

# PML-Containing Nuclear Bodies: Their Spatial Distribution in Relation to Other Nuclear Components

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**Abstract** The PML protein is a human growth suppressor concentrated in 10 to 20 nuclear bodies per nucleus (PML bodies). Disruption of the PML gene has been shown to be related to acute promyelocytic leukaemia (APL). To obtain information about the function of PML bodies we have investigated the 3D-distribution of PML bodies in the nucleus of T24 cells and compared it with the spatial distribution of a variety of other nuclear components, using fluorescence dual-labeling immunocytochemistry and confocal microscopy. Results show that PML bodies are not enriched in nascent RNA, the splicing component U2-snRNP, or transcription factors (glucocorticoid receptor, TFIIF, and E2F). These results show that PML bodies are not prominent sites of RNA synthesis or RNA splicing. We found that a large fraction of PML bodies (50 to 80%) is closely associated with DNA replication domains during exclusively middle-late S-phase. Furthermore, in most cells that we analysed we found at least one PML body was tightly associated with a coiled body. In the APL cell line NB4, the PML gene is fused with the RAR $\alpha$  gene due to a chromosomal rearrangement. PML bodies have disappeared and the PML antigen, i.e., PML and the PML-RAR fusion protein, is dispersed in a punctated pattern throughout the nucleoplasm. We showed that in NB4 cells the sites that are rich in PML antigen significantly colocalize with sites at which nascent RNA accumulates. This suggests that, in contrast to non-APL cells, in NB4 cells the PML antigen is associated with sites of transcription. The implications of these findings for the function of PML bodies are consistent with the idea that PML bodies are associated with specific genomic loci. © 1996 Wiley-Liss, Inc.

**Key words:** nuclear bodies, PML, confocal microscopy, image restoration, RNA

The PML protein is closely related to acute promyelocytic leukaemia (APL) [reviewed by Grignani et al., 1994; Warrel et al., 1993]. This disease is characterized by a t(15;17) chromosome translocation, which fuses the gene for PML to that for the alpha-retinoic acid receptor (RAR $\alpha$ ) [de Thé et al., 1991; Goddard et al., 1991; Kakizuka et al., 1991; Kastner et al., 1992]. The hematopoietic stem cells that express the fusion gene do not differentiate beyond the promyelocytic stage, resulting in promyelo-

cytic leukaemia. Evidence has been presented that PML is a growth suppressor [Ahn et al., 1995; Koken et al., 1995; Mu et al., 1994] and a suppressor of cell transformation [Liu et al., 1995].

PML is a RING-finger motif protein [Borden et al., 1995; Goddard et al., 1991; Reddy et al., 1992] concentrated in a specific type of nuclear bodies, i.e., PML bodies, containing various other antigens of unknown function [Ascoli and Maul, 1991; Fusconi et al., 1991; Szostecki et al., 1987]. Several viral proteins are targeted to these structures; examples are the adenovirus oncoprotein E1A [Carvalho et al., 1995; Puvion-Dutilleul et al., 1995], the immediate early gene 1 product (ICP0) of herpes simplex virus [Maul et al., 1993], the IE1 protein of cytomegalocytic virus [Kelly et al., 1995], and the SV40 large T antigen [Carvalho et al., 1995]. Association with such viral proteins causes a dramatic redistribution of PML body proteins, including PML itself.

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Similarly, fusion of PML protein with the transcription factor RAR $\alpha$  results in fragmentation of PML bodies and dispersion of the antigen throughout the nucleoplasm [Daniel et al., 1993; Dyck et al., 1994; Koken et al., 1994; Weis et al., 1994]. Interestingly, treatment of such cells with retinoic acid causes reformation of PML bodies and remission of the leukaemia [Dyck et al., 1994; Koken et al., 1994; Weis et al., 1994]. Possibly, disruption of PML bodies by sequestration of the PML protein in other protein complexes results in a loss of the growth suppressing properties of the protein. These observations show that PML bodies have important, though still poorly understood functions in the cell nucleus.

PML bodies belong to a morphologically defined class of nuclear structures: the nuclear bodies [for reviews see Bouteille et al., 1974; Brasch and Ochs, 1992]. These are electron-dense, often spherical structures in nuclei [Bouteille et al., 1974; de Thé et al., 1960]. PML bodies have a diameter of 0.3 to 1.0  $\mu\text{m}$  and are tightly associated to the nuclear matrix [Stuurman et al., 1992]. Typically, a mammalian nucleus contains 10 to 20 PML bodies [Stuurman et al., 1992]. This is true for human tissue as well as all cell lines tested with one exception, i.e., the promyelocytic cell line NB4 [Dyck et al., 1994; Koken et al., 1994; Stuurman et al., 1992; Weis et al., 1994]. PML bodies are also recognized by several other monoclonal and polyclonal antibodies [Ascoli and Maul, 1991; Fusconi et al., 1991; Szostecki et al., 1987].

