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Nuclear DNA helicase II is recruited to IFN-α–activated transcription sites at PML nuclear bodies

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It is known that nuclear DNA helicase II (NDH II) links CREB-binding protein directly to RNA polymerase II holoenzyme, and that this interaction is essential for gene activation by CREB. Here, we report for the first time that some NDH II/RNA helicase A is a component of promyelocytic leukemia nuclear bodies (PML NBs). An autoimmune serum specific for PML NBs was identified and used in immunoprecipitation experiments. NDH II was present in the immunoprecipitates as shown by mass spectrometry and by immunoblotting. Immunofluorescence and ultrastructural studies showed that NDH II colocalizes with a small subset of PML NBs in control cells, however, colocalizes with practically all bodies in interferon-α–stimulated cells. After interferon stimulation, more PML NBs were found to contain newly synthesized RNA, as indicated by bromouridine incorporation. PML NBs also contain RNA polymerase II. The association of NDH II with PML NBs was transcriptionally dependent, and NDH II was present in all bodies with nascent RNA. Blocking of mRNA synthesis caused NDH II relocalization from nucleoplasm to nucleoli. Based on the data, we suggest that NDH II recruitment to PML NBs is connected with transcriptional regulation of interferon-α–inducible genes attached to PML NBs.

Introduction

The nucleus of the eukaryotic cell is a complex organelle compartmentalized into structural and functional domains. The promyelocytic leukemia nuclear bodies (PML NBs)* are nuclear multiprotein structures that are tightly bound to the nuclear matrix (Chang et al., 1995). They are also referred to as nuclear bodies, nuclear domain 10, Kr bodies, or promyelocytic leukemia oncogenic domains (Ascoli and Maul, 1991; Dyck et al., 1994; Koken et al., 1994; Weis et al., 1994). Cells typically contain 10–30 PML NBs per nucleus with diameters between 0.2 and 1 μm, although their number and size change during the cell cycle (Koken et al., 1995; for review see Zhong et al., 2000a).

The promyelocytic leukemia gene product (PML) is necessary for the proper formation of PML NBs (Ishov et al., 1999). It is a ubiquitously expressed matrix-associated nuclear phosphoprotein in which overexpression induces growth suppression (Mu et al., 1994; Chang et al., 1995). The PML gene was originally cloned as the t(15;17) chromosomal translocation partner of the retinoic acid receptor (RARα) in acute promyelocytic leukemia in which fusion genes encoding PML-RARα and RARα-PML fusion proteins are generated (de The et al., 1991; Kakizuka et al., 1991; Melnick and Licht, 1999). PML and PML-RARα proteins have been shown to modulate the activity of a set of downstream target genes, although it is not clear whether this is a direct or indirect effect on transcription (Doucas et al., 1993; Guiochon-Mantel et al., 1995; Wang et al., 1998). As PML is invariably associated with the PML NBs in all cell types studied so far, it has become a defining marker for this structure.

The PML NBs contain several other proteins in addition to PML. The first identified biochemical component of PML NBs was the Sp100 nuclear matrix–associated protein, an autoantigen in some patients with primary biliary cirrhosis (Szostecki et al., 1990). This protein is an IFN-inducible acidic protein that may transactivate a variety of promoters (Guldner et al., 1992; Xie et al., 1993). Other components of PML NBs identified so far include Sp140 (Bloch et al.,

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*Abbreviations used in this paper: AMD, actinomycin D; BLM, Bloom's syndrome protein; BrU, bromouridine; BrUTP, bromouridine triphosphate; CBP, CREB-binding protein; NDH II, nuclear DNA helicase II; PML, promyelocytic leukemia protein; PML NB, promyelocytic leukemia nuclear body; POL, polymerase; RA, retinoic acid; RARα, retinoic acid receptor.

Key words: interferon; nuclear DNA helicase II; PML nuclear body; RNA helicase A; RNA polymerase II

1996), ISG20 (Gongora et al., 1997), SUMO-1 (Boddy et al., 1996; Sternsdorf et al., 1997), Int-6 (Desbois et al., 1996), Bloom's syndrome protein (BLM; Ishov et al., 1999), Daxx (Ishov et al., 1999; Zhong et al., 2000b), CREB-binding protein (CBP; LaMorte et al., 1998), pRB (Alcalay et al., 1998), and p53 (Zhong et al., 2000a).

Type I and II IFNs dramatically increase the transcription of the PML gene through an IFN-stimulated response element present in the PML promoter (Lavau et al., 1995; Stadler et al., 1995). IFNs also increase the expression of other PML NB components, such as Sp100 and ISG20, as well as the number of PML NBs (Guldner et al., 1992; Gongora et al., 1997). This suggests a role for PML and PML NBs as a part of the antiviral defense machinery activated by IFNs in viral infections.

Although the biochemical function of PML and PML NBs remains largely unknown, consistent evidence is accumulating for a role of PML in transcription regulation. PML is a member of the RING finger family of proteins that also includes other proteins involved in cellular transformation and in the regulation of transcription, such as transcriptional coactivator BRCA 1, the breast cancer–specific tumor suppressor protein (Chapman and Verma, 1996; Somasundaram et al., 1997). PML interacts with CBP. It enhances transcriptional activation by CBP (LaMorte et al., 1998; Doucas et al., 1999; von Mikecz et al., 2000; Boisvert et al., 2001), and also by certain nuclear receptors, including RARα/RXRα, which act as retinoic acid (RA)-dependent transcriptional activators (Liu et al., 1996b; Zhong et al., 1999). The analysis of $PML^{-/-}$ mice and cells has shown that the presence of PML is crucial for the tumor growth–inhibitory activity of RA and for RA induction of myeloid differentiation, as well as for the RA-dependent transcriptional activation of $p21^{WAF/CIP1}$gene (Wang et al., 1998). It was shown that $p21^{WAF/CIP1}$gene regulates cell cycle progression and cellular differentiation, and can be activated by nuclear receptors, including RARα/RXRα (Liu et al., 1996a; Casini and Pelicci, 1999). PML is also involved in the regulation of major histocompatibility complex expression (Zheng et al., 1998). The evidence for a role of PML NBs in transcription comes from the demonstration of nascent RNA polymerase (POL) II transcripts within this nuclear body (LaMorte et al., 1998). However, more recently, nascent RNA was detected in the periphery of PML NBs, indicating that the surroundings of the PML NBs are the sites of transcriptional activity (Boisvert et al., 2000).

In this paper, we show that the nuclear DNA helicase II (NDH II) is a member of the PML NBs proteins. Furthermore, evidence is presented that the association of NDH II with PML NBs is transcriptionally dependent, and that the nascent POL II transcripts are localized at the same PML NBs where NDH II is recruited in INF-α–stimulated cells. Our results also demonstrate functional differences between individual PML NBs within one nucleus, as active transcription takes place only at a subset of PML NBs. We propose that NDH II recruitment to PML NBs is connected with transcriptional regulation of INF-α–inducible genes attached to PML NBs.

Results

Human autoimmune serum X103 immunoprecipitates protein complex of PML NBs

By screening a collection of human autoimmune sera using indirect immunofluorescence on HeLa cells, a serum (X103) showing a distinct nuclear pattern of brightly labeled spots was identified (Fig. 1 A). Double labeling of HeLa cells with X103 serum and mAb 5E10 against PML (Fig. 1 B) identified the spots as PML NBs showing complete colocalization. IFN treatment (1,000 U/ml IFN-α for 24 h), which has been shown to increase the number of PML NBs due to the IFN-induced up-regulation of PML and Sp100 (Guldner et al., 1992; Lavau et al., 1995; Grotzinger et al., 1996), resulted in an increase in the number of X103-specific nuclear structures (Fig. 1, C and D). An immunoprecipitation was performed using X103 serum coupled with protein G–Sepharose; the immunoprecipitates were analyzed by SDS-PAGE followed by Western blot or mass spectrometry. From two X103-positive bands in Western blots, IFN-α treatment led to an increase of a signal of one antigen (Fig. 1 E, lanes 1 and 2) migrating at ~80 kD. The second antigen (signal of which was not increased by IFN-α treatment; data not shown) was identified by mass spectrometry as the E2 component of the pyruvate dehydrogenase multienzyme complex located in mitochondria. It is known as M2 mitochondrial autoantigen in patients with primary biliary cirrhosis (Coppel et al., 1988). The IFN-α–stimulated antigen was identified as Sp100 protein (Fig. 1 E, lane 3), a well-known component of PML NBs in which expression is highly up-regulated by IFNs (Guldner et al., 1992; Grotzinger et al., 1996). The electrophoretic mobility of Sp100 protein is highly aberrant, and it has been previously reported to be faster than 100 kD (Szostecki et al., 1990; Sternsdorf et al., 1997).

Because Sp100 protein is constitutively present in PML NBs, we questioned whether the anti-Sp100 antibody (X103) immunoprecipitates the protein complex of PML NBs. The immunoprecipitates were screened for the presence of other PML NBs proteins by immunoblotting. The blot shows (Fig. 1 F) that PML protein as well as BLM (well-known components of PML NBs) coimmunoprecipitate together with Sp100 from both control and IFN-α–treated cells. Like Sp100, PML is highly stimulated by IFN-α but BLM protein is not, which corresponds with the literature data (Fig. 1 F, lanes 4 and 5). These results clearly show that the protein complex of PML NBs is specifically immunoprecipitated by anti-Sp100 antibody (X103).

NDH II is a component of PML NBs

Apart from the proteins already characterized as PML NBs associated, a new protein was identified in immunoprecipitated protein complexes when cells were stimulated by IFN-α (Fig. 1 G). The protein migrating at 140 kD was identified by mass spectrometry as NDH II, alternatively named RNA helicase A, a highly conserved member of the DEXH superfamily of helicases (Lee and Hurwitz, 1993). This result was confirmed by Western blot of the same immunoprecipitates using anti–NDH II antibody (Fig. 1 H). As

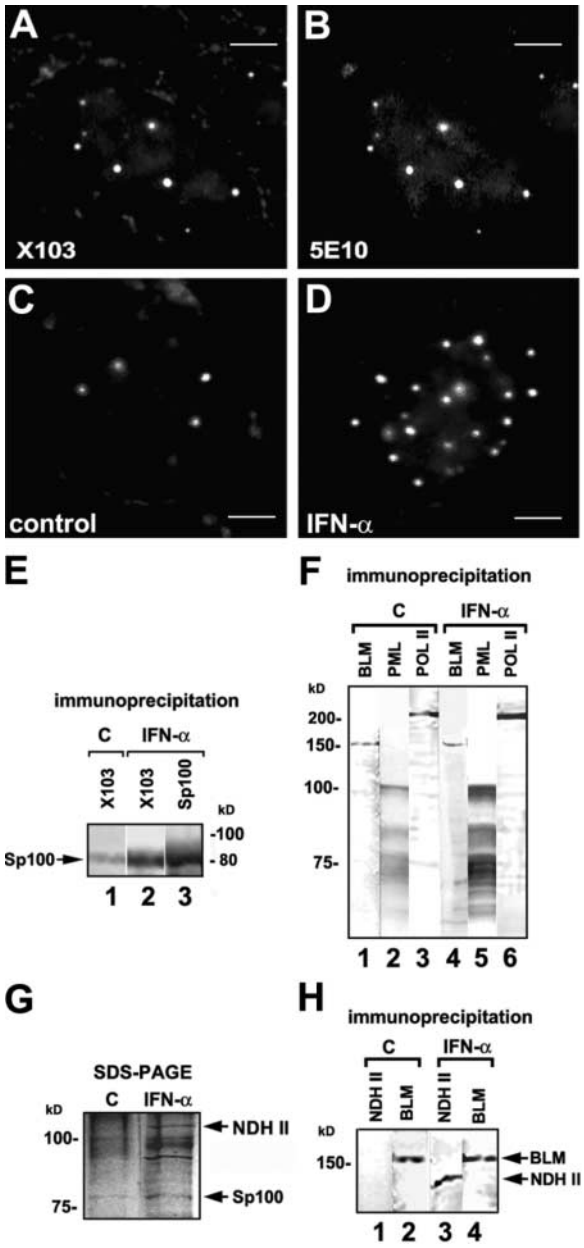

Figure 1. **NDH II is present in PML NBs upon IFN-α induction.** (A and B) Double immunostaining of HeLa cells using autoimmune X103 serum (A) and mAb 5E10 (B) recognizing PML protein. PML and X103 antigen colocalize in PML NBs. (C and D) Effect of IFN-α treatment on X103-specific antigen. Immunofluorescent localization of X103 antigen in HeLa cells: (A) control cells; (B) after treatment with IFN-α (1,000 U/ml for 24 h). Bars, 2 μm. (E–H) Immunoprecipitation using X103 serum coupled with protein G–Sepharose followed by Western blot. The immunoprecipitates from control and IFN-α–treated cells (1,000 U/ml for 24 h) were analyzed using antibodies indicated above the blot. (E) IFN-α treatment led to an increase of a signal of X103-specific antigen migrating at ~80 kD (lanes 1 and 2) identified as Sp100 (lane 3). (F) PML protein (lanes 2 and 5), BLM (lanes 1 and 4), and POL II (lanes 3 and 6) are present in the immunoprecipitates from both control and IFN-α–treated cells. PML expression is highly enhanced by IFN-α (lane 5) but BLM is not (lane 4). (G) 10% SDS-PAGE of immunoprecipitates from control and IFN-α–treated cells. NDH II (140 kD) coimmunoprecipitates with the PML NBs complex after IFN-α stimulation. The gel was stained with Coomassie brilliant blue R-250 for total protein. (H) Western blot of the same immunoprecipitates using anti–NDH II antibody confirmed its presence in immunoprecipitates from IFN-α–treated cells (lane 3) but not in control cells (lane 1). As a control, anti-BLM antibody was used to detect BLM protein (lanes 2 and 4) that is present in both cases.

shown, NDH II is present in immunoprecipitates from IFN-α–treated cells (Fig. 1, lane 3) but is not detectable in immunoprecipitates from control cells (Fig. 1 H, lane 1). On the other hand, BLM protein, used here as a control, is present in both cases (Fig. 1, lanes 2 and 4). Moreover, when immunoprecipitation using anti–NDH II antibody coupled with protein G–Sepharose was performed and the immunoprecipitates were analyzed by SDS-PAGE followed by Western blot, Sp100 coimmunoprecipitated with NDH II (data not shown). We conclude from these experiments that NDH II is a component of the PML NBs complex after IFN-α stimulation.

IFN-α treatment enhances colocalization of NDH II and PML NBs

To confirm the localization of NDH II in PML NBs in situ, we used confocal laser scanning microscopy of control and IFN-α–stimulated HeLa cells (1,000 U/ml IFN-α for 24 h) using the anti-Sp100 antibody (X103) and the anti–NDH II antibody. Fig. 2 A shows that the nuclear distribution of NDH II in control cells can be observed as a fine granular nucleoplasmic staining with a few larger foci. Some (but not all) of the foci colocalize with PML NBs. Similarly, some of the PML NBs do not colocalize with NDH II at all. After IFN-α stimulation (Fig. 2 B), NDH II is detected in most PML NBs. Nevertheless, a considerable amount of NDH II remains distributed throughout the nucleoplasm. Immunoelectron microscopy also clearly demonstrated the presence of NDH II in PML NBs in IFN-α–stimulated cells (Fig. 3 A). The ratio of colocalization of PML NBs with NDH II foci in control and IFN-α–stimulated cells was quantified by measuring the fluorescence intensities overlap along profiles spanning the PML NBs (Fig. 4). In control cells (Fig. 5), NDH II colocalizes with only 19% of PML NBs, 28% of PML NBs colocalize partially, and ~53% do not colocalize with NDH II foci. In contrast, in IFN-α–treated cells NDH II colocalizes with 79% of PML NBs, ~2% colocalize partially, and 19% of PML NBs do not colocalize with NDH II. Thus, we conclude that NDH II is present in a subset of PML NBs in control cells growing in standard conditions. However, IFN-α stimulation causes recruitment of NDH II into the majority of PML NBs.

NDH II association with PML NBs is interrupted by transcriptional inhibition

It has been shown previously that NDH II, functioning as a bridging factor between the transcriptional coactivator CBP and POL II, cooperates with CBP in mediating transcriptional activation of target genes via CREB (Nakajima et al., 1997). In addition, several studies suggest that PML and PML NBs may play a role in transcription events (Vallian et al., 1997; LaMorte et al., 1998), although no direct interaction between PML and the transcription complex has been demonstrