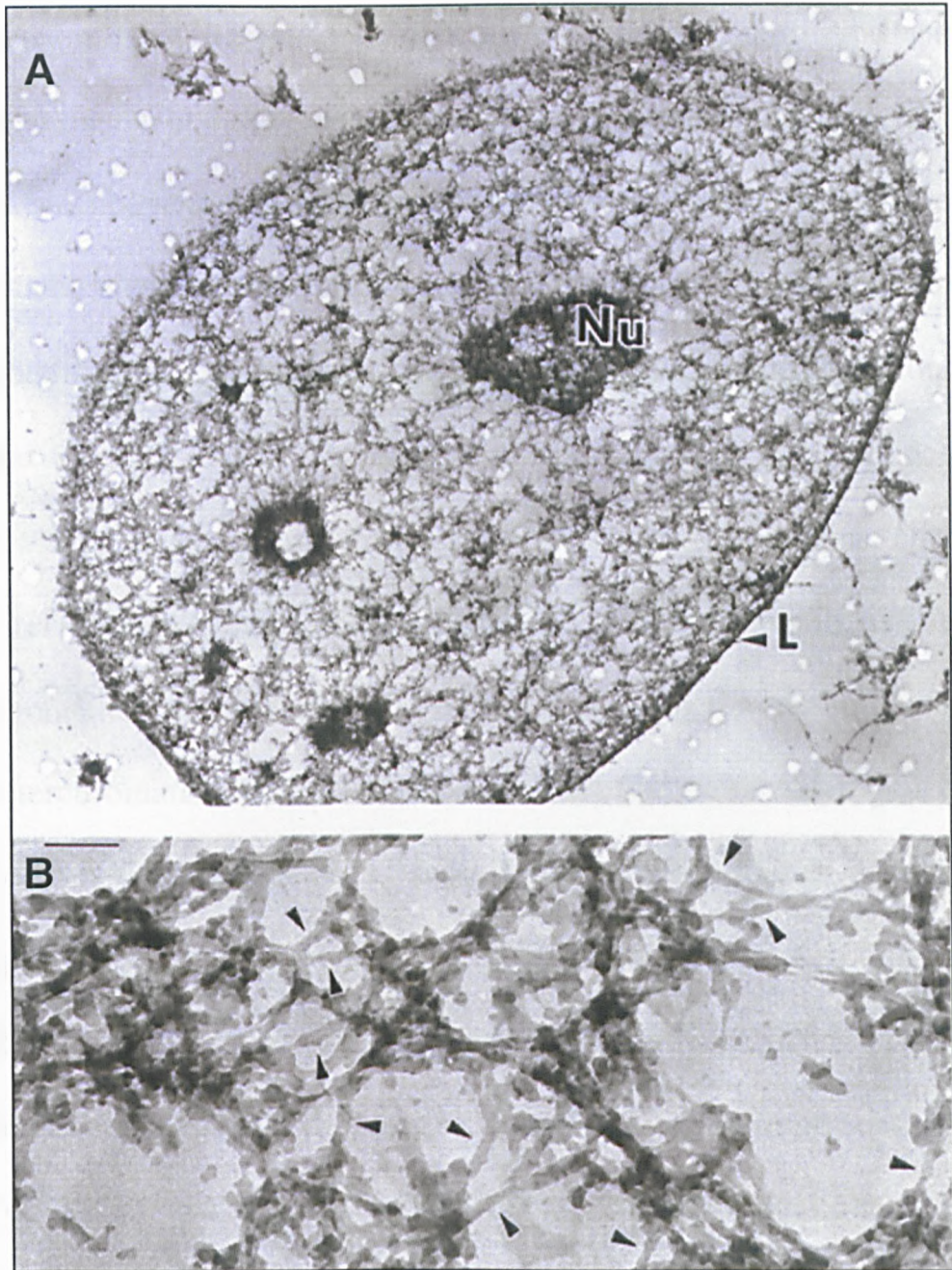


Fig.21: Nuclear matrix. (A) Electron microscopy image of a nucleus, after Dnase digestion of chromatin and high salt extraction of soluble nuclear components.(B)Particular of the highly-branched, anastomosing residual network, called nuclear matrix). Nu=nucleolus, L=nuclear lamina. (From Nickerson et al, 1997)

Fibrils are typically 3 to 5 nm in diameter, often irregularly coiled. ICGs are composed of 20 to 25 nm diameter particles interconnected by anastomosing fibrils, distinct from the perichromatin fibrils before mentioned (fig.21B). The nuclear matrix contains abundant RNA, lipids and retains less than 5% of nuclear DNA and 10% of nuclear proteins. Biochemically, it is composed of a complex set of proteins, some of which are cell-type specific whereas others appear to be ubiquitous (Fey and Penman, 1987). The latter are represented by polypeptides with apparent molecular weight between 30 and 70 Kds: lamins, cheratins, intermediate filament-like proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs), enriched in the pericromatin fibrils. The Interchromatin Granule Clusters (IGCs) are more heterogeneous in composition than fibrils, showing a certain degree of cell-type specificity. Several proteins, as splicing factors, transcription factors, RNA Pol II large subunit, have been found to be enriched in the IGCs fraction, suggesting the possibility that IGCs could represent the most "dynamic" fraction of the nuclear matrix, directly linked to gene expression (Mancini et al., 1994; Mortillaro et al., 1996; Pederson, 1998). Recently, a subclass of IGCs has been biochemically and functionally characterized as centers of pre-RNA synthesis and processing (Mintz et al., 1999). The presence of actin-binding-proteins in several biochemically isolated nuclear complexes (Peterson et al.,

1998) together with the demonstration of direct interactions between proteins of the basal transcription machinery and nuclear matrix structural components (Bruno et al., 1999) further underlies the importance of the “nuclear skeleton” for the organization and modulation of nuclear activities.

Signals able to promote targeting of proteins to the nuclear matrix have been described. These signals (nuclear matrix targeting signals, NMTSS) are distinct from the nuclear localization signals (NLSs) and there is no obvious consensus sequence (McNeil et al., 1998; Zeng et al., 1997). The association of proteins (such as transcription factors, polymerases, splicing factors) with the "nuclear scaffold" is developmentally regulated. The interaction of the NMP-2/AML transcription factor with the nuclear matrix represents a paradigmatic example of this phenomenon (Lindenmuth et al., 1997): in developing osteoblasts, the NMP-2/AML factor is tightly anchored to the nuclear scaffold and shows high DNA binding activity. In mature osteocytes, the same factor is much less active in DNA binding and almost entirely excluded from the interior part of the nucleus. The qualitative and quantitative composition of nuclear matrix is different in normal and transformed cells (Alberti et al., 2000), further suggesting that the spatial organization of the nucleus plays an important role in the regulation of cellular functions.

By DNase digestion of isolated nuclei and subsequent high salt extraction of the nuclease digested material, we have been able to collect five fractions, corresponding, respectively, to:

1. Cytoplasmic proteins;
2. nucleoplasmic proteins (released by DNase treatment of intact nuclei), e.g: mainly histone H1, several transcription factors
3. nucleoplasmic proteins (by ammonium sulfate precipitation), e.g: histone H2 A and B, H3, H4 , HMG proteins and transcription factors
4. Outer nuclear matrix proteins (by NaCl 2M precipitation)
5. Inner Nuclear matrix proteins. (structural proteins and intermediate filaments).

We tested the efficiency of the fractionation by screening the obtained subcellular fractions, by Western Blot technique, for the presence and subcellular distribution of proteins whose localization was known, e.g β -tubulin for the cytoplasmic fraction (fr. 1); HMGI(I)Y protein for the nucleoplasmic fraction (fr. 3); lamin B1 for the nuclear matrix fraction (fr. 5). We assessed the presence in the nucleoplasmic fractions of histones, by Coomassie staining of 15% polyacrylamide gels of the obtained subnuclear fractions (fig.22A and B, respectively).

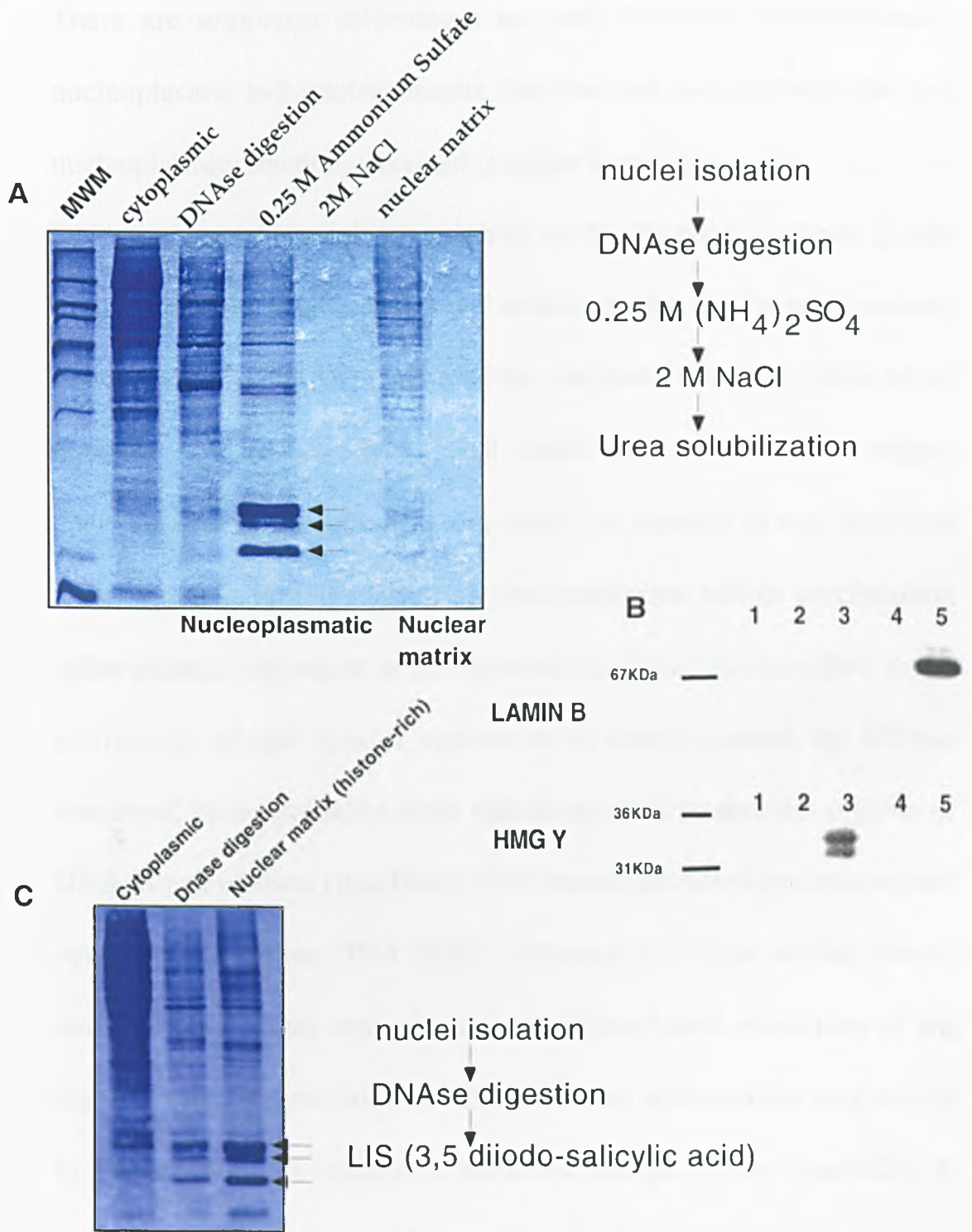


Fig.22: Subcellular fractionation experiments. Coomassie staining(A,C) of fraction obtained with two different techniques. Western Blot (B) of the same fractions represented in panel A , with antibodies against known subnuclear antigens. Arrows indicate histones.

There are important differences not only between the cytoplasmic, nucleoplasmic and nuclear matrix fractions but even between the two nucleoplasmic fractions obtained (number 2 and 3).

DNase-released proteins, enriched in the fraction number 2, are proteins whose "nuclear retention" would require an "intact chromatin environment". The subversion of the chromatin structure, induced by nuclease digestion (in isotonic salt conditions), is sufficient to trigger the release of those proteins from nuclei, in absence of any increased ionic strength. Proteins requiring the ammonium sulfate precipitation (after nuclease digestion) to be extracted could be "less sensitive" to the subversion of the overall chromatin structure exerted by DNase treatment, being probably more tightly attached to specific regions of DNA. Some of them (like HMG (I)Y) remain anchored to these regions even in the cell-free DNA "halo", obtained by *in situ* nuclear matrix isolation procedures and ammonium sulfate-based extraction of the digested nuclear material. The latter technique is essentially very similar to the biochemical extraction methods, except for the possibility to perform indirect immunofluorescence studies *in situ*, with a certain degree of preservation of the overall nuclear morphology. Complete digestion and removal of the chromatin and soluble nuclear proteins have been assessed by the absence of DAPI staining in the final

preparations and by immunolocalization of both soluble HMGI(Y) and nuclear matrix-associated polypeptides.

Due to the existing debate about the influence of the isolation method on the qualitative and quantitative composition of nuclear matrix (Martelli et al., 1999; Pederson, 2000), in particular due to the utilization of "precipitation-prone" conditions (Pederson, 1998), we have performed experiments with other different nuclear matrix isolation techniques.

Similar results have indeed been obtained using the LIS (3,5 diiodosalicylic acid lithium salt) extraction (Izaurrealde et al., 1988; Mirkovitch et al., 1984). LIS acts in a detergent-like manner, leading to the isolation of nuclear matrices characteristically enriched in histones (fig.22C). It is of note that the isolation takes place in isotonic, not precipitation-prone salt conditions.

Subnuclear distribution of PML, PML/RAR α and RAR α

We performed experiments to evaluate, biochemically, the subcellular distribution of PML, RAR α and PML/RAR α .

Both transiently transfected cell lines (Hela, Phenix) and stably expressing cell lines (U937 cells) were used, with analogous results. When possible, we used cell lines endogenously expressing the proteins of interest.

As already described (Chang et al., 1995 and fig.23A), the PML protein is, in asynchronous cell populations, fully retained in the nuclear matrix fraction.

For the first time, we showed that RAR α is a protein mainly associated with the nuclear matrix. Very little amounts of RAR α are present in the nucleoplasmic fraction (fig.23 B). As it is possible to observe in the figure, a significant amount of RAR α is represented in the cytoplasmic fraction: interestingly, cytoplasmic RAR α migrates in a SDS-PAGE gel quite slower than the one associated with the nuclear /nuclear matrix fraction. This phenomenon has been observed constantly and in different cell lines. Probably, a postranslational modification (ie, a phosphorylation event...) modulates the cytoplasmic/nuclear distribution of the protein.

To study the subnuclear distribution of PML/RAR α , we have used, in parallel with U937 stable expressing cells, the NB4 cell line, derived

from an APL patient and spontaneously expressing the leukemic fusion protein. PML/RAR α is represented in both nucleoplasmic and nuclear matrix fractions (fig.23C). In presence of similar levels of expression, the relative amount of PML/RAR α in the nucleoplasmic fractions (2-3) is higher than that of RAR α . A semi-quantitative analysis of the subnuclear distribution of PML3, RAR α and PML/RAR α revealed that the fusion protein is represented for the 30-35% in the nucleoplasmic fractions, while only 10-15% of RAR α is present in this fractions. Thus, it is possible to conclude that the PML/RAR α fusion protein is partially "delocalized" with respect to its physiological counterparts, being more 'soluble' than both PML and RAR α .

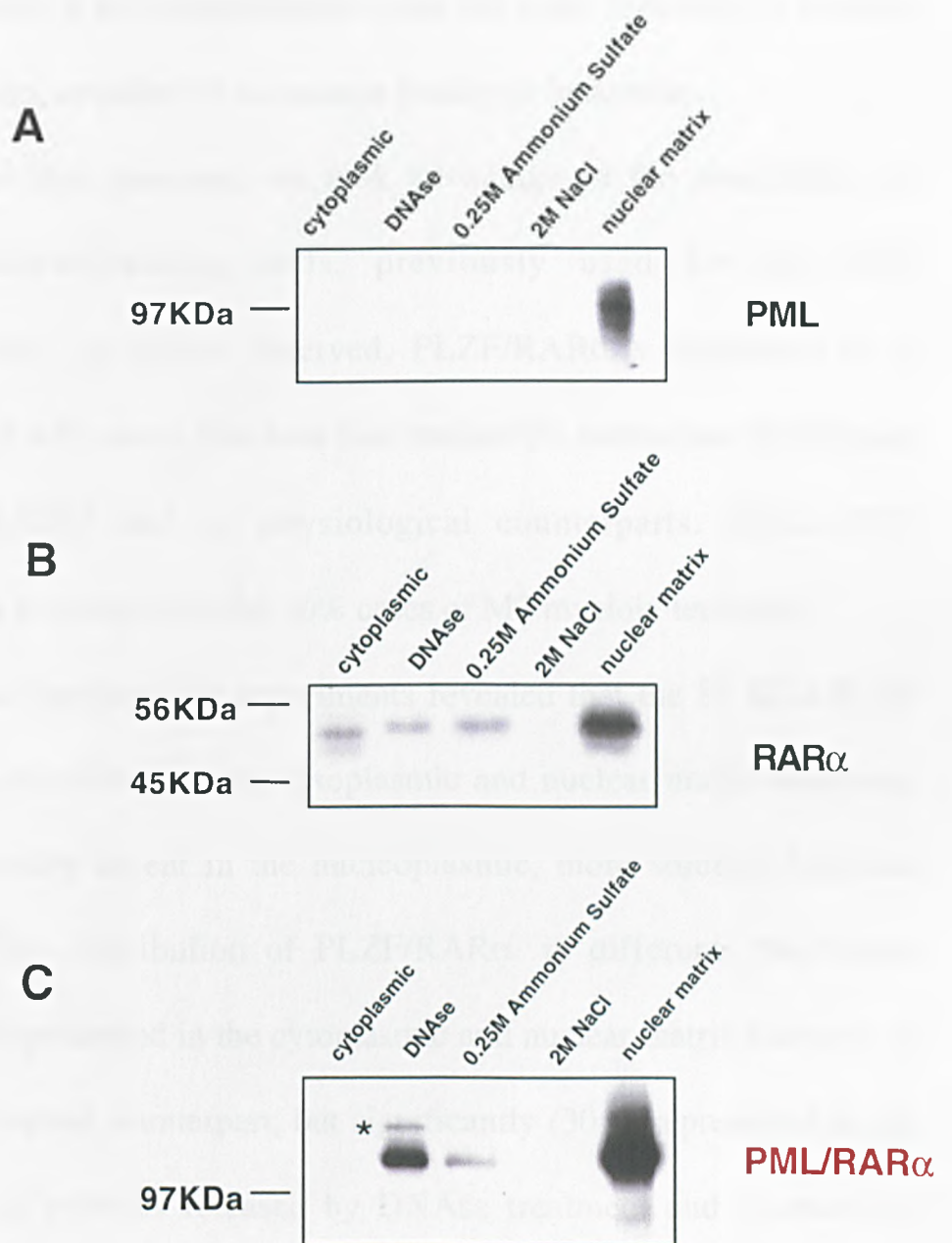


Fig.23: Subnuclear distribution of PML/RAR α and its physiological counterparts. Immunoblot analysis with α PML (A), α RAR α (panel B and C) of fractions obtained by DNase digestion and high salt extraction of isolated nuclei from cells expressing stably the indicated proteins. Asterisk indicates an anti RAR α , immunoreactive aspecific band.

Subnuclear distribution of other AML fusion proteins

We evaluated if the delocalization observed could represent an isolated phenomenon, or rather be a common finding in leukemias.

To answer this question, we took advantage of the availability of PLZF/RAR α expressing cells, previously used for the SEC experiments. As before observed, PLZF/RAR α is expressed in a minority of APL cases. We have also studied the subnuclear distribution of AML1/ETO and its physiological counterparts. AML1/ETO expression is observed in the 40% cases of M2 myeloid leukemia.

Subnuclear fractionation experiments revealed that the 87 KDa PLZF protein is distributed in the cytoplasmic and nuclear matrix fractions, being virtually absent in the nucleoplasmic, more soluble, fractions (fig.24). The distribution of PLZF/RAR α is different: the fusion protein is represented in the cytoplasmic and nuclear matrix fractions as its physiological counterpart, but significantly (30%) represented in the fractions of proteins released by DNase treatment and Ammonium sulfate extraction.

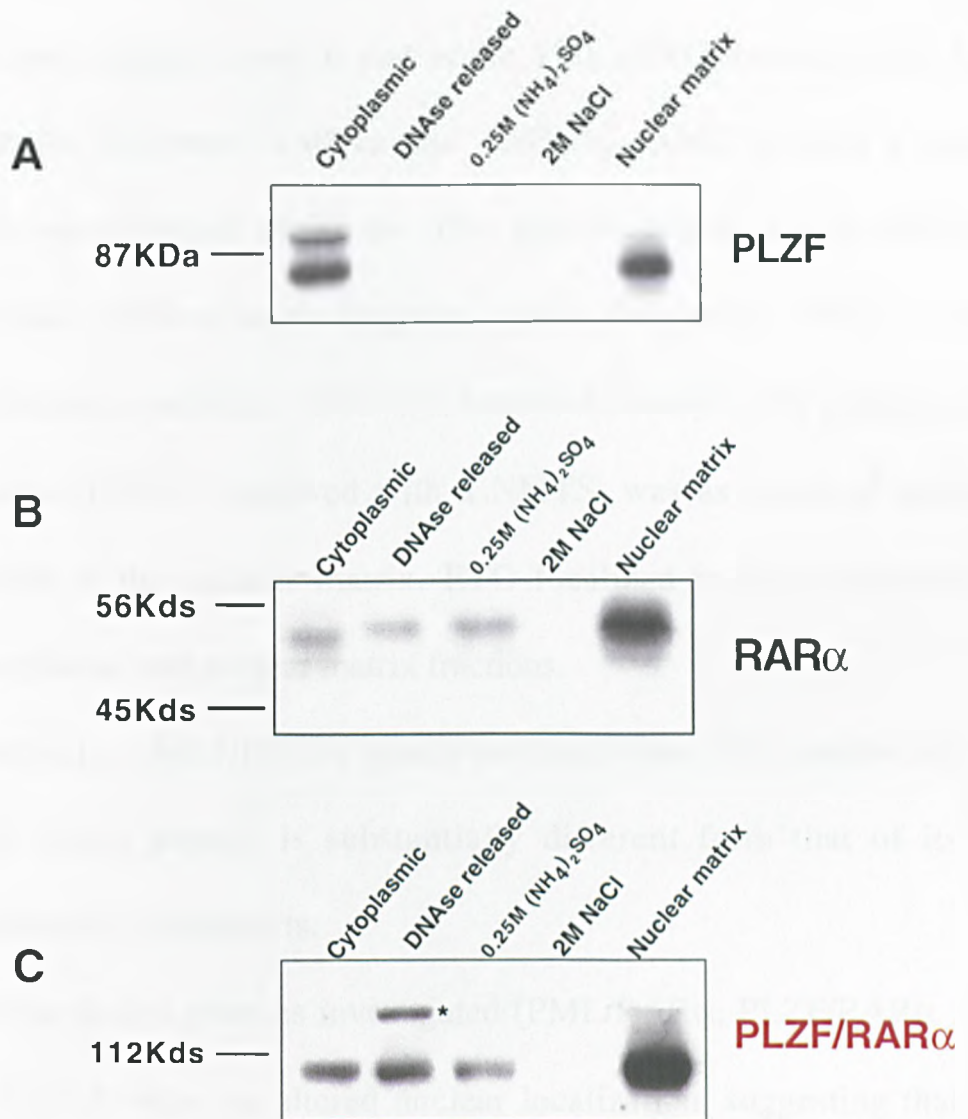


Fig.24: Subcellular distribution of PLZF/RAR α and its physiological counterparts .Immunoblot analysis with α PLZF (A), α RAR α (B and C) of fractions obtained by DNase digestion and high salt extraction of isolated nuclei from cells expressing stably the indicated proteins. Asterisk indicates an anti RAR α , immunoreactive aspecific band.

Studies on the subnuclear distribution of AML1/ETO, a non-APL related leukemic fusion protein, raised the possibility that the observed phenomenon could be extended to non APL fusion protein. The AML1 transcription factor, which is part of the AML1/ETO fusion protein, has two major isoforms: AML1a and AML1b. AML1a lacks a short carboxy-term region of 31 aa. This region, conserved in AML1b, represents a nuclear-matrix-targeting-signal (Zeng et al., 1997). In our experimental conditions, AML1a is localized mainly in the cytoplasmic fractions. AML1b, endowed with a NMTS, was as expected mainly localized in the nuclear matrix. ETO localized in the cytoplasmic, nucleoplasmic and nuclear matrix fractions.

Interestingly, AML1/ETO is mainly nucleoplasmic. The distribution of the fusion protein is substantially different from that of its physiological counterparts.

All of the fusion proteins investigated (PML/RAR α , PLZF/RAR α , AML1/ETO) show an altered nuclear localization, suggesting that their compartmentalization may play a role in their leukemogenetic potential.

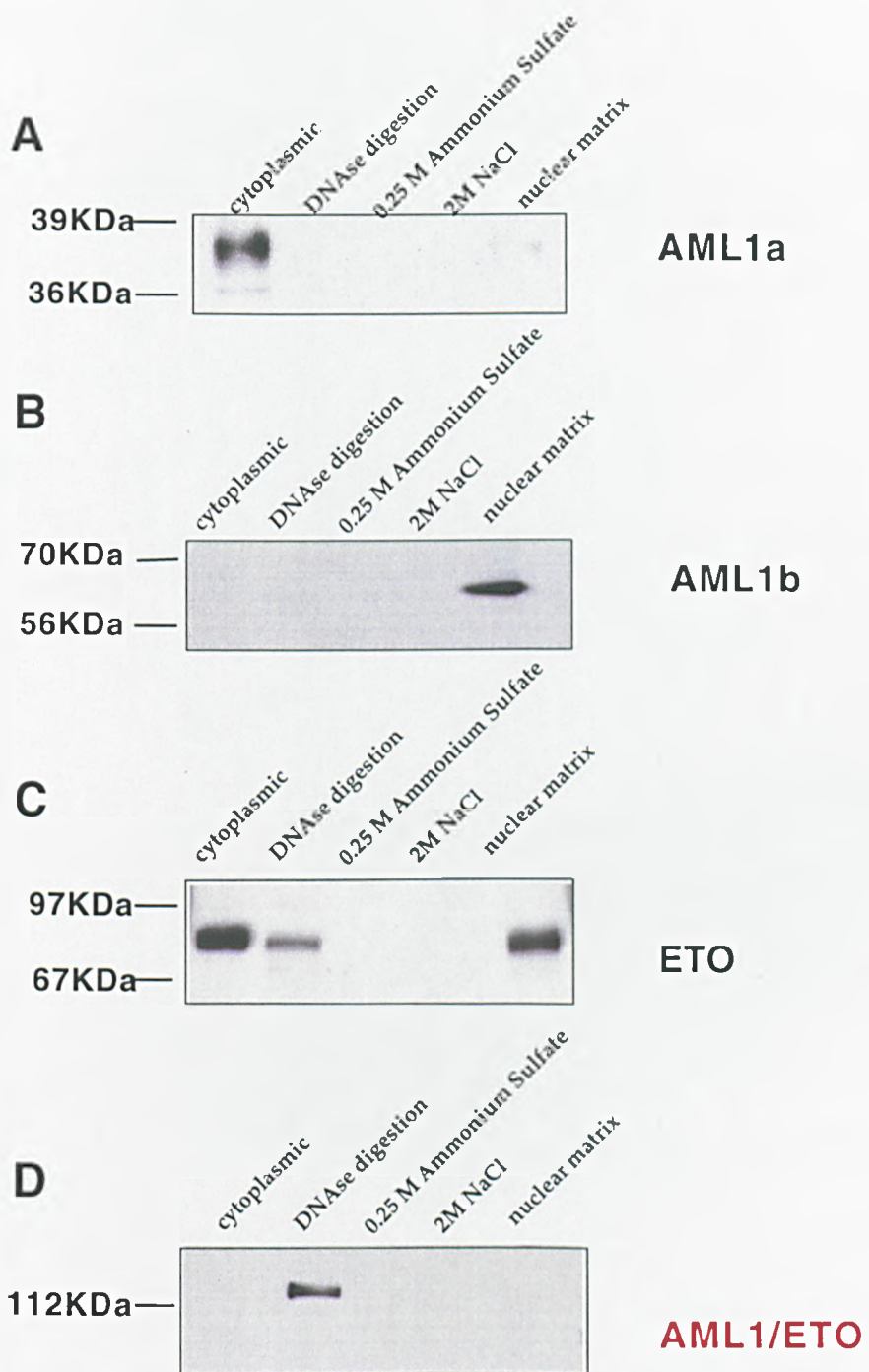


Fig.25: Subcellular distribution of AML1/ETO and its physiological counterparts. Immunoblot analysis with anti-AML1 (A and B), anti-ETO (C and D) of fractions obtained by DNase digestion and high salt extraction of isolated nuclei from cells expressing stably the indicated proteins.

The Ser/Pro rich region of PML: a possible nuclear matrix targeting signal (NMTS). In several nuclear matrix targeted proteins, residues have been identified as responsible of this phenomenon (DeFranco and Guerrero, 2000; McNeil et al., 1998; Zeng et al., 1997): these regions, unlike the nuclear localization signals, are variable and specific for each proteins. With the intent to determine residues necessary and sufficient for targeting the physiological counterparts of leukemogenic proteins to the nuclear matrix, (eventually deleted or rearranged in the chimera), we have studied the distribution of the following PML and PML/RAR α mutants:

- PML-P/R: this is a PML deletion mutant lacking the Ser-Proline rich carboxyterminal region of wtPML3, which is lost in the generation of PML/RAR α chimeric protein. This protein contains exactly the portion of PML retained in the chimeric fusion protein
- PML Ser4-: in the carboxy terminal Ser/Pro rich region, four serine residues (the four serine phosphorylated by the Protein kinase Casein Kinase II) have been mutagenized to alanine residues.
- PML/ Δ RAR α : In the PML/ Δ RAR α fusion protein, the "C" domain, corresponding to the RAR α DNA Binding Domain, has been deleted.

Interestingly, the PML-P/R protein is significantly represented, unlike the PML wt protein, in the nucleoplasmic fractions (fig.26A and B,

respectively). As before observed, this mutant contains exclusively the portion of PML wt retained in the translocation product, thus lacking the COOH Ser/Pro rich domain. It is likely that the missing region is important for the complete nuclear matrix targeting of the wtPML protein.

The Ser/Pro rich region of wt PML is a region, as its denomination suggests, enriched in serine residues. We studied the subnuclear distribution of the PMLSer4- mutant, in which four serine residues, represented in the COOH Ser/Pro rich region have been replaced with alanine residues. This mutant, PMLSer4-, shows a subnuclear distribution essentially identical to the PML-P/R deletion mutant. (fig.27C)

Indeed, while the wt PML protein is completely associated with nuclear matrix, the PMLSer4- mutant is partially delocalized in the nucleoplasmic fractions, thus effectively suggesting that phosphorylation of serine residues in the carboxyterminal region of PML may play a role in the interaction of PML with the nuclear matrix. In the PML/RAR α molecule, the Ser/Pro rich region of wtPML is lost. The loss of this nuclear matrix targeting signal could be responsible for the partial loss of the nuclear matrix targeting of the chimeric protein, that could depend entirely on RAR α sequences (see below).

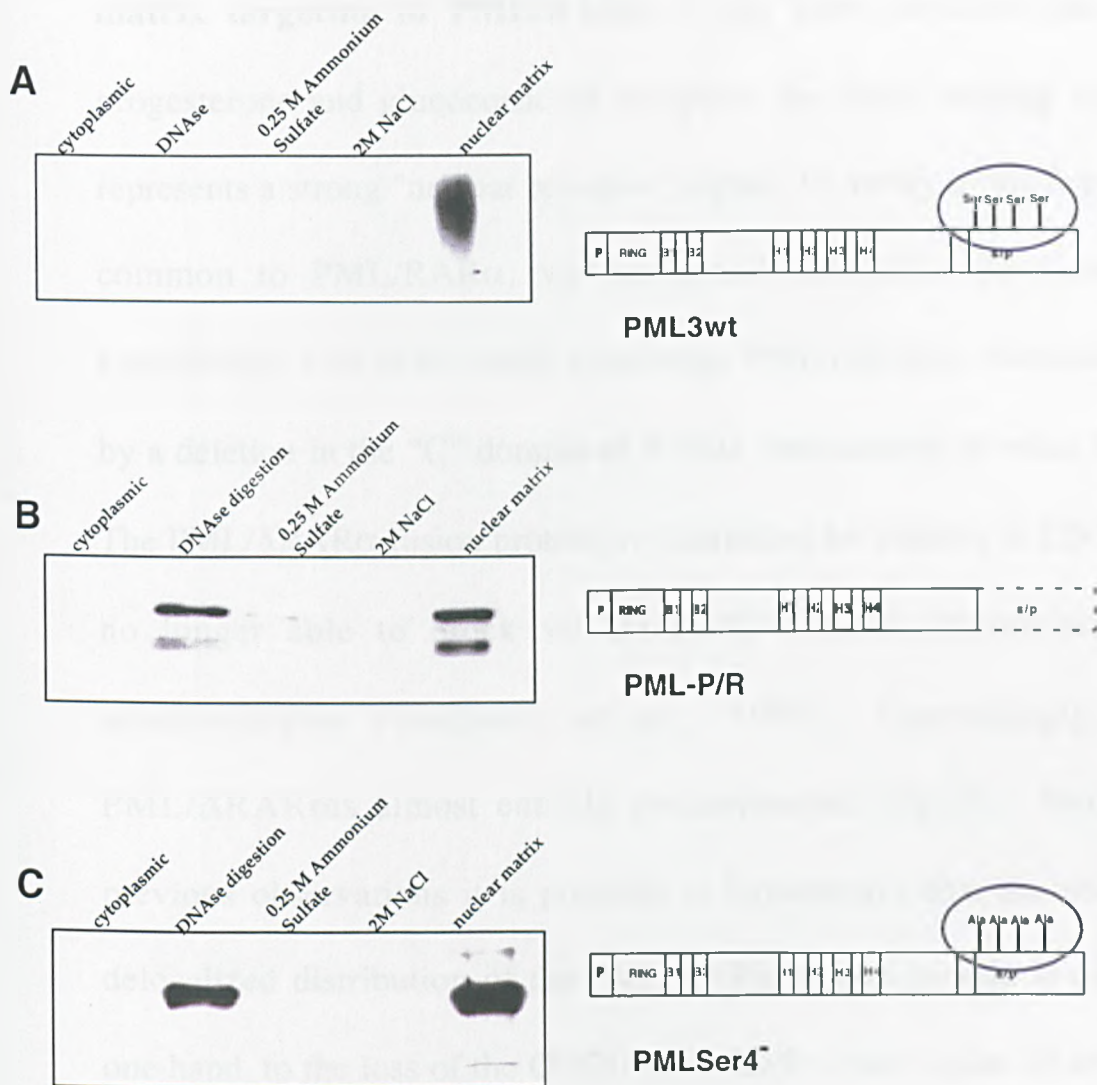


Fig.26: Subnuclear distribution of PML mutants. Western blot with α PML of fractions obtained by DNase digestion and high salt extraction of isolated nuclei from cells expressing stably the indicated proteins.

The DNA Binding Domain of RAR α is required for the nuclear matrix targeting of PML/RAR α . It has been reported that, for progesterone and glucocorticoid receptors, the DNA binding domain represents a strong "nuclear retention" signal. To verify if this feature is common to PML/RAR α , we performed subnuclear fractionation experiments with cells stably expressing PML/ Δ RAR α , characterized by a deletion in the "C" domain of RAR α (maintaining an intact NLS). The PML/ Δ RAR α fusion protein is defective for binding to DNA and no longer able to block vit D3/TGF β -induced differentiation of promyelocytes (Grignani et al., 1996). Interestingly, the PML/ Δ RAR α is almost entirely nucleoplasmic (fig.27). From the previous observations it is possible to hypothesize that the partially delocalized distribution of the PML/RAR α fusion protein is due, on one hand, to the loss of the COOH term Ser/Pro rich region of wt PML and, on the other hand, to the presence in the fusion protein of a NMTS, requiring or corresponding to the RAR α DNA Binding Domain. It is of note, however, that the NMTS in PML/RAR α is less efficient in NM retention than in the context of wtRAR α .

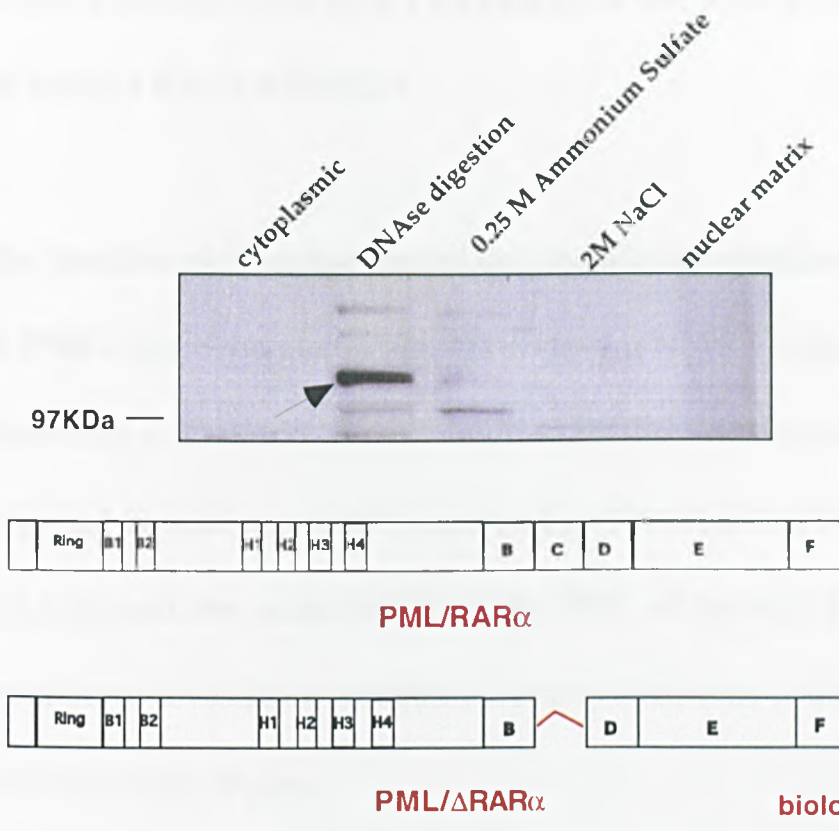


Fig.27: The RAR α DBD promotes targeting of PML/RAR α . to the nuclear matrix Western blot with α RAR antibody of fractions obtained by DNase digestion and high salt extraction of isolated nuclei from cells expressing **PML/ Δ RAR α** , a DBD deletion mutant of PML/RAR α . Arrow indicates the band corresponding to PML/ Δ RAR α .

FUNCTIONAL CHARACTERIZATION OF THE PML SER/PRO RICH REGION

The Ser/Pro rich region modulates the oligomerization state of PML. Since the nuclear matrix targeting of PML mutants lacking or harboring a mutated version of the Ser/Pro rich region of PML is impaired but not completely lost, we hypothesized that this region could not represent the main NMTS of the PML wt protein. For this reason, we tried to investigate whether accessory functions could eventually be ascribed to this region.

From our observations, based on gel filtration chromatography experiments, we have found that the PML3 wt protein exhibits an oligomerization state different from that of PML/RAR α . By comparison with gel filtration high molecular weight markers (Dextran Blue and thyroglobulin), the size of the PML and PML/RAR α complexes in nuclear extracts obtained from stably expressing cell lines, is respectively several millions of Daltons and 800-600 kDa (Fig. 28A and B, respectively).

Since in PML/RAR α the Ser/Pro rich region of PML is invariably lost, we performed size exclusion chromatography experiments in order to evaluate if the reduced oligomerization state of the fusion protein can be linked to the loss of the before mentioned region.

Interestingly, both the deletion mutant (PML-P/R) and the point mutation mutant (PML Ser4-) show an oligomerization state consistently and significantly reduced, respect to the wt PML protein (fig.28 C and D).

As previously shown, the wt PML protein migrates in gel filtration at an apparent molecular weight of several millions of Daltons, while PML-P/R and the PMLSer4- mutants migrate at approximately 600-800 kDa.

Interestingly, the oligomerization state of PML mutated in the Ser/Pro rich region is very similar to that of PML/RAR α .

The last finding suggests that the coiled-coil of PML, conserved in PML/RAR α , PML-P/R and PMLSer4-, mediates the acquisition of an initial oligomerization state, maintained in the fusion protein, and further modulated, in the wt PML molecule, by the presence of an intact COOH terminus.

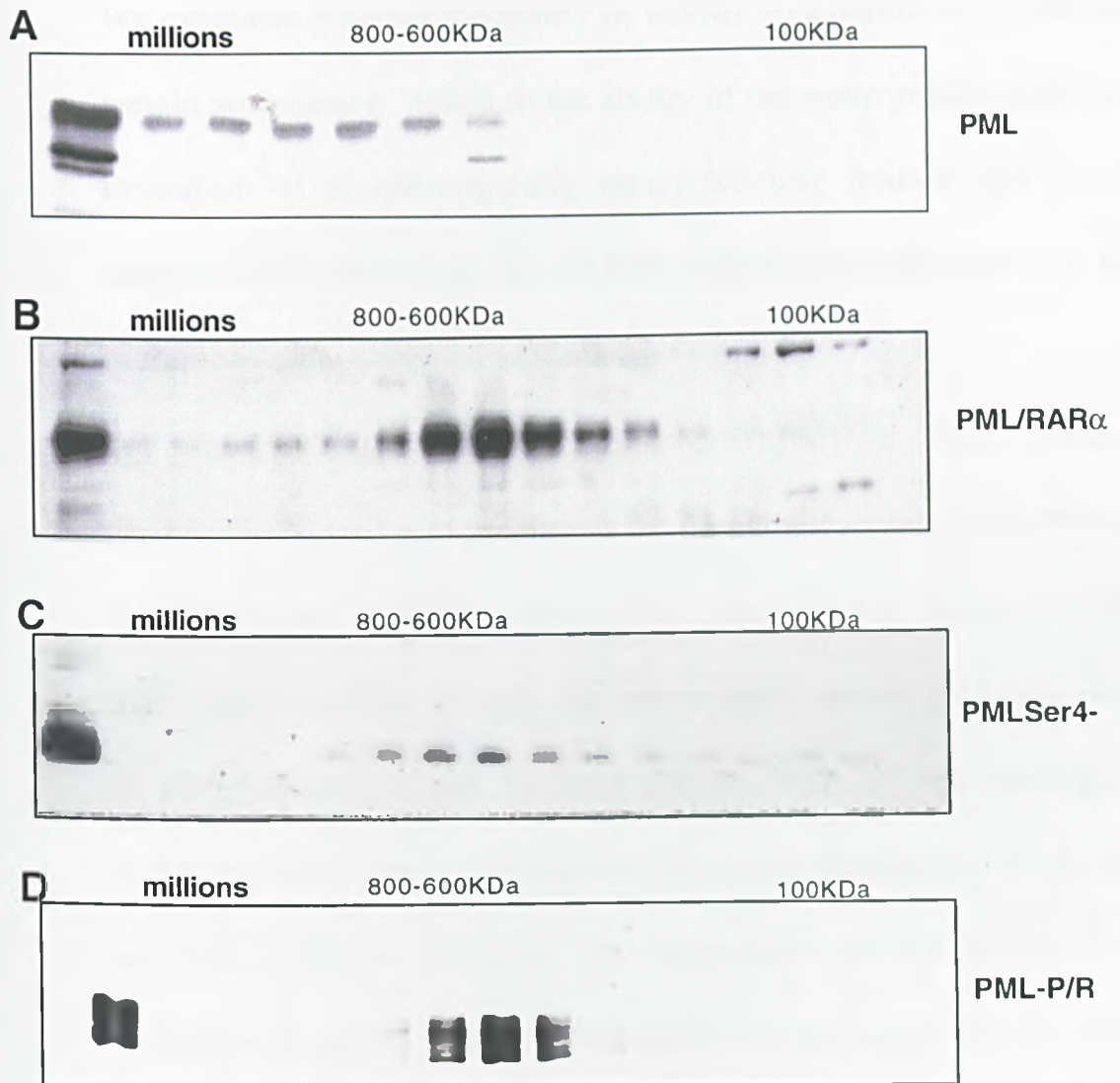


Fig.28: The ser/pro rich region influences the oligomerization state of PML. Gel-filtration analysis of nuclear extracts from cells expressing, respectively, wtPML (A), PML/RAR α (B), PMLser4- (C), PML-PR (D).

The Ser/Pro-rich region is required for the “functional’ integrity of Nuclear Bodies.

We evaluated whether the ability of wtPML to assemble high molecular weight structures is linked to the ability of the same protein to drive the formation of morphologically intact Nuclear Bodies. We checked therefore the ability of the COOH oligomerization-defective PML mutants to organize Nuclear Bodies.

We evaluated, by DNA microinjection and indirect immunofluorescence experiments, the potential capacity of wt PML and the COOH term mutants to reconstitute intact Nuclear Bodies in PML^{-/-} MEF cells. In PML^{-/-} cells, the exogenously added wild-type protein is able to reconstitute Nuclear Bodies (fig.29) morphologically indistinguishable from the nuclear structures represented in the PML^{+/+} cells. On the contrary, the expression of the PML-P/R and PMLSer4⁻ is able to trigger the formation of few, bigger Nuclear Bodies (fig. 30 and 31, respectively). Notably, while the "reconstituted" PODs are characteristically resistant to the *in situ* removal of the chromatin and high salt extraction of the nucleoplasmatic proteins, the structures originated from the oligomerization-defective mutants are almost completely removed by this procedure, leading to the formation of nuclear scaffolds with no or very faint signal for PML.

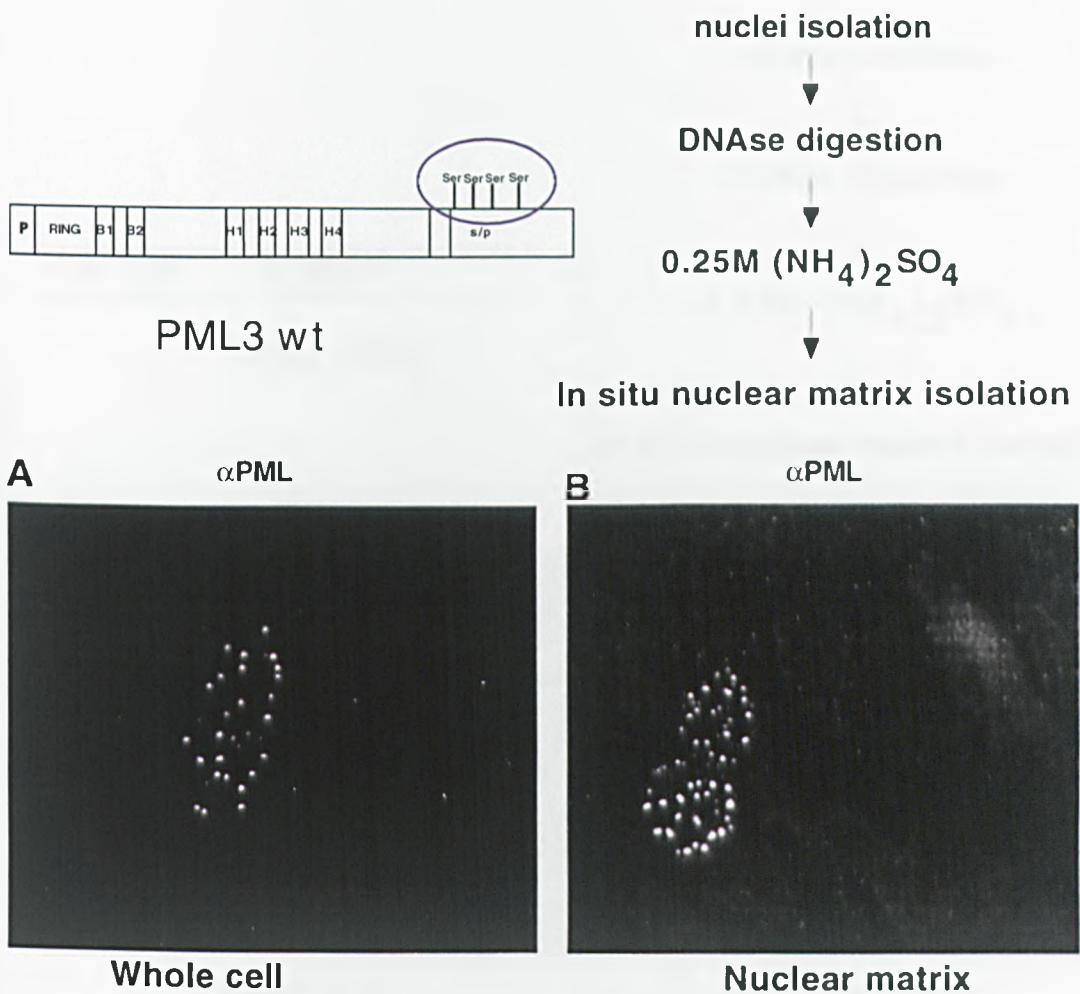


Fig.29: PML is able to drive assembly of matrix-anchored Nuclear Bodies. PML ^{-/-} MEFs were microinjected with an expression vector for wtPML3 and subjected to indirect immunofluorescence with an αPML antibody (PGM3). (A) In intact, fixed cells, reconstituted Nuclear Bodies are evident. (B) After in situ extraction of DNA and soluble nuclear proteins, reconstituted Nuclear Bodies are still evident.

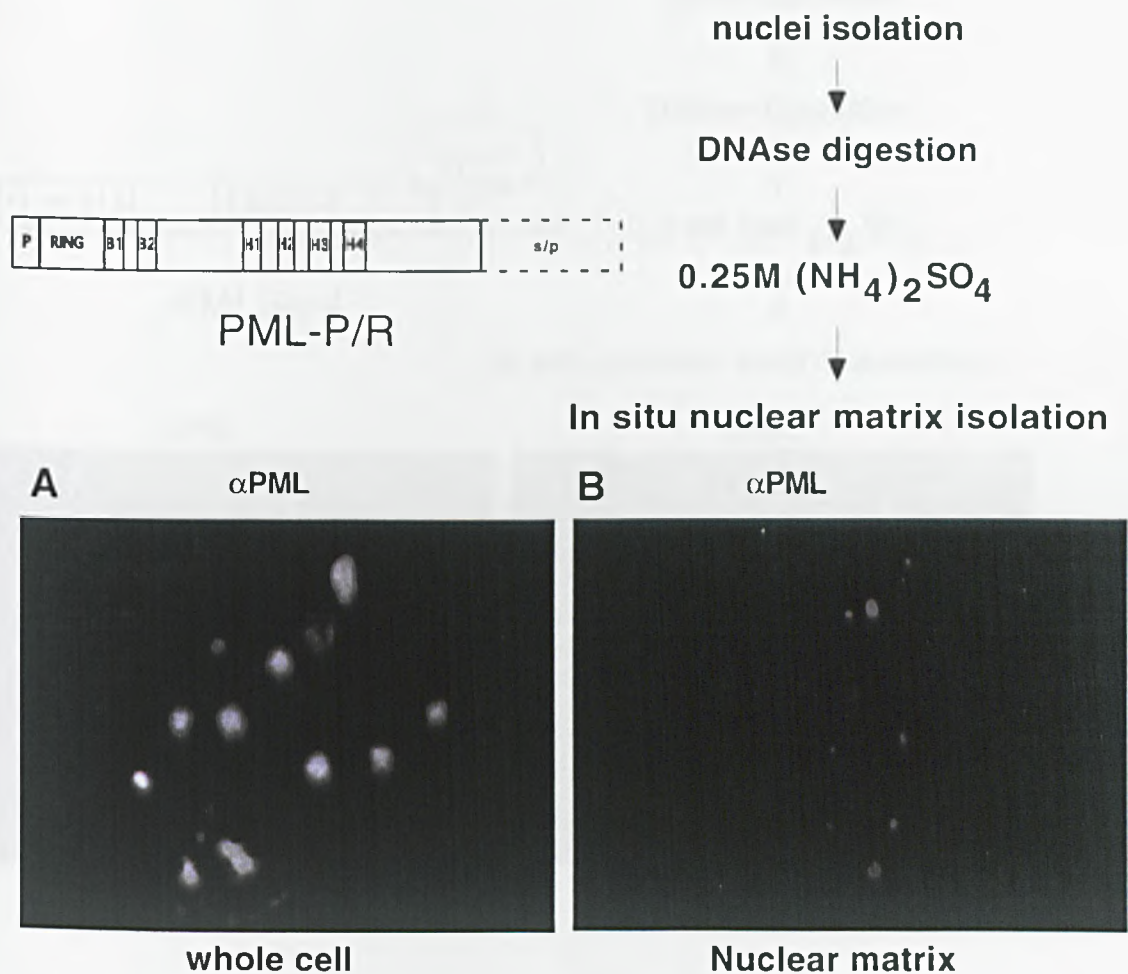


Fig.30 : PML-P/R is not able to drive assembly of matrix-anchored Nuclear Bodies. PML^{-/-} MEFs were microinjected with an expression vector for the mutant PML-PR and subjected to indirect immunofluorescence with an αPML antibody (PGM3). (A) In intact, fixed cells, enlarged, irregular Nuclear Bodies are evident. (B) After in situ extraction of DNA and soluble nuclear proteins, reconstituted Nuclear Bodies are not more evident.

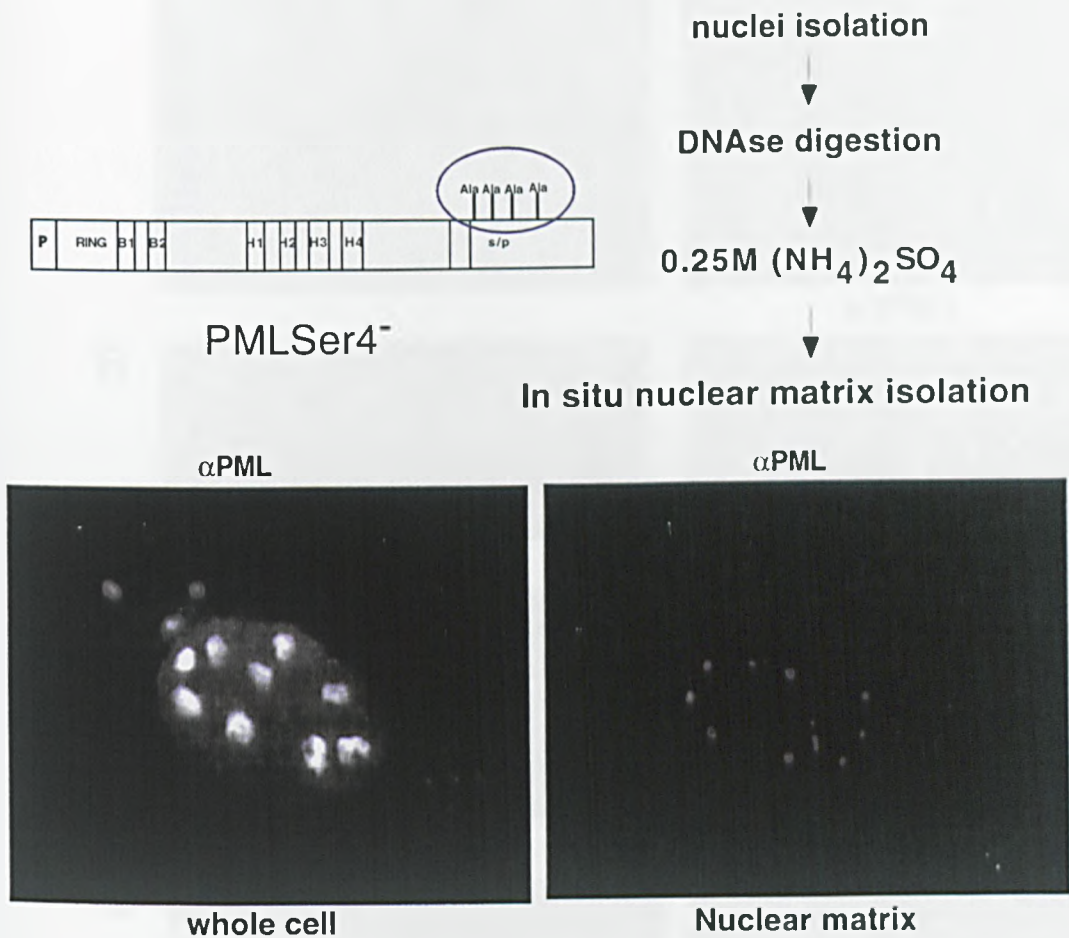


Fig.31: The mutant PMLSer4⁻ is not able to drive assembly of matrix-anchored Nuclear Bodies. PML^{-/-} MEFs were microinjected with an expression vector for the mutant PMLSer4⁻ and subjected to indirect immunofluorescence with an αPML antibody (PGM3). (A) In intact, fixed cells, enlarged, irregular Nuclear Bodies are evident. (B) After in situ extraction of DNA and soluble nuclear proteins, reconstituted Nuclear Bodies are not more evident.



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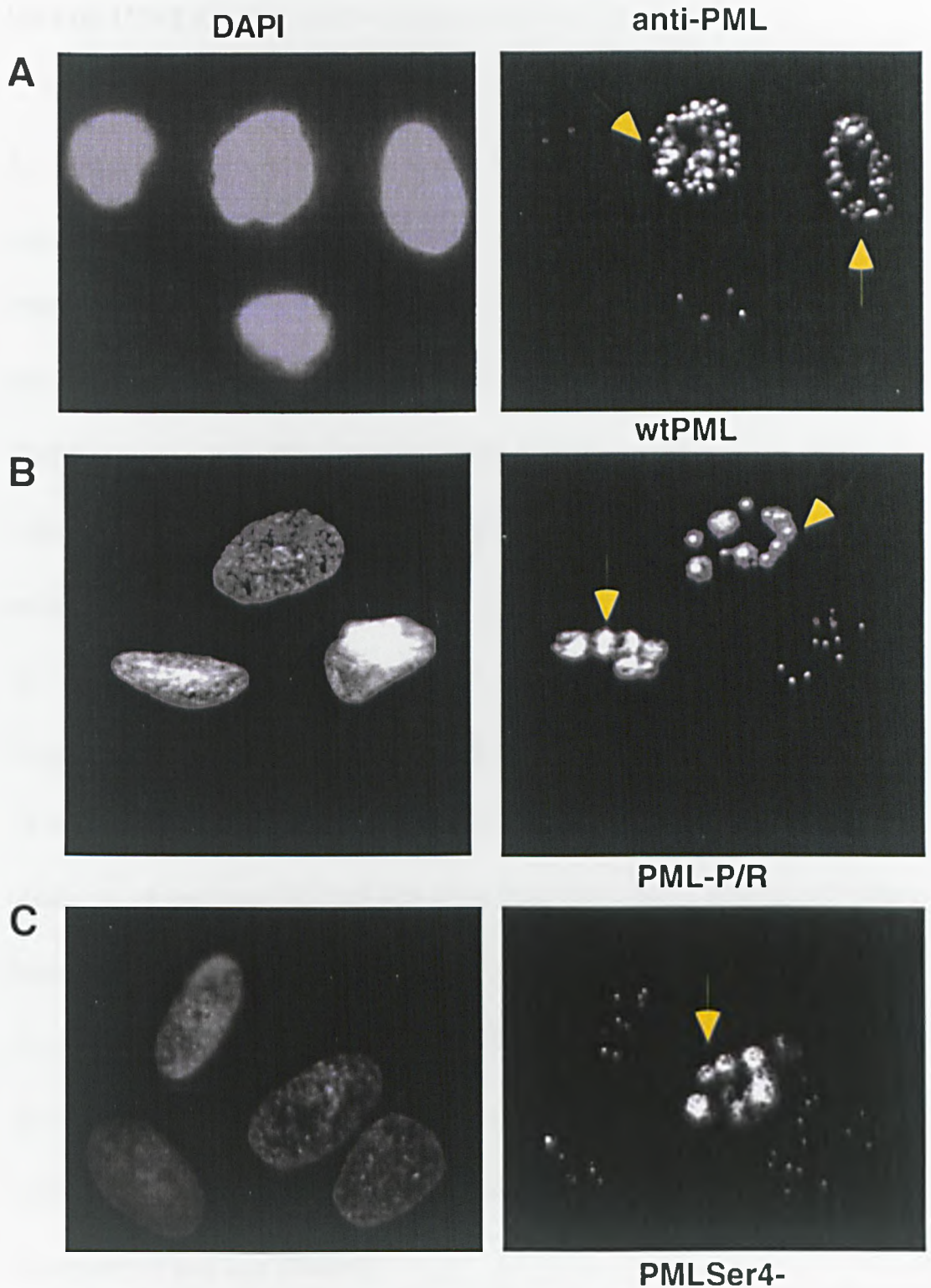


Fig.32: Expression of PML mutants in a PML +/+ context leads to alterations of Nuclear Bodies. HeLa cells were microinjected with expression vectors for PMLwt(A), PML-P/R (B), and PMLSer4- (C). DAPI staining of corresponding cells is in the left panels. Arrows indicate microinjected cells.

FRAGMENTATION OF NUCLEAR BODIES IN APL

As before observed, oligomerization state and nuclear matrix association of the described PML3 mutants is pretty similar to PML/RAR α .

Interestingly, unlike PML/RAR α , expression of PML-P/R and PMLSer4- does not affect the integrity of Nuclear Bodies in a PML +/- cellular context. This suggests that domains in the RAR α moiety of the chimera are responsible for this effect.

At least two mechanisms could explain the Nuclear Bodies fragmentation triggered by PML/RAR α :

i) oligomerization of RAR α mediated by the coiled-coil region of PML leads to alterations of the RA pathway, of which Nuclear Bodies fragmentation could represent a consequence;

ii) specific targeting of RAR α domains to the PML compartment leads to the recruitment, via RAR α -mediated protein-protein interactions, of cofactors responsible for modification(s) able to destabilize the structure of Nuclear Bodies.

To verify the first hypothesis, we tested the effect of the expression of the CC-RAR α fusion protein on the integrity of Nuclear Bodies.

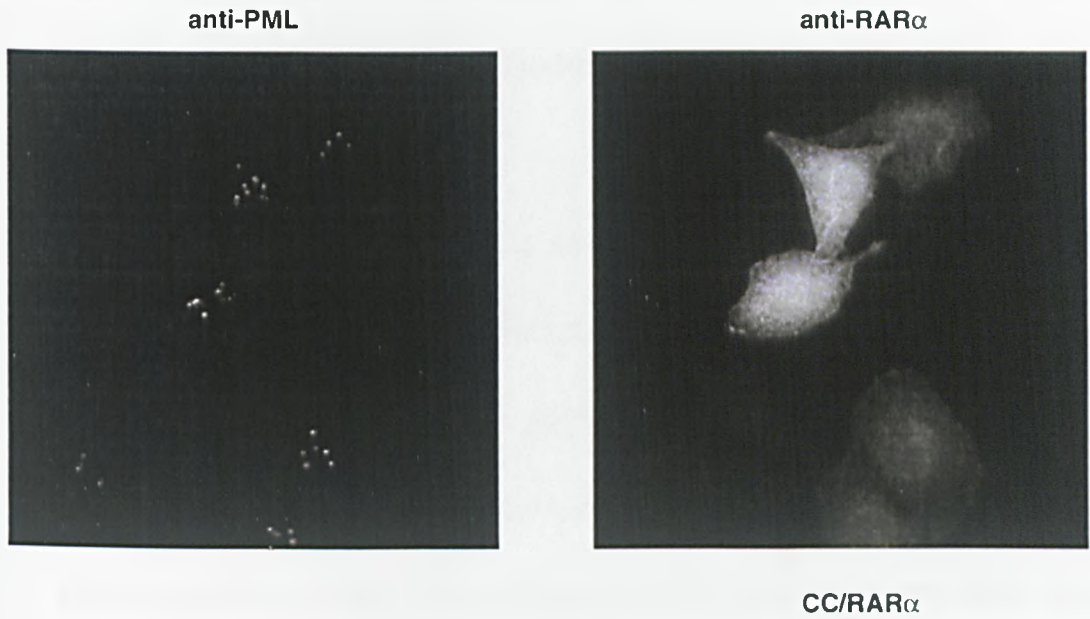


Fig.33: Expression of CC-RAR α does not lead to Nuclear Bodies fragmentation. Indirect Immunofluorescence with α -RAR α and α -PML antibodies of cells expressing CC-RAR α .

CC-RAR α (in which the isolated coiled coil region of PML is fused to RAR α) is able to oligomerize and repress transcription. Notably, CC-RAR α is not able to destroy Nuclear Bodies, (fig. 33) and shows a nuclear diffuse pattern. This suggests that oligomerization of RAR α *per se* is not sufficient to destabilize the Nuclear Bodies, in absence of proper targeting to Nuclear Bodies of regions of PML absent in CC-RAR α .

Experiments were performed to assess the nuclear localization of PML/RAR α AHT. The presence, in the hinge region of RAR α , of triple point mutations makes the AHT mutant not more able to recruit corepressor molecules and repress transcription (Horlein et al., 1995). Oligomerization state and subnuclear distribution of the AHT mutant are similar to that of PMLRAR α (fig34A and B, respectively).

Interestingly, expression of the AHT mutant does not destroy Nuclear Bodies. As in the case of PML-P/R and PMLSer4- expression, they appear intact and enlarged (fig. 35), suggesting that recruitment of HDACs may play a role in the disruption of Nuclear Bodies.

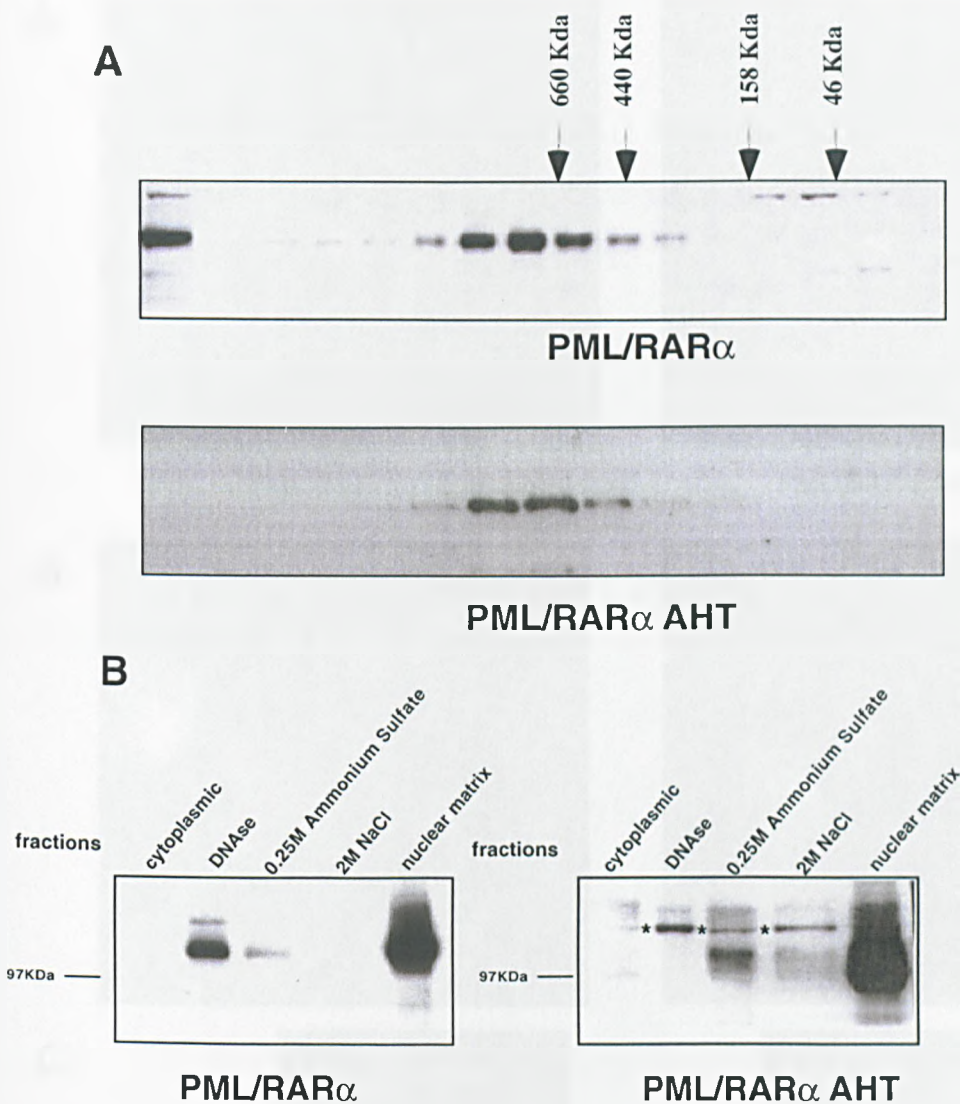


Fig.34. Subnuclear distribution and oligomerization state of PML/RAR α AHT is identical to that of PML/RAR α . (A) SEC analysis of nuclear extract from cells expressing , respectively, PML/RAR α (upper panel) and PML/RAR α AHT (lower panel). (B) Subnuclear distribution of PML/RAR α compared to PML/RAR α AHT. Asterisk indicates a α RAR α specific, immunoreactive band.

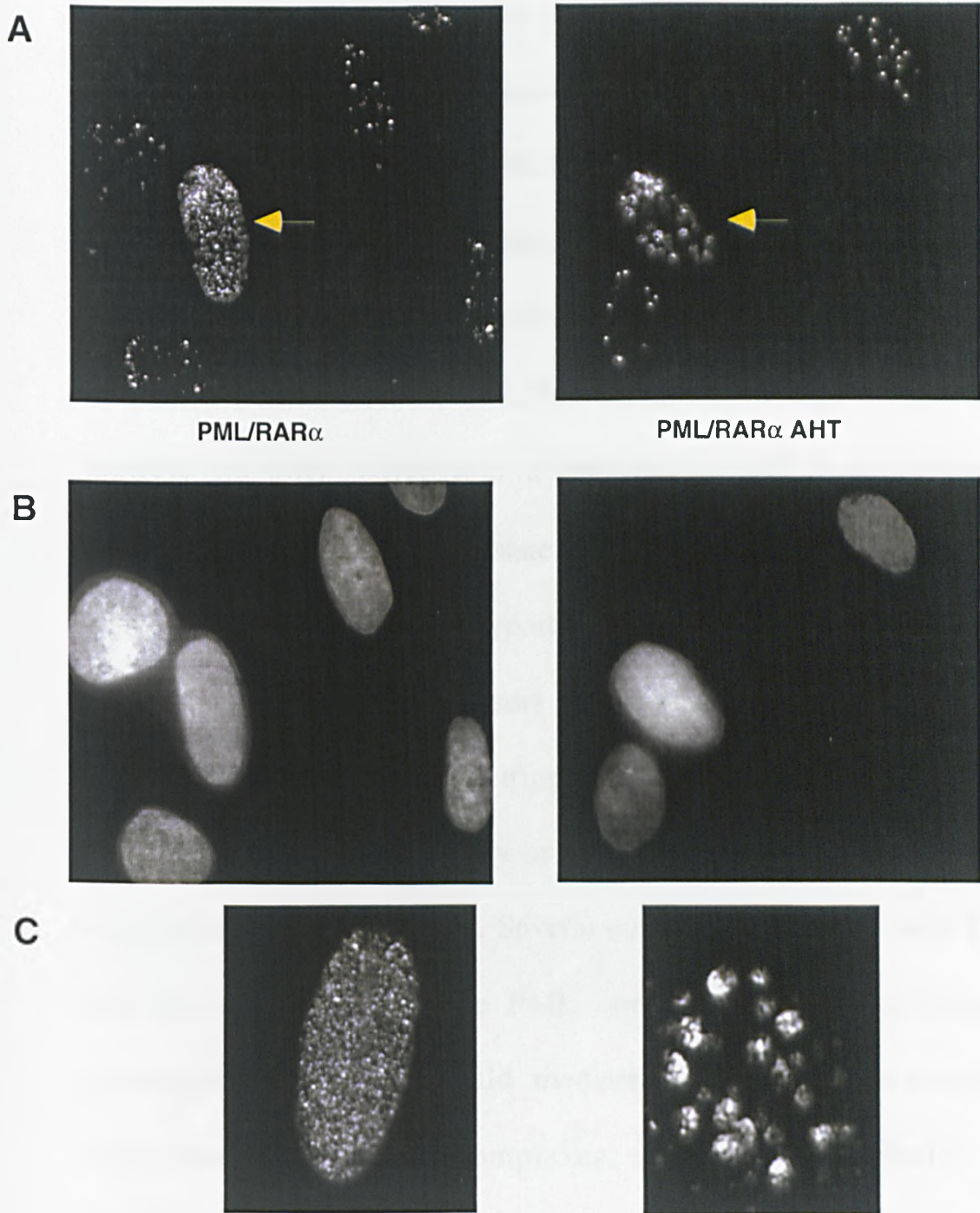


Fig.35: PML/RAR α AHT expression does not cause Nuclear Bodies fragmentation. (A) Indirect immunofluorescence with anti-PML antibodies of HeLa cells microinjected with expression vectors for PML/RAR α (left) and PML/RAR α AHT (right). Arrows indicate microinjected cells (B) DAPI staining of the corresponding cells described in A.(C) Magnified images representative of the state of Nuclear Bodies upon expression of PML/RAR α (left) and PML/RAR α AHT (right).

In order to verify if the fragmentation of Nuclear Bodies could involve changes in the oligomerization state of PML, we performed SEC analysis of nuclear extracts derived from cells cotransfected with both PML3 and PML/RAR α and PML3 and PML/RAR α AHT. Expression of PML/RAR α changed the oligomerization state of the coexpressed PML3, whose elution profile “shifts” from a region of several millions of Daltons, in absence of PML/RAR α expression (fig.36A), to 600-800kDa (fig.36B). Expression of PML/RAR α AHT does not lead to changes in the oligomerization state of PML. (fig.36C).

The last findings raise the hypothesis that aberrant recruitment to the PML compartment of corepressors and associated deacetylase activities mediated by PML/RAR α can trigger fragmentation of the Bodies and that this event is either directly or indirectly related to changes in the oligomerization state of PML. Several corepressors interact with PML and are represented in the PML compartment in physiological conditions. PML/RAR α could mediate an abnormal targeting of additional corepressor complexes, altering dramatically the stoichiometry and representation of those factors into the Nuclear Bodies. Alternatively, factors different from corepressors and able to posttranslationally modify PML or key components of the Nuclear Bodies could be recruited by PML/RAR α . The AHT mutant could be impaired in recruiting these molecules.

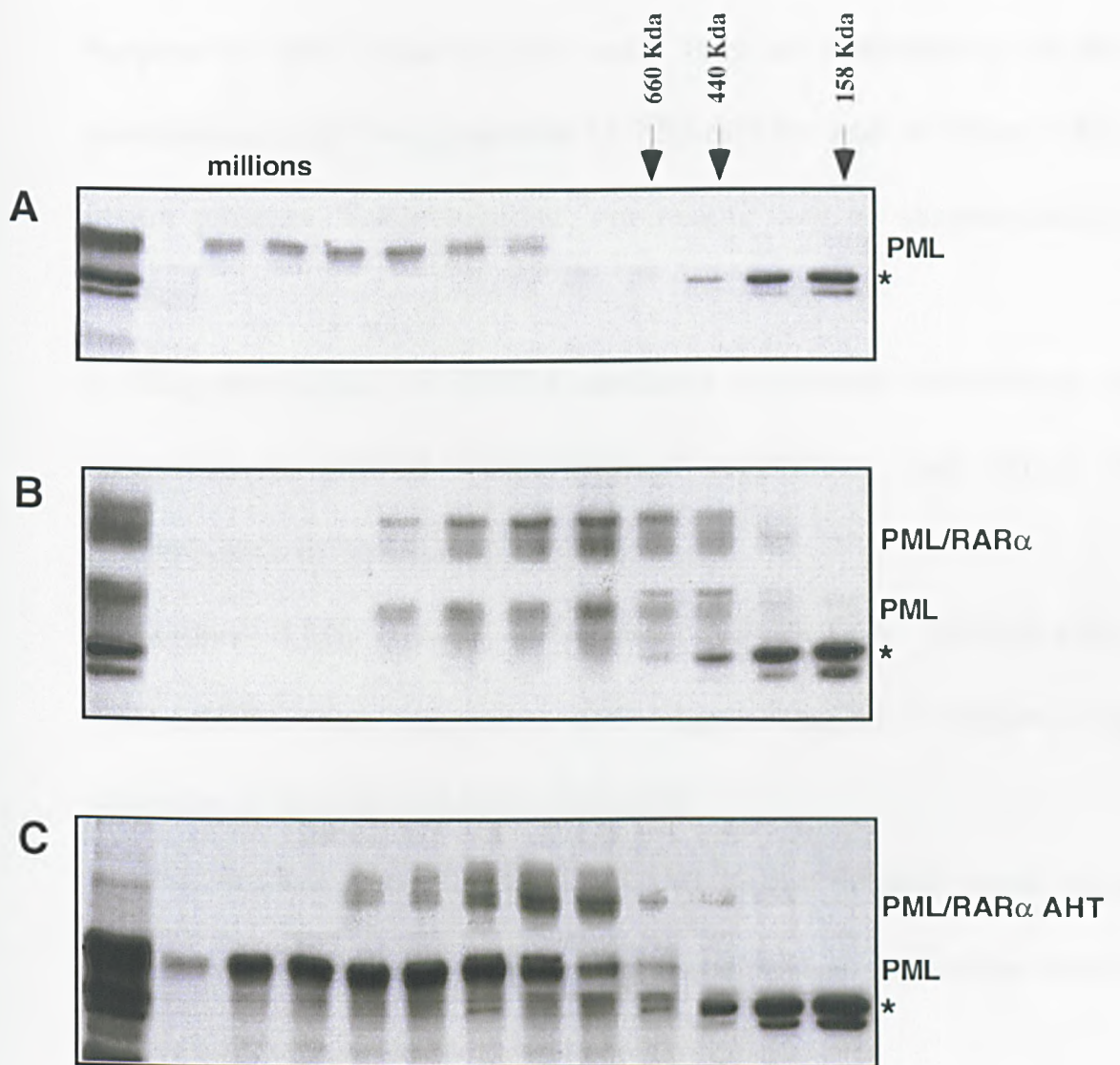


Fig.36: PML/RAR α and PML/RAR α AHT expression exert different effectson the oligomerization state of PML. SEC analysis of nuclear extracts derived from cells transfected, respectively, with PML (A), PML + PML/RAR α (B), PML + PML/RAR α AHT (C). Asterisk indicates an anti-PML immunoreactive, protein.

CONCLUSIONS AND GENERAL CONSIDERATIONS

Purpose of this experimental work was to characterize at the biochemical level the properties of PML/RAR α and of other AML fusion proteins. Taken together, our results may be summarized as follows:

- i) Oligomerization of RAR α mediates increased recruitment of corepressors, altered transcriptional regulation, and block of hematopoietic differentiation;
- ii) Other AML fusion proteins (PLZF/RAR α , NPM/RAR α , AML1/ETO) form oligomers, and oligomerization is required for activation of their leukemogenic potential;
- iii) PML/RAR α , and all of the other AML fusion proteins tested, show an altered subcellular distribution and association to the nuclear matrix with respect to their physiological counterparts;
- iv) In the case of PML/RAR α , this altered distribution is linked to an alteration in oligomerization potential as compared to wild-type PML protein, due both to the loss of a Ser/Pro rich region located in the C-terminus of PML, and to the acquisition of RAR α sequences;
- v) PML/RAR α is able to alter oligomerization of wild-type PML, and this phenomenon correlates with the fragmentation of PML Nuclear Bodies.

These results suggest that unique changes in the biochemical properties of AML fusion proteins may constitute a unifying mechanism linking their altered subcellular localization, and their capacity to block hematopoietic differentiation.

We will discuss the main conclusions that can be driven from these results in the following sections.

Oligomerization of transcription factors in AMLs.

Transcriptional regulation is characterized by a high degree of crosstalk between transcription factors that bind different or overlapping DNA sequences in the context of a promoter. These interactions can positively or negatively regulate the transcriptional rate of target promoters. One mechanism through which modulation can take place is the qualitative and quantitative modification of the availability of cofactors.

Recruitment of chromatin modifying activities (histone deacetylases/ acetylases, topoisomerases, helicases, DNA methyltransferases) to a specific target promoter is a key feature of transcriptional regulation, resulting in localized changes in the chromatin structure that becomes more/less permissive for the binding of basal transcription factors and RNA polymerase II. Localization and subnuclear compartmentalization of transcription factors and cofactors appear thus of fundamental importance in the “economy” of transcriptional regulation.

In the experimental work described here we have performed a biochemical isolation and characterization of PML/RAR α high molecular weight complexes. We provide evidence that the leukemic fusion protein is represented in oligomeric structures *in vivo*. We have established that the ability of the fusion protein to repress transcription through an abnormal recruitment of corepressors and its ability to block differentiation of hematopoietic precursors, both phenomena pathogenetically linked to the development of a leukemic state, strictly correlate with its oligomerization state. Oligomerization of interaction surfaces for DNA/protein creates novel biochemical conditions of increased affinity and avidity for co-factors, altering the physiological balance that governs the transcriptional program of a non-neoplastic cell. During granulocytic differentiation, RAR α acts in two different ways (Kastner et al, 2001): in absence of exogenous retinoic acid, RAR α limits activation of the differentiation program, probably by recruiting corepressors to target promoters; in presence of exogenous retinoic acid, it sensitizes granulocyte precursors to the differentiative stimulus. Physiological levels of retinoic acid trigger release of corepressor complexes and recruitment of coactivators. The oligomeric conformation of RAR α in APL increases significantly the transcriptional repression, rendering the cells sensitive only to pharmacological concentrations of

retinoic acid. In other words, oligomerization is sufficient to activate the oncogenic potential of RAR α .

Several other leukemic fusion proteins form HMW structures: PLZF/RAR α , NPM/RAR α and AML1/ETO. We demonstrate that AML1/ETO HMW structures are composed of oligomers and that this phenomenon is related to the altered transcriptional abilities and capacity to block hematopoietic development of the fusion protein.

Oligomerization of transcription factors takes place also in physiological conditions: examples are p53, STAT5, Groucho, TEL, Sp1 (Chen et al., 1998; Jeffrey et al., 1995; John et al., 1999; Jousset et al., 1997). Notably, a single point mutation that prevents STAT5 tetramerization decreases levels of STAT5-mediated transcriptional activation. For other leukemia-associated fusion proteins, oligomerization constitutes a mechanism of oncogenic activation. In the literature, we found some cases where this mechanism may be in action. Fusion of a truncated leukemia oncogene, MLL, to bacterial β -galactosidase (a tetrameric protein) caused leukemia in mice (Dobson et al., 2000). The TEL oligomerization domain is required for transcriptional repression of TEL-AML1 (Jousset et al., 1997; Uchida et al., 1999). Notably, the portion of AML1 retained in this fusion protein includes a region recently shown to recruit corepressors (Levanon et al., 1998; Uchida et al., 1999). The oligomerization domain of TEL is also found in other

leukemia-associated fusion proteins, where it leads to the constitutive activation of the associated tyrosine kinase (Lacronique et al., 1997), a frequent mechanism of oncogene activation in human tumors. Therefore, oligomerization appears to be a mechanism of oncogene activation for both tyrosine kinases and transcription factors. Interestingly, oligomerization inhibitory peptides are able to revert *in vitro* the transforming phenotype of BCR/ABL, a tyrosine kinase fusion protein found in chronic myelogenous leukemia (Guo et al., 1998). Oligomerization is therefore a putative target for therapy of APL, and of other AMLs (such as those expressing AML1/ETO) shown to involve this mechanism of oncogene activation.

Altered subcellular distribution and association with NM of AML fusion proteins.

We have evaluated the influence of the altered biochemical properties of PML/RAR α on the subcellular localization of the protein: we found that the fusion protein is differently compartmentalized respect to its physiological counterparts. Furthermore, we extended this observation to the distribution of another APL fusion protein, PLZF/RAR α , and of another AML fusion protein, AML1/ETO.

Nuclear topology changes in physiological (cell differentiation, senescence, apoptosis) and pathological conditions (inflammation,

neoplasia). An example is the dramatic changes in nuclear volume/morphology that accompany granulocytic differentiation of hematopoietic precursor cells. Big, round-shaped nuclei are substituted by characteristically poly-lobate structures, typical of mature cells. It is easy to imagine how these changes in nuclear volume and morphology can influence the distribution of transcription factors and cofactors. Several evidences of specific, differentiation-related, changes in the compartmentalization of nuclear factors have been collected: i) during neuronal differentiation, the ETO transcription factor is localized in the nucleus in embryonal neuronal cells, while in mature Purkinje cells it is essentially cytoplasmic (Sacchi et al., 1998) ii) the YY1 transcription factor is tightly nuclear matrix anchored in immature osteoblasts. In mature osteocytes, it lacks the nuclear matrix association and becomes distributed in the soluble nuclear fraction (Bushmeyer and Atchison, 1998). The localization of transcriptional cofactors is also variable during development and upon specific stimuli: EGF and other mitogenic stimuli trigger the cytoplasmic relocation of SMRT, which is predominantly nuclear in untreated cells (Hong and Privalsky, 2000; Hong et al., 1998).

We propose, based on morphological and biochemical criteria, that the chromosomal translocation alters the spatially and temporally regulated “positioning” of the involved fusion proteins, and that rearrangement of

internal critical domains renders the localization/function no longer subjected to physiological modulation. The altered localization of the chimera may further induce, through aberrant recruitment of factors whose localization is tightly controlled during the differentiation programme, important alterations of many cellular events.

Thus, “to not be in the right place at the right time” exerts profound effects in the cell, contributing to the determination of the leukemic phenotype.

Analysis of the determinants of the different subnuclear distribution of PML/RAR α with respect to its physiological counterparts allowed us to identify a COOH term region of PML which is important for the nuclear matrix association of the protein. PML/RAR α lacks the COOH term region of PML. The fusion protein is still partially able to interact with the NM, but this is due exclusively to the presence of a NMTS (nuclear matrix targeting signal), corresponding to or requiring the DNA Binding Domain (DBD) of RAR α . Infact, deletion of the RAR α DBD makes the protein entirely nucleoplasmatic. Lack of the PML COOH term region is therefore functionally substituted, in the context of the fusion protein, by the acquisition of the RAR α DBD. This may explain why the subcellular distribution of PML/RAR α is more similar to wtRAR α than to wtPML.

The Ser/Pro rich region is target of posttranslational modifications: mutations in four serine residues in this region greatly affect the nuclear

matrix targeting of PML, suggesting a direct or indirect involvement of a phosphorylation or dephosphorylation event. The studies performed also provided us the possibility to establish an indirect link between control of oligomerization and nuclear matrix association of the protein, since the described mutations in the COOH term region have negative effects on the PML oligomerization state. As for PML mutants in the serine residues, PML/RAR α lacking the COOH term region of PML shows a reduced oligomerization state and an altered association with the NM compared to wtPML.

Several hypotheses can be formulated to explain relationships between the oligomerization state and the nuclear matrix association of PML. We believe that the model formulated to explain the function of NuMA offers interesting similarities with our own situation.

NuMA is a nuclear protein whose overexpression in Hela cells leads to the formation of regular nuclear networks that fill the nucleus, are stable to detergent extraction, and can be visualized by electron microscopy. In interphase cells NuMA is a component of the nuclear matrix (Luderus et al., 1994). Overexpression of NuMa in Hela cells results in the formation of a “lattice” like structure, stable to detergent extraction, with a quasi-hexagonal organization.(Gueth-Hallonet et al., 1998) The assembly of NuMA into lattices seems to involve at least three steps: (i) dimer formation; (ii) formation of multiarm oligomers; and (iii)

interaction of head and tail domains from different oligomers to form lattices. The first two steps occur by self assembly and require neither post-translational modifications nor additional proteins since they can be achieved starting from recombinant NuMA purified from E.Coli (Harborth et al., 1999). It has been hypothesized that in NuMA there are distinct domains responsible for both the formation of oligomers first, and then of more complex, lattice-like structures. Interestingly, NuMA assembles into the described higher order structures when it is dephosphorylated in a mitotic extract from HeLa cells (Saredi et al., 1997). So, a dephosphorylation event is needed to drive or to accelerate the assembly of the lattice-like complex, supra-oligomeric structures. Like for NuMA, a postranslational event is required also for the regulation of PML oligomerization, and the Ser/Pro rich COOH region appears to be the target of this modification. The coiled-coil region of PML ensures a basal oligomerization state to the molecule: modulation of the “initial” oligomerization state by postranslational modifications (Phosphorylation, acetylation, etc.) involving, at least partially, the Ser/Pro rich region, could promote a “supraoligomerization” of the protein with consequent formation of bigger, more complex structures that are required for the stable association with NM, and possibly (see below) for the correct assembly of Nuclear Bodies.

It would be interesting, in the future, to investigate whether the Ser/Pro

rich region of PML represents a classic nuclear matrix targeting signal, or if it represents a specialized domain, whose function requires a PML-specific context. In particular, ability of the isolated region (alone or in combination with other domains) to target a Gal4 DBD to the interior part of the nucleus and the transcriptional consequences of this phenomenon have to be tested in future experiments.

Interesting avenues of research emerged from the studies performed: the serine residues in the Ser/Pro rich region of PML that we have demonstrated to be important for the NM targeting of PML are target of phosphorylation by Protein kinase Casein Kinase II (CKII). This observation suggests that the oligomerization state of the PML protein can be dynamically modulated by posttranslational modification events. Casein Kinase II is a ubiquitous kinase, involved in the transduction of proliferative signals. In the last years, CKII activity has been demonstrated to be involved in many stress-signaling pathways (Gerber et al., 2000; Gotz et al., 2000; Sayed et al., 2000). It is possible that the kinase could act as a “sensor”, to transduce extracellular signals to Nuclear Bodies. Changes in the oligomerization of PML could thus represent adaptive responses to environmental changes.

Oligomerization potential and nuclear matrix association.

PML mutants impaired in the ability to oligomerize and fully associate with the nuclear matrix reconstitute, in PML $-/-$ MEFs, Nuclear Bodies that appear few in number, enlarged, irregular and not resistant to the NM isolation procedure. There are two not mutually exclusive possibilities: that i) acquisition of a proper oligomerization state is a necessary event for the physiological “nucleation “ of PML associated domains and that ii) the loss of the carboxyterminal region of wtPML by itself affects the stability of the protein-protein interactions required for the assembly of such macromolecular complexes. Preliminary observations from our group indicate that the PML COOH mutants are still able to recruit several known Nuclear Bodies components, but the stoichiometry of interaction appears greatly reduced with respect to the wtPML (data not shown). In the future, we plan to evaluate the biological consequences of the altered properties of the PML COOH mutants, and in particular their capacity to retain the growth suppressor activities of the wt protein and the ability to cooperate with Ras in inducing replicative senescence.

PML/RAR α expression drives, in PML $+/+$ and PML $-/-$ cells, the assembly of NM-associated, PML independent domains. These domains are different from the altered Nuclear Bodies reconstituted by PML COOH mutants: this finding implies that the RAR α portion of the

fusion protein plays an active role in their formation, suggesting that the oligomerization of RAR α domains confers to the fusion protein novel and unique properties, with respect to wtPML and wtRAR α (which shows a nuclear diffuse distribution).

Fragmentation of Nuclear Bodies in APL

The fusion protein exerts negative effect on the oligomerization of wtPML. Not unexpectedly, this phenomenon strictly correlates with the fragmentation of Nuclear Bodies and recruitment of wtPML in the PML/RAR α associated domains. This effect requires oligomerization and targeting to Nuclear Bodies of RAR α . In particular, the integrity of the hinge region of RAR α , which has been shown to be required for the recruitment of corepressor complexes, is required. It is possible to speculate that aberrant recruitment, through the RAR α moiety, of activities that introduce post-translational modifications of PML (or other components of Nuclear Bodies) could promote changes in the oligomerization state of PML and destabilization of such structures.

The presence of PML/RAR α in the nucleus is probably able to superimpose an altered stoichiometry to the multiple wtPML interactions; this event acts synergistically with the perturbations linked to the altered transcription, due to the RAR α oligomerization. PML by itself has been shown to “promote” RA activated transcription of target genes (Wang et

al., 1998). Indeed, it is of note that the alteration of the RA signaling pathway is not sufficient for the onset of APL: transgenic mice of RAR α with an E domain mutation which inhibits endogenous RAR α activity in a dominant negative manner show a normal phenotype, while transgenic mice of both normal PML/RAR α and PML/RAR α with a mutation in the same site of RAR α developed APL (Shao et al., 1997) (Kogan et al., 2000).

In summary, at least a dual mechanism of action characterize the PML/RAR α pro-leukemic activity: on one hand, i) the dramatic subversion of the RA signaling; on the other hand, ii) the alteration of the oligomerization/function of wt PML. The experimental work here described suggests that altered regulation of the oligomerization state of both PML and RAR α is likely to represent an unifying biochemical mechanism impinging on the process of the pathogenesis of APL.

EXPERIMENTAL PROCEDURES

Cell lines and culture conditions.

U937 cells were maintained in RPMI 1640 medium supplemented with 10% foetal calf serum, Penicillin/Streptomycin and Glutamine, in humidified atmosphere at 5% CO₂. NB4 cells were cultured in the same conditions. PML^{+/+} and ^{-/-} MEFs were cultured in DMEM medium supplemented with 10% North American foetal calf serum plus Penicillin/Streptomycin and glutamine, in humidified atmosphere at 9% CO₂. Hela cells were cultured in DMEM medium supplemented with 10% BCS serum, plus Penicillin/Streptomycin and Glutamine, in humidified atmosphere at 5% CO₂. 293T cells were cultured in DMEM medium supplemented with 10% North American FCS plus Penicillin/Streptomycin and Glutamine, in humidified atmosphere at 5% CO₂. All U937 clones used were stable expressing clones. Expression of the desired protein is under the control of metallothionein promoter. Expression of the protein was obtained by treatment of cells for 8-12 hours with 100 μ M ZnSO₄. Due to the higher levels of expression, for the U937 expressing RAR α induction was performed at a ZnSO₄ concentration of 50 μ M. Levels of expression of the proteins of interest have been checked and compared by Western Blot and Indirect immunofluorescence.

Microinjection experiments

Briefly, adherent cells were seeded on 0.1% gelatin coated glass coverslips 24hrs before microinjection. Microinjection was performed with an Axiovert 100 (Zeiss) and cDNA was injected in the nucleus at a concentration of 30-50ng/ul. After 3-5 hours, cells were washed two times in PBS 1X and fixed.

Western Blot and immunoprecipitations.

For Western Blot, the following antibodies were used: goat polyclonal anti HMG(I)Y (Santa Cruz Biotech.); mouse monoclonal antiLaminB1 (Calbiochem). Rabbit polyclonal antibodies against AML1 and against ETO; a rabbit polyclonal anti RAR α ; an hybridoma supernatant against PML (PGM3), a mouse polyclonal antiCC of PML and a rabbit polyclonal antiPLZF antisera were kindly provided by Dr. Barbara Verducci, at European Institute of Oncology. Antisera against RAR α and PML (PGM3) were also used for immunoprecipitation experiments.

SEC analysis

Gel filtration chromatography experiments were performed at 4C, using an FPLC system with two p-500 pumps (Amersham Pharmacia Biotech) equipped with a 24 ml Superose 6 HR 10/30 column,

equilibrated unless otherwise indicated with a running buffer containing 20mM Tris-Cl pH 7.4, 10% glycerole, 0.4 M KCl , 2mM DTT, 10 ugr/ml Aprotinin, 10ugr/ml Leupeptin, 1mM PMSF. Forty 0.6 ml fractions were collected, at a flow rate of 0.2ml/min. 0.5 –1 milligrams of sample were loaded for each run. Apparent size of nuclear complexes was *bona fide* determined by comparison to known gel-filtration grade molecular weight markers.

When indicated, before gel filtration chromatography, Heparin affinity chromatography was performed, using a P-500 pump, with a flow rate of 0.5 ml/min at 4 C. Analytical gel filtration chromatography was performed using a SMART system (Amersham Pharmacia Biotech) equipped with a 24 ml Superose 6 HR 3.2 column. 100–500 nanograms of protein were loaded for each run. 24 fractions of 100ul were collected, at a flow rate of 50ul/min.

Preparation of nuclear extracts.

U937 cells stably expressing the protein of interest were collected by centrifugation at 300g at room temperature, then washed two times with PBS1X() and then incubated on ice with five bed volumes of hypotonic

solution1 (20mM Tris-Cl pH 7.4, 10% glycerole, 2mM DTT, 10 ugr/ml Aprotinin, 10ugr/ml Leupeptin , 1mM PMSF) for ten minutes. Then swollen cells were collected, resuspended in the same solution at a density of 10^7 cells/ml and homogenized in a Dounce homogenizer (type B pestle, Wheaton, USA) with 25-40 strokes, on ice. Breakage of nuclei was assayed under microscope. After homogeneization, nuclei were centrifuged at 900g, at 4 C, for ten minutes. Supernatant, containing mainly cytoplasmic proteins and membranes, was discarded. Nuclei were washed again with sol 1 (plus 0.1% NP-40) and then centrifuged as before. Nuclear pellet was resuspended on ice with ice cold Extraction Buffer (20mM Tris-Cl, pH 8.8, 10% glycerole, 2mM DTT, 10ugr/ml Aprotinin, 10ugr/ml Leupeptin , 1mM PMSF). KCl was added dropwise to a final 0.8M concentration, in three - four times, with gentle agitation. After salt addition, nuclei were extracted for 30' in high salt containing Extraction Buffer. Nuclear suspension was then ultracentrifuged in an Optima Beckman ultracentrifuge, at 50000rpm, at 2C, for 1 hour. After ultracentrifugation, nuclear pellet was discarded and the soluble nuclear extract stored at - 80C or used immediately.

Biochemical purification of PML/RAR α HMW complexes

Nuclear extracts from U937-PML/RAR α cells were diluted to a final 0.2M KCl concentration with Extraction Buffer, then passed two times through a 5ml Heparin-Sepharose column (Amersham Pharmacia Biotech) controlled by a P-500 peristaltic pump (Amersham Pharmacia Biotech). The flow-through fraction, containing, by Western Blot analysis, most of the nuclear PML/RAR α was then concentrated with a Centricon system (Centricon 30, with a molecular weight cut-off of 30 Kda) and then loaded on a 24ml- Superose 6 HR 10/30 gel filtration column (Amersham Pharmacia Biotech), equilibrated with 0.4M KCl containing-running buffer (20mM Tris-Cl pH 7.4, 10% glycerole, 0.4 M KCl, 2mM DTT, 10 μ g/ml Aprotinin, 10 μ g/ml Leupeptin, 1mM PMSF) Both the Heparin affinity chromatography and the gel filtration chromatography were performed at 4C. Forty 0.6ml fractions were collected. The PML/RAR α -containing fractions (fractions 18-22) were diluted with incubation buffer (Hepes 20 mM pH 7.4, EDTA 1 mM, DTT 1 mM, aprotinin and leupeptin 10 μ g/ml, pepstatin 2 μ g/ml, 1 mM PMSF, glycerol 10%, MgCl₂ 3mM, NaF 5 mM, NP40 0.1%) to a final 0.1M KCl and then incubated for 1 hour at 4°C with biotinylated RARE double-stranded oligonucleotide coupled to streptavidine-agarose beads (7 μ g/mg starting material). Beads were washed extensively with incubation buffer (containing 0.1 M KCl). Elution was

performed in buffer containing 1M KCl. As controls, we used either streptavidine-agarose beads alone, or performed the incubation with RARE-containing beads in the presence of a 100 fold excess RARE competitor in solution.

In vivo crosslinking and PML/RAR α complexes isolation

U937-PML/RAR α expressing cells were grown for 1 hour in medium devoid of cysteine and methionine, and then for 8 hours in the presence of ³⁵-S labelled cysteine and methionine mix (Amersham). Before harvesting, cells were incubated for 30 minutes at room temperature in PBS plus 0.1 mM DTBP (Dimethyl 3,3'-dithiobispropionamide-2HCl, Pierce). Nuclei were isolated as previously described, except for the absence of reducing agents in the hypotonic solution 1.

Isolated nuclei were extracted in modified RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 1% Sodium deoxycholate, 0.2% SDS, 2 mM EDTA, 5 mM NaF, aprotinin and leupeptin 10 μ g/ml, pepstatin 2 μ g/ml, 1 mM PMSF, 100 mM Tris-Cl pH 7.4). The extracts were collected and analysed by SEC on a Superose 6 HR 10/30 column (Pharmacia Biotech) previously equilibrated with running buffer (20mM Tris-Cl pH 7.4, 10% glycerole, 0.4 M KCl, 2mM DTT, 10 μ g/ml Aprotinin, 10 μ g/ml Leupeptin, 1mM PMSF). Forty 0.6ml fractions have been collected. High molecular weight fractions containing PML/RAR α

were subsequently immunoprecipitated with an anti-PML monoclonal antibody (PGM3). After extensive washings with the gel-filtration running buffer supplemented with 0.1% NP-40, immunoprecipitates were eluted by incubation of the beads with 1% SDS at room temperature for 10 minutes. An aliquot of the eluate (approximately 1/5 of the material) was loaded on a 2.4ml analytical Superose 6 HR column mounted on a SMART FPLC system (Amersham Pharmacia Biotech). Aliquots of 100ul were collected, denatured in Laemmli buffer and analyzed by SDS-PAGE and autoradiography.

In vivo crosslinking procedures

Asynchronously growing U937 PML/RAR α expressing cells were collected by centrifugation at 300g, at RT for 5 minutes. After removal of medium, cells were resuspended in PBS1X containing 0.1 mM DTBP (Pierce), a thiol-sensitive, bifunctional crosslinker. The DTBP molecule is able to covalently link primary amines of two different proteins. Since the molecule possesses an internal diameter of approximately 11.6 Å, The crosslinker can act as a covalent bridge between two interacting proteins. The presence, as before mentioned, of a disulfide bond in the center of the molecule, makes the crosslinking reversible by reducing agents as DTT or B-mercaptoethanol. In order to reduce as much as possible the aspecific crosslinking of nuclear

proteins, several pilot experiments were devoted to identify the minimum concentration of DTBP effective on PML/RAR α HMWC. We decided to treat the cells with 0.1mM DTBP for 30 minutes at room temperature. SDS-PAGE and colloidal Coomassie staining of 4-15% gels run in not reducing conditions revealed that in these conditions at least macroscopically there is no significant artefactual aggregation/precipitation of proteins, compared to extract derived from untreated cells. Nuclei were isolated as before described, except for the absence of reducing agents in the hypotonic solution 1 used. Isolated nuclei were extracted with modified RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 1% Sodyum deoxycholate, 0.2% SDS, 2 mM EDTA, 5 mM NaF, aprotinin and leupeptin 10 μ g/ml, pepstatin 2 μ g/ml, 1 mM PMSF, 100 mM Tris-Cl pH 7.4). After 20 minutes on ice, the nuclear suspension was ultracentrifuged in an Optima Beckman ultracentrifuge, at 50000rpm, at 2C, for 1 hour. The soluble supernatant was then diluted with dilution buffer to lower the concentration of detergents, and immunoprecipitated with anti PML antibodies. As a control, immunoprecipitation was performed in the same conditions with an, unrelated antibody (anti-HA, BABCO). After extensive washing immunoprecipitated material was eluted with Laemmli buffer and analyzed by SDS-PAGE and Western Blot with polyclonal anti RAR α antibody in both reducing and not reducing conditions. As very high

molecular weight SDS-PAGE markers we used the crosslinked Phosphorylase B (SIGMA), which produce a ladder, evident after Ponceau staining of the nitrocellulose filters, ranging from 80 KDa to 600 KDa.

Pull-down assays and co-immunoprecipitation experiments

GST-NcoR pull-down assays were performed incubating the indicated amounts of GST-NCoR (attached to a constant amount of glutathione-agarose beads, Amersham) in the presence of ³⁵-S labelled, *in vitro* translated proteins. Coimmunoprecipitation experiments were performed as follows : after incubation for 1 hour at 4C , beads were gently collected by centrifugation and washed four times with incubation buffer (Hepes 20 mM pH 7.4, EDTA 1 mM, DTT 1 mM, 100mM NaCl, aprotinin and leupeptin 10 µg/ml, pepstatin 2µg/ml, 1 mM PMSF, glycerol 10%, MgCl₂ 3mM, NaF 5 mM, NP40 0.1%) and eluted by Laemmli buffer for SDS-PAGE analysis and autoradiography.

Characterization of the isolated CC domain of PML.

The GST-CC was purified from BL21 cells, incubated with prescission protease (Pharmacia) to cleave the GST moiety and yield CC, that was subsequently purified by Mono-Q, and then analysed by gel filtration chromatography (Superose 12 column, SMART system, Pharmacia Biotech). *In vitro* cross-linking was performed in

cross-linking buffer (Hepes 20 mM, pH 7.4, NaCl 150 mM, EDTA 1 mM, DTT 1 mM), in the presence of cross-linker (BS³, Pierce at 0.08, 0.4, 2 mM). After incubation, the reactions were quenched with Tris (50 mM, pH 6.8), and then samples were analyzed by SDS-PAGE.

Transactivation assay

Transient transfections of HeLa were performed by calcium phosphate. PML/RAR α (or RAR α) expression vector did not repress transcription from a RARE-less reporter, and constructs carrying the AHT mutation, which abrogates NCoR binding did not repress activation of the RARE-G5-TATA promoter by GAL4-VP16 (not shown). Luciferase activity was measured with a Lucy1 luminometer (Anthos).

In vitro differentiation of murine hematopoietic progenitors

Murine hematopoietic progenitors were purified from the bone marrow of BALB-C mice on the basis of the absence of lineage differentiation markers (lin⁻). Purified cells were pre-stimulated for two days in medium containing IL-3 (20 ng/ml), IL-6 (20 ng/ml) and SCF (100 ng/ml), and then attached to Retronectin (Takara Shuzo)-coated plates. Cells were incubated with the supernatant from Phoenix packaging cells transfected with the indicated retroviral constructs. GFP⁺, infected cells

were sorted by FACS, and seeded in methylcellulose plates supplemented as above, plus G-CSF (60 ng/ml) and GM-CSF (20ng/ml). After 8-10 days, differentiation was measured by FACS analysis. Uninfected cells and cells infected with the control vector behaved identically (not shown).

Subcellular fractionation procedures

U937 cells were harvested by centrifugation, washed twice with PBS1X at room temperature, then resuspended in five bed volumes of sol 1 (20 mM PIPES, pH 6.8, 1 mM EGTA, 1 mM MgCl₂, Aprotinin 10ug/ml, Leupeptin 10ug/ml, Pepstatin 2 ug/ml, PMSF 1 mM, NaF 50 mM) and incubated for five minutes on ice. Triton X-100 was added at a final concentration of 0.5%. Cells were gently resuspended and left on ice for additional 5 minutes. Then cells were centrifuged at 900g, at 4C for 5 minutes, and supernatant (containing cytoplasmic proteins) was collected as fraction 1. After a washing with Sol1 without detergent, nuclear pellets were resuspended in 5 bed volumes of Sol2.(100 mM KCl, 300 mM Sucrose, 10 mM PIPES pH 6.8, 3 mM MgCl₂, 1 mM EGTA, Aprotinin 10ug/ml, Leupeptin 10ug/ml, Pepstatin 2 ug/ml, PMSF 1 mM, NaF 50 mM). RNase-free DNase (Roche) was added at a final concentration of 100ug/ml and the nuclear suspension incubated for 45 minutes at 30 C with gentle agitation. The nuclear suspension was centrifuged at 4C (1500 g, at 4C), the supernatant was collected as

fraction 2. The nuclear pellet was again resuspended with Sol2 . Ammonium Sulfate was added to a final concentration of 0.25M (from a 4M stock solution) and the nuclear suspension left for 10 minutes at room temperature, with gentle agitation. After another round of centrifugation (1500 g, at RT), supernatant was collected (fraction 3) and the nuclear pellet resuspended in Sol2 and left on ice. Immediately NaCl was added at a final concentration of 2M and the suspension incubated for ten minutes on ice. After centrifugation (4000g, at 4C) the supernatant, designated as fraction 4, was collected and the remaining nuclear scaffolds resuspended in Laemmli buffer and boiled.

We are able to collect five fractions, corresponding, respectively, to:

1. Cytoplasmic proteins;
2. nucleoplasmic proteins (released by DNase treatment of intact nuclei), e.g: histone H1 and several transcription factors
3. nucleoplasmic proteins (by Ammonium Sulfate precipitation), e.g: histone H2,3,4 ,HMG proteins and many transcription factors
4. Outer nuclear matrix proteins (by NaCl 2M precipitation)
5. Inner Nuclear matrix proteins .(structural proteins and intermediate filaments)

We have tested the quality of the fractionation by screening the subcellular fractions, by Western Blot technique, for the presence and subcellular distribution of proteins whose localization was known, as

the tubulin for the cytoplasmic fraction, the HMGI(Y) protein for the nucleoplasmic fraction (fr. 3); the lamin B2 for the nuclear matrix fraction(fr. 5). We assessed the presence, in the nucleoplasmic fractions, of histones, by Coomassie staining of 15% polyacrylamide gels of the obtained subnuclear fractions. (fig.22)

LIS (3,5 diiodosalicylic acid lithium salt)- based subnuclear fractionation.

Briefly, cells were collected by centrifugation and resuspended in Sol 1. (20 mM PIPES, pH 6.8, 1 mM EGTA, 1 mM MgCl₂, Aprotinin 10ug/ml, Leupeptin 10ug/ml, Pepstatin 2 ug/ml, PMSF 1 mM, NaF 50 mM). Triton X-100 was added at a final concentration of 0.5 % and cells left on ice for five minutes. In some cases 1% digitonin (Sigma) have been used, with analogous results. Cells were centrifuged as before indicated and the supernatant collected as cytoplasmic fraction After resuspension in DNase containing solution 2 (100 mM KCl, 300 mM Sucrose, 10 mM PIPES pH 6.8, 3 mM MgCl₂, 1 mM EGTA, Aprotinin 10ug/ml, Leupeptin 10ug/ml, Pepstatin 2 ug/ml, PMSF 1 mM, NaF 50 mM) , LIS (3,5 diiodosalicylic acid lithium salt, Sigma) was added at room temperature at a final concentration of 25mM., with gentle agitation. After 20 minutes incubation, supernatant was collected by

centrifugation (4000g, RT) and the nuclear scaffolds resuspended in Laemmli buffer and boiled.

In situ subnuclear fractionation and nuclear matrix isolation

For *in situ* fractionation of adherent and not adherent cells, a slightly modified protocol has been used. For non-adherent cells (U937, NB4) extraction has been performed in tubes instead of on glass coverslips. Nuclear scaffolds collected by centrifugation were finally “attached” on glass coverslips by Cytospin centrifugation, at 800g for 3 minutes, at RT after incubation with primary and secondary antibody.

Adherent cells were seeded on glass coverslips coated with 0.1 % gelatin (Sigma) 18 hours before harvesting. Cells were then washed with PBS1X twice. After washing, cells were incubated with cold solution 1 (20 mM PIPES, pH 6.8, 300mM sucrose, 1 mM EGTA, 1 mM MgCl₂, Aprotinin 10ug/ml, Leupeptin 10ug/ml, Pepstatin 2 ug/ml, PMSF 1 mM, NaF 50 mM) supplemented with 0.5 % Triton X-100 and left at 4C with gentle agitation for ten minutes. Supernatant was removed by gentle aspiration and cells washed once with solution 1 without detergent (washing solution) After removal of the washing solution, solution 2 (100 mM KCl, 300 mM Sucrose, 10 mM PIPES pH 6.8, 3 mM MgCl₂, 1 mM EGTA, Aprotinin 10ug/ml, Leupeptin 10ug/ml, Pepstatin 2 ug/ml, PMSF 1 mM, NaF 50 mM) supplemented

with RNase-free DNase was gently added and coverslips left for 45 minutes at room temperature. After removal of the DNase-containing solution, solution 2 supplemented with 0.25M Ammonium Sulfate was gently added and cells extracted for 10 minutes at RT). After ammonium Sulfate extraction, coverslips were washed twice with cold PBS1X, prior to fixation. Effective removal of chromatin was assessed by the absence of DAPI staining and disappearance of the immunofluorescent signal for chromatin bound proteins (e.g HMG(I) Y proteins).

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