To obtain information about the function of PML bodies we compared the 3D-distribution of a variety of nuclear components and functions with that of PML bodies in a bladder carcinoma cell line (T24), using double immunolabeling in combination with confocal microscopy. We did similar experiments in the promyelocytic leukaemia cell line NB4 in which the PML antigen, i.e., the PML protein and the PML-RAR $\alpha$  fusion product, is dispersed in the nucleoplasm, instead of being concentrated in PML bodies. Our results show that PML bodies are major sites neither of transcription nor of splicing. Strikingly, PML bodies are found preferentially associated with middle-late replication domains in S-phase nuclei, suggesting association with specific genomic loci. In contrast, in promyelocytic cells the spatial distribution of the PML antigen partially coincides with that of nascent pre-mRNA. This suggests that the dispersed PML

protein and/or the PML-RAR $\alpha$  fusion protein in these mutant cells is involved in regulation of transcription.

## MATERIALS AND METHODS

### Cell Cultures

T24 (human bladder carcinoma, ATCC HB4) cells were grown at 37°C in a 10% CO<sub>2</sub> atmosphere in DMEM (Gibco, Breda, The Netherlands), supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) (Boehringer, Almere, The Netherlands), 2 mM L-glutamine (Gibco), 100 IU/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin (Gibco). NB4 cells [Lanotte et al., 1991] were grown at 37°C under a 5% atmosphere in RPMI (Gibco), supplemented with 20% heat-inactivated FCS and the additions indicated above.

### Immunocytochemistry

Cells were grown on coverslips for 48 h and fixed in 2% formaldehyde in PBS (140 mM NaCl, 2.7 mM KCl, 6.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4) for 15 min at room temperature. Subsequently, coverslips were incubated as follows: 2  $\times$  5 min in PBS, 5 min in PBS containing 0.5% Triton X-100, 2  $\times$  5 min in PBS, 10 min in PBS containing 100 mM glycine, 10 min in PBS containing 10% BSA (Sigma, Bornem, Belgium), 5 min in PBG (PBS containing 0.5% [w/v] BSA and 0.1% [w/v] gelatine from cold water fish skin [Sigma]). Incubations with the primary antibody were done in PBG, overnight at 4°C. The coverslips were subsequently washed 4  $\times$  5 min in PBG and incubated with the corresponding secondary antibodies for 1.5 h in PBG at room temperature.

The following combinations of primary and secondary antibodies were used. To label PML bodies and PML-RAR $\alpha$ -containing domains, we used the mouse monoclonal antibody 5E10 (1/3 dilution), which recognizes both PML and the PML-RAR $\alpha$  fusion protein [Koken et al., 1994]. For PML bodies we also used the human autoimmune serum SUN3 (1/500 dilution), which recognizes Sp100, another protein located in PML bodies [Stuurman et al., 1992; Szostecki et al., 1987]. Rabbit serum against p80coilin (1/10,000 dilution, kindly provided by Dr. A.I. Lamond) was used to visualize coiled bodies. U2-snRNP, a component of the splicing machinery, was labeled with the mouse monoclonal antibody 4G3 ([Habets et al., 1989] 1/5 dilution). BrdU incor-

porated into newly synthesized DNA and BrU incorporated into nascent RNA were detected with a rat monoclonal antibody against BrdU (Seralab, Crawley Down, UK; 1/500 dilution) [Wansink et al., 1993, 1994]. The glucocorticoid receptor was labeled with the rabbit polyclonal antibody pab57 ([Cidlowski et al., 1990]; 1/500 dilution). For all mouse monoclonal antibodies a donkey anti-mouse antibody, conjugated with TRITC or DTAF (Jackson, West Grove, PA), was used as a secondary antibody. The secondary antibody for human autoimmune antibodies was a goat anti-human antibody, conjugated with TRITC (Nordic Immunochemicals), while for the rabbit antibodies a donkey anti-rabbit antibody, conjugated with FITC or TRITC (Jackson, West Grove, PA), was used. Finally, the secondary antibody for the rat anti-BrdU/BrU monoclonal antibody was a biotin-conjugated donkey anti-rat antibody (Jackson), followed by streptavidin, conjugated to FITC (Jackson) for transcription or a FITC-conjugated donkey anti-rat antibody (Amersham, Little Chalfont, UK) for replication. In all experiments DNA was stained with 0.4  $\mu\text{g}/\text{ml}$  Hoechst 33258 (Sigma) in PBS. Coverslips were mounted in PBS pH 8, containing 90% glycerol and 1 mg/ml p-phenylenediamine (Sigma).

#### **BrUTP Labeling of Nascent RNA in Permeabilized Cells**

This procedure has been described in detail by Wansink et al. [1993, 1994]. Briefly, cells are detergent permeabilized and incubated for 30 min in a medium that supports run-on transcription. In this medium UTP was replaced by BrUTP. Nascent RNA (mainly hnRNA synthesized by RNA polymerase II) that had incorporated BrU, was visualized by indirect immunofluorescent labeling, using a first antibody that specifically recognizes BrUTP and BrdUTP. Controls in which (1) BrUTP was replaced by UTP, (2) one of the NTPs was omitted, or (3) that were treated with RNase-A after the run-on experiment, showed only weak background labeling, demonstrating that the labeling procedure only visualizes RNA.

#### **In Vivo Labeling of Replication-Sites With BrdU**

T24 cells were grown on coverslips. To label nascent DNA in vivo, BrdU (Sigma) was added to the culture medium during 10 min at a final concentration of 10  $\mu\text{M}$ . Coverslips were washed twice with culture medium for 1 min at 37°C and the cells were fixed and permeabilized as described above. Prior to immunolabeling, DNA

was denatured in 2N HCl at 37°C for 30 min after which the coverslips were washed twice with PBS.

#### **Confocal Laser Scanning Microscopy**

Images were collected with a Leica confocal laser scanning microscope, equipped with a 488/514 dual-band argon-ion laser and an oil-immersion objective ( $\times 100$ , N.A. = 1.32). Fluorescence was detected using a 525DF10 bandpass filter for FITC or DTAF and a 550 nm long pass filter for TRITC. Pairs of images were collected simultaneously in the green and red channels. Images were collected as  $512 \times 512 \times 32$  voxel images (sampling distance 49 nm lateral and 208 nm axial).

#### **Image Processing and Image Analysis**

For image processing and analysis we used the software package SCIL-IMAGE developed at the University of Amsterdam [Ten Kate et al., 1990; Van Balen et al., 1994]. Optical crosstalk was corrected for as described previously [Manders et al., 1992]. To enhance the effective resolution of the confocal images, we applied a novel image restoration technique, which removes diffraction-induced distortions in the image. Briefly, the point-spread function (PSF) of the microscope was determined using images of 0.23  $\mu\text{m}$  fluorescent (FITC) latex beads, using identical optical conditions as used for imaging of cell nuclei. This experimentally obtained PSF was used to correct images of cell nuclei by an iterative constrained Tikhonov-Miller deconvolution algorithm [Van der Voort and Strasters, 1995].

To quantitatively analyse the relationship between the spatial distributions of two complex nuclear 3D-distributions we used a cross-correlation method, recently developed by Van Steensel et al. [Van Steensel et al., 1996]. Briefly, in this method the two 3D-images are shifted with respect to each other over a distance of  $\Delta n$  voxels along the x or y axis. For each shift ( $\Delta n = 1, 2, 3$ , etc.) the overlap is calculated, expressed as Pearson's coefficient  $r_p$  [Gonzalez and Wintz, 1987]. When  $r_p$  is plotted vs.  $\Delta n$ , a correlation function (CCF) is generated. As demonstrated elsewhere [Van Steensel et al., 1996],  $r_p$  is independent of  $\Delta n$  if the two distributions are unrelated. In that case any overlap between the two distributions is not more and not less than what is expected for two unrelated distribu-

tions. In contrast, if  $r_p$  shows a maximum at  $\Delta n = 0$  the overlap between the distributions is more than expected for unrelated distributions. Likewise, if the CCF shows a minimum around  $\Delta n = 0$ , the two distributions (partially) exclude each other.

## RESULTS

To obtain insight into the function of PML bodies we used immunofluorescence microscopy and compared their spatial distribution in interphase nuclei of the human bladder carcinoma cell line T24 with the distribution of several other nuclear components: (1) nascent RNA, (2) transcription factors (glucocorticoid receptor, TFIID, E2F), (3) the splicing component U2-snRNP, (4) coiled bodies, and (5) nascent DNA at different stages in S-phase. Furthermore, we have analysed the distribution of the PML antigen in the APL cell line NB4 in relation to that of glucocorticoid receptors and nascent RNA. The 3D-distribution of the immunofluorescent labeling patterns in the nucleus were analysed by confocal laser scanning microscopy (CLSM), using a novel image restoration technique that considerably improved the effective resolution of the images [Van der Voort and Strasters, 1995].

### PML Bodies Do Not Contain Measurable Amounts of Nascent RNA

We investigated whether PML bodies are involved in transcription, by analysing whether they contain nascent RNA. To this end, we labeled nascent RNA *in situ* in permeabilized cells in the presence of BrUTP, followed by fluorescent labeling using an antibody that recognized incorporated BrU [Wansink et al., 1993]. Nascent RNA was found in numerous small domains scattered throughout the nucleoplasm and is excluded by the nucleoli (Fig. 1A) as has been shown earlier [Jackson et al., 1993; Wansink et al., 1993]. Careful visual inspection of the 3D-images showed that nascent RNA was almost never found in PML bodies. Therefore, it is unlikely that PML bodies are major sites of transcription.

### Transcription Factors Are Not Enriched in PML Bodies

Since the PML protein has transcription factor-like features [Borden et al., 1995; Goddard et al., 1991; Reddy et al., 1992], we investigated

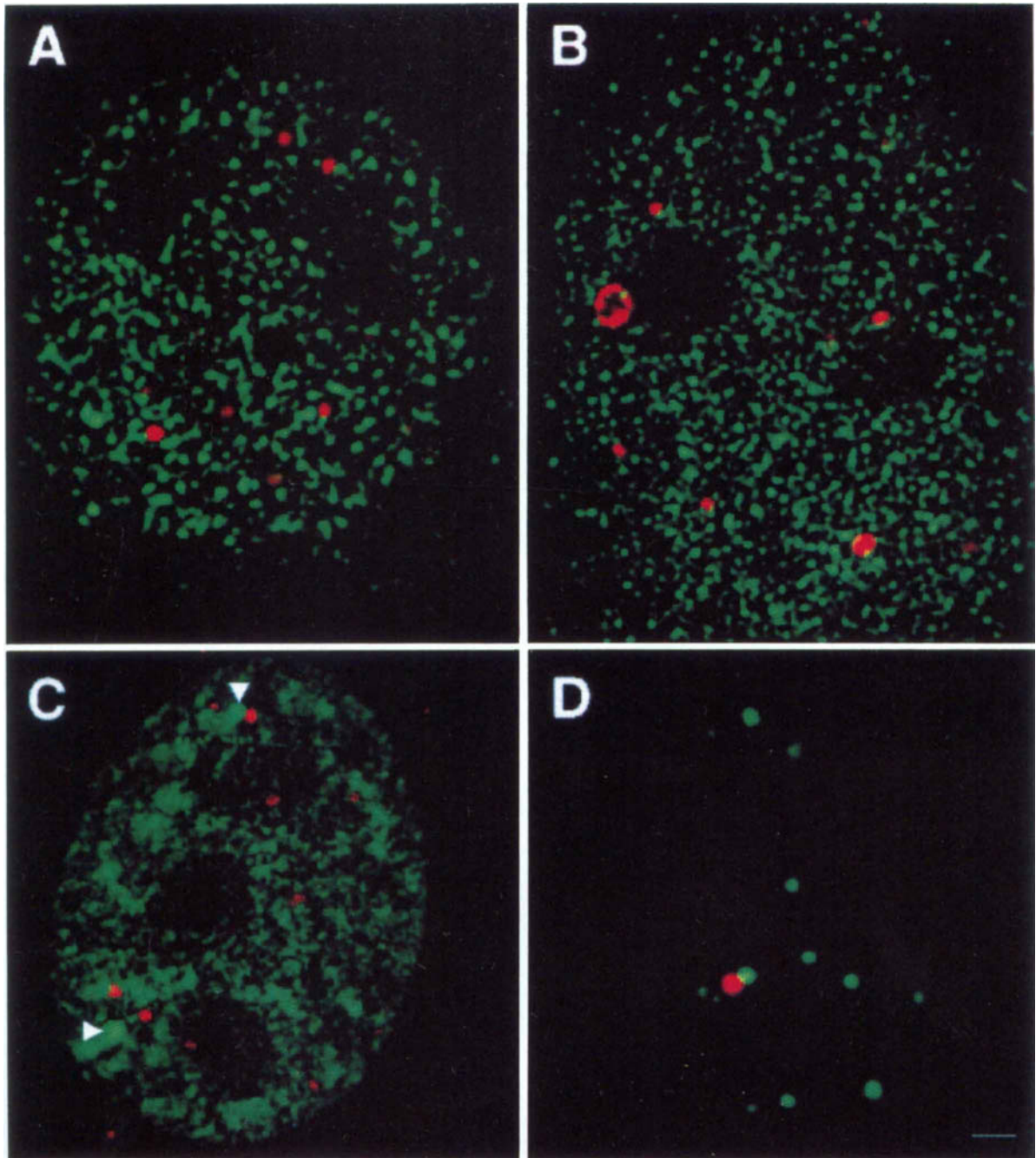
whether PML bodies are enriched in one of several transcription factors. Figure 1B compares the nuclear distribution of glucocorticoid receptors (GR) and PML bodies. To ensure nuclear localization of the GR, the cells were cultured in medium containing dexamethasone (0.1  $\mu$ M) for 1 h. GRs are found in numerous domains scattered throughout the nucleoplasm and are excluded by nucleoli (Fig. 1B) as shown earlier [Van Steensel et al., 1995ab]. Although now and then GR-antigen was found inside a PML body, no systematic relationship between the two distributions was observed. We have also done double labeling experiments with anti-PML and antibodies against the general transcription factors TFIID and E2F (data not shown). Their distributions in the nucleus were similar to that of the GR. Like the GR, the spatial distribution of these two transcription factors was not related to that of PML bodies in any recognizable way.

### PML Bodies Do Not Contain the Splicing Components U2-snRNP

To investigate a possible role of PML bodies in splicing we compared the spatial distribution of PML bodies with that of the splicing component U2-snRNP. Figure 1C shows double labeling with anti-U2-snRNP and anti-PML. In the U2-snRNP labeling pattern three different types of nuclear domains can be distinguished that are enriched in this splicing component. First, brightly-labeled spherical foci are observed that correspond to coiled bodies (arrowheads in Fig. 1C) [Carmofonseca et al., 1991; Raška et al., 1990]. Second, strongly labeled speckled nuclear domains are present that correspond mostly to clusters of interchromatin granules [Fakan et al., 1984; Spector et al., 1991]. Finally, a less intense, reticular labeling pattern throughout the nucleoplasm is observed [Spector et al., 1991], which at least in part corresponds to perichromatin fibrils [Fakan, 1994]. PML bodies were always found in nuclear areas that are devoid of U2-snRNP labeling. This result is in agreement with that of Stuurman et al. [Stuurman et al., 1992], who showed that another splicing component (U1-snRNP) is also absent from PML bodies. Therefore, PML bodies are not major sites of RNA splicing.

### PML Bodies and Coiled Bodies Are Paired

From the data in the previous section it is already clear that PML bodies and coiled bodies



**Fig. 1.** Comparison of the spatial distribution of the PML antigen in T24 cells with the distribution of nascent RNA. (A), the glucocorticoid receptor (B), the splicing factor U2-snRNP (C), and coiled bodies (D). All images represent a single optical section through about the centre of the cell nucleus. A: RNA was labeled during 30 min by incorporation of BrUTP in permeabilized T24 cells in a run-on transcription assay, followed by immunodetection using an anti-BrdU antibody (green image). PML bodies were visualized with mAb 5E10 (red image). No correlation is observed between the two distributions. B: Dexamethasone-stimulated (100 nM, 1 h) T24 cells were double labeled with an antiserum against the glucocorticoid receptor

(Ab 57; green image) and with mAb 5E10, that labels PML bodies (red image). No correlation is observed between the two distributions. C: T24 cells were labeled with a monoclonal antibody against U2-snRNP (mAb 4G3; green image) and a human autoimmune serum recognizing PML bodies (SUN3; red image). No correlation is observed between the two distributions. D: T24 cells were labeled with an anti-p80coilin antibody (rabbit anti-p80; red image) and with mAb 5E10, recognizing PML (green image). Note that one coiled body is located next to one PML body, an observation made in most cells that we analysed. Bar = 1  $\mu$ m.

are different nuclear structures (Fig. 1C). From our labeling studies with anti-U2-snRNP we obtained the impression that a coiled body was sometimes adjacent to a PML body. To investigate a possible spatial relationship between coiled bodies and PML bodies more closely, we labeled coiled bodies with an antibody against p80coilin, while PML bodies were labeled with the monoclonal antibody 5E10. As expected, coiled bodies and PML bodies are clearly distinct structures (Fig. 1D). T24 cells contain one to four coiled bodies per nucleus. In each of the 15 nuclei that were investigated one coiled body was always found adjacent to a PML body.

We estimated the probability  $p$  that a randomly positioned coiled body is paired with a randomly localized PML body to be about 1 event in 25 nuclei. The probability  $p$  is calculated with  $p = (4/3)\pi(d)^3 \times n \times m/v$ , where  $d$  is the distance between the centres of a paired PML/coiled body structure,  $n$  is the average number of PML bodies per nucleus,  $m$  is the average number of coiled bodies per nucleus, and  $v$  is the volume of a nucleus in  $\mu\text{m}^3$ . If  $d = 0.5 \mu\text{m}$  (coiled bodies and PML bodies each have an average diameter of about  $0.5 \mu\text{m}$ ),  $n = 20$ ,  $m = 2$ , and  $v = 500 \mu\text{m}^3$ ,  $p$  will be  $1/25$ . Therefore, the observed pairing of at least one coiled body with a PML body in each of the 15 analysed nuclei is highly significant. Although the function of neither coiled bodies nor PML bodies is understood, these observations suggest a functional relationship between these two types of nuclear bodies.

#### **PML Bodies Are Often Found Adjacent to Replication Domains in Middle-Late S-Phase**

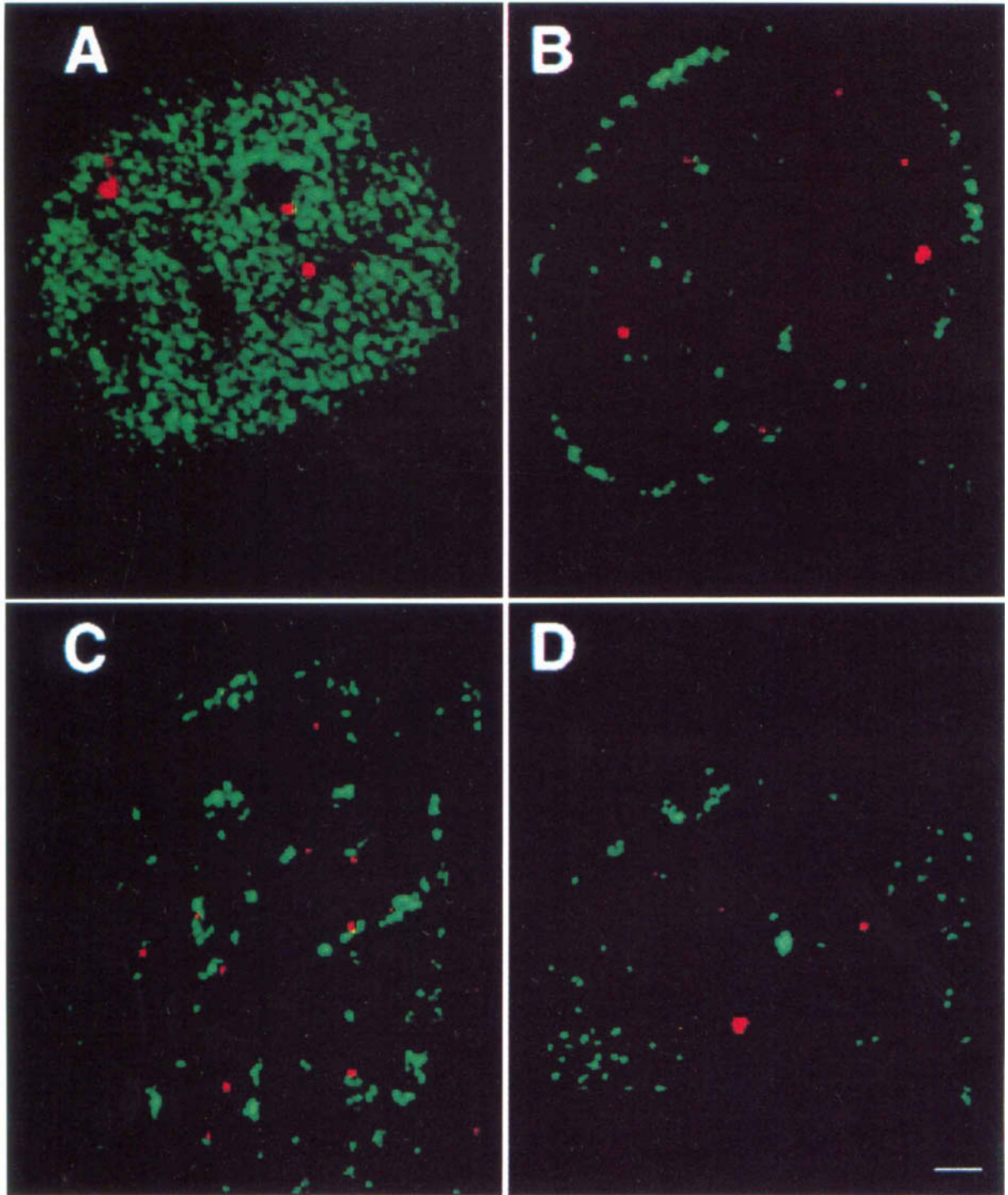
We have analysed whether a spatial relationship exists between PML bodies and DNA replication domains. To this end nascent DNA was visualized by incorporating the thymidine-analogue BrdU into nascent DNA in S-phase cells *in vivo*, followed by fluorescent labeling of incorporated BrdU using an antibody that specifically recognizes this nucleotide. Nascent DNA is found in numerous domains throughout the nucleus, showing a distinct spatial distribution that changes as S-phase progresses [Manders et al., 1992; Nakamura et al., 1986; Nakayasu and Berezney, 1989; OKeefe et al., 1992]. We distinguished four typical replication patterns, i.e., early, middle, middle-late, and late S-phase, respectively. Examples of such patterns are shown in Figure 2. Early S-phase is characterized by

numerous small domains of nascent DNA (Fig. 2A), which are scattered throughout the nucleus. Typical of middle S-phase (Fig. 2B) are the replication sites that are found mainly at the nuclear periphery and around nucleoli. In mid-late S-phase, replication sites form chain-like structures (Fig. 2C), whereas late S-phase is characterized by a few large replication domains (Fig. 2D).

During the entire replication process PML bodies are excluded from nuclear domains that contain newly synthesized DNA (Fig. 2). Strikingly, in middle-late S-phase PML bodies are often found next to a DNA replication domain (Fig. 2C). Such a relationship was not observed in other stages of S-phase. In T24 cells synchronized through mitotic shake-off, we analysed this relation more precisely. In each middle-late S-phase nuclei that we analysed ( $n = 10$ ), 50 to 80% of the PML bodies were found directly adjacent to a replication domain. Evidently, throughout S-phase PML bodies never contain detectable amounts of nascent DNA. Except for middle-late S-phase, no perceivable relationship between PML bodies and replication domains exists. In middle-late S-phase a significant fraction of the PML bodies is found directly next to a replication domain.

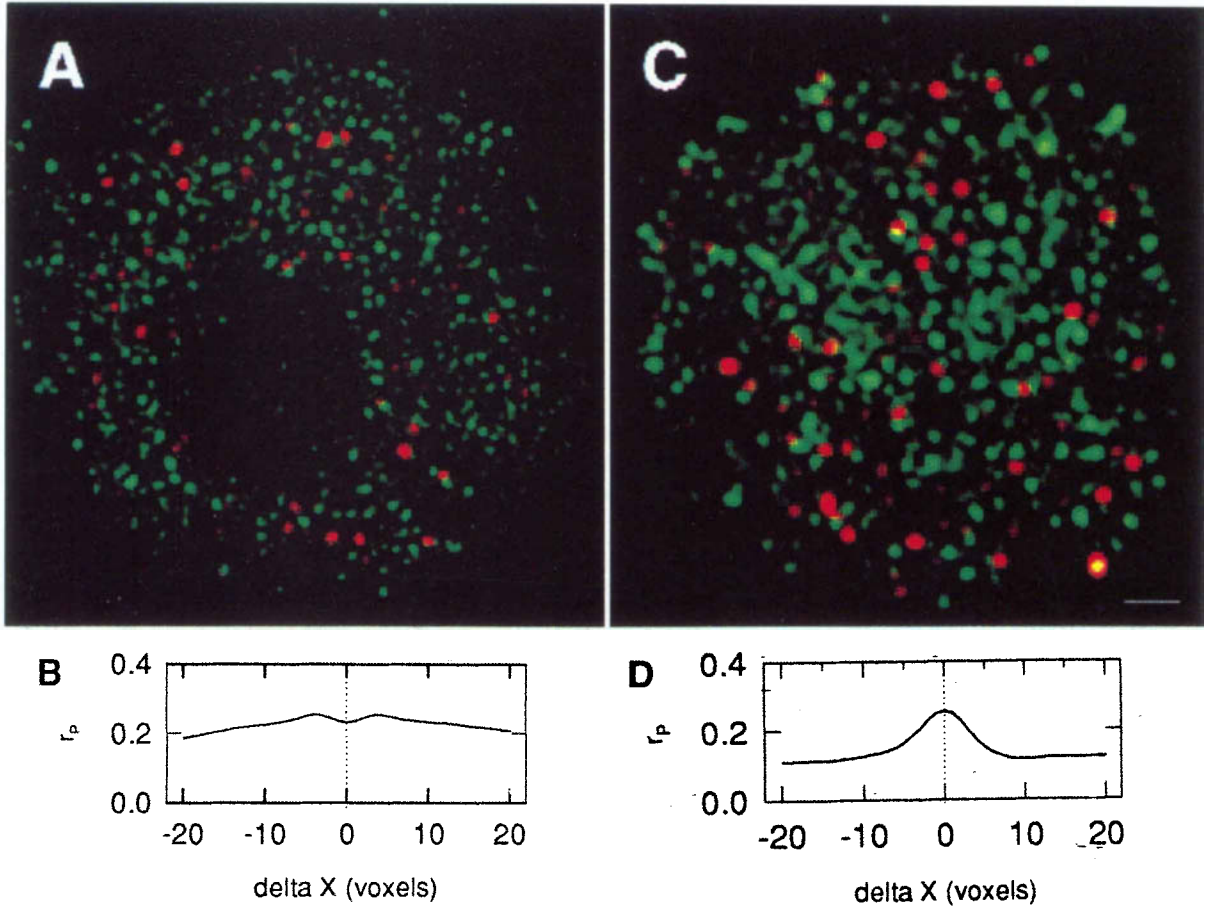
#### **In the Promyelocytic Leukaemic Cell Line NB4, the PML Antigen Does Not Colocalize Significantly With the Glucocorticoid Receptor**

In the acute promyelocytic leukaemic (APL) cell line NB4 the PML gene is fused to the gene of the  $\alpha$ -retinoic acid receptor (RAR $\alpha$ ). Expression of the PML-RAR $\alpha$  fusion protein causes disruption of PML bodies and dispersion of the PML antigen into a much larger number of small nuclear domains that are scattered throughout the nucleoplasm [Dyck et al., 1994; Koken et al., 1994; Weis et al., 1994]. The PML-RAR $\alpha$  protein has been reported to act as a transcription factor [Doucas et al., 1993; Kakiyama et al., 1991; Rousselot et al., 1994]. Therefore, we investigated whether another transcription factor, the glucocorticoid receptor, is present in the same nuclear domains as PML-RAR $\alpha$ . In NB4 cells, stimulated with  $0.1 \mu\text{M}$  dexamethasone during 1 h, a polyclonal antibody against the GR detects a large number of GR-containing nuclear domains throughout the nucleoplasm (Fig. 3A), very similar to what is observed in Figure 1B for T24 cells. We employed a newly developed cross correlation analysis [Van



**Fig. 2.** Comparison of the distribution of PML bodies in nuclei of T24 cells with that of sites of replication at different stages of S-phase. Replication sites were labeled by in vivo incorporation of BrdU for 10 min. Incorporated BrdU was visualized by fluorescent labeling with an anti-BrdU antibody (green image). PML bodies were labeled with mAb 5E10 (red image). Four phases in the replication process are shown: (A) early S-phase,

(B) middle S-phase, (C) middle-late S-phase, and (D) late S-phase. No correlation between the spatial distribution of replication sites and that of PML bodies is observed in A, whereas PML bodies and replication sites seem to exclude each other in B and D. In C a significant fraction of the PML bodies is located next to replication domains. Bar = 1  $\mu$ m.



**Fig. 3.** Comparison of the spatial distribution of the PML antigen in the APL cell line NB4. **A:** NB4 cells were dexamethasone stimulated as for Figure 1B and double labeled with an antiserum against the glucocorticoid receptor (Ab 57; green image) and with mAb 5E10, that recognizes the PML antigen (red image). No significant correlation is observed between the two distributions as could be demonstrated by the cross corre-

lation analysis shown in **B**. **C:** In the APL cell line NB4 nascent RNA was labeled as described for Figure 1A (green image). The PML antigen was visualized with mAb 5E10 (red image). Cross correlation analysis of the two distributions shows a significant, positive relationship (i.e., overlap) between the two distributions, reflected by a distinct maximum in the cross correlation function shown in **D**. Bar = 1  $\mu\text{m}$ .

Steensel et al., 1996] to investigate whether any relationship between these two complex 3D-distributions can be detected. Figure 3B shows the cross correlation function (CCF) that is obtained by comparing the 3D-distribution of the PML antigen and that of the glucocorticoid receptor. The degree of overlap between the two distributions (quantitatively expressed as the overlap coefficient  $r_p$ ) is essentially independent of the displacement along the x-axis (shift expressed in voxels  $\Delta x$ ). This indicates that the nuclear distributions of the glucocorticoid receptors and of the PML antigen show an overlap that is not more or less than is expected for two completely unrelated spatial distributions.

#### In the Promyelocytic Leukaemic Cell Line NB4, the PML Antigen Partially Colocalizes With Nascent RNA

Since the PML-RAR $\alpha$  protein has been reported to act as a transcription factor [Doucas et al., 1993; Kakizuka et al., 1991; Rousselot et al., 1994], we compared the nuclear distribution of the PML antigen in NB4 cells with that of nascent RNA. The result is shown in Figure 3C. Visual inspection indicates that PML antigen-containing domains often at least partially overlap with sites of transcription. A more objective analysis was carried out by determining the cross correlation function (Fig. 3D). The overlap ( $r_p$ ) between the two distributions shows a maxi-



mum around  $\Delta\chi = 0$ . This indicates that the overlap between the distribution of PML-containing domains and the domains that contain nascent RNA is more than expected for two unrelated distributions. This indicates that many of the scattered nuclear domains that contain the PML antigen are involved in transcription, in contrast to PML bodies in non-leukaemic cells.

## DISCUSSION

To gain insight into the function of the nuclear bodies that contain the PML protein (PML bodies) we have compared their spatial distribution in interphase nuclei of cultured T24 cells with that of a variety of other nuclear components: (1) the splicing factor U2-snRNP, (2) several transcription factors, (3) nascent RNA, (4) nascent DNA at different stages in S-phase, and (5) p80coilin, a landmark for another type of nuclear body, the coiled body. We used fluorescence dual-labeling immunocytochemistry and confocal microscopy, in combination with a novel image-restoration technique, which removes diffraction-induced distortions in the images [Van der Voort and Strasters, 1995]. This procedure significantly increases the effective resolution of 3D-images and allows a better comparison between spatial distributions.

Our results strongly suggest that PML bodies are neither sites of transcription nor of RNA splicing, because no nascent RNA is detectable in PML bodies and splicing components U2-snRNP (this paper) and U1-snRNP [Stuurman et al., 1992] are absent. This conclusion is strengthened by our observation that none of the transcription factors that we investigated (GR, TFIIH, E2F) were found to be accumulated in PML bodies. Recently, we have analysed the spatial distribution of a number of general and specific transcription factors and RNA polymerase II in mammalian interphase nuclei. None of these proteins showed a distribution similar to PML bodies.

In the promyelocytic leukaemic cell line NB4 the situation is different. Here, a significant fraction of the large number of sites at which the PML antigen, i.e., the PML protein and the PML-RAR $\alpha$  fusion product, is found in the nucleoplasm colocalizes with sites at which nascent RNA accumulates. The positive correlation between the complex distribution of the PML antigen and that of nascent RNA was

concluded from a cross correlation analysis [Van Steensel et al., 1996] of the 3D-images. The same procedure showed that in NB4 cells no relationship exists between the distribution of the PML antigen and that of the glucocorticoid receptor. These two observations are consistent with the observation of Van Steensel et al. [Van Steensel et al., 1995b], who showed that no relationship is observed between the punctate 3D-distributions of the glucocorticoid receptor and of nascent RNA. These results show that in NB4 cells the PML protein and/or the PML-RAR $\alpha$  fusion protein is probably involved in transcriptional activation. Whether this is mediated by the PML protein, by the PML-RAR $\alpha$  fusion protein, or by a complex of both proteins [Kastner et al., 1992; Perez et al., 1993], remains to be established.

The function of PML bodies is still unknown. Two observations may shed light on this problem. First, in all cells that we have analysed at least one coiled body was always found in close proximity of a PML body. This suggests a relationship between the (still unknown) function of these two types of nuclear bodies. It should be noted, however, that since there are many more PML bodies than coiled bodies in any cell, many of the PML bodies are not in contact with a coiled body. Such pairing of nuclear bodies has also been observed earlier by electron microscopy and by immunofluorescent microscopy [Ascoli and Maul, 1991; Brasch and Ochs, 1992]. A second observation is that in middle-late S-phase many PML bodies (50–80% of all PML bodies) are found next to and in contact with a replication domain. This pairing suggests that protein factors that are present in PML bodies either are involved in replication during that particular stage of S-phase, or are specifically associated with a type of chromatin that is replicated relatively late in S-phase.

What may be the function of PML bodies? Here, our finding that PML bodies are not related to transcription and that PML bodies are often associated with domains containing late replicating DNA, is important. The PML protein is a growth inhibitor [Ahn et al., 1995; Koken et al., 1995; Liu et al., 1995; Mu et al., 1994]. Since PML has transcription factor-like structural features [Borden et al., 1995; Goddard et al., 1991; Reddy et al., 1992], it may be that it is part of a protein complex that is associated with DNA. Another protein of this complex

is the human auto-immune antigen Sp100 [Sternsdorf et al., 1995; Szosteki et al., 1990]. These observations are consistent with the idea that PML bodies, as visualized by fluorescence microscopy and electron microscopy [Stuurman et al., 1992], are protein complexes associated with specific loci of the human genome, possibly affecting the transcriptional activity of these loci, resulting in late replication. Interaction with the PML-RAR $\alpha$  fusion protein may disrupt the protein complex and change the activity of the loci to which PML bodies are associated, resulting in APL. It is intriguing that a variety of viral proteins (adenovirus oncoprotein E1A [Carvalho et al., 1995; Puvion-Dutilleul et al., 1995], the immediate early gene 1 product (ICP0) of herpes simplex virus [Maul et al., 1993], the IE1 protein of cytomegalocytic virus [Kelly et al., 1995], and the SV40 large T antigen [Carvalho et al., 1995]) induce a similar disruption of PML bodies. It is unclear what loci may be repressed by PML bodies. In this context it is important to note that coiled bodies are often associated with the locus that codes for U2-RNA of snRNPs [Frey and Matera, 1995; Smith et al., 1995]. Our observation, showing that a coiled body is often joined by a PML body, suggests that a PML body is associated with the same locus or a locus nearby. Identification of the target genes for PML bodies is the key to understanding their function.

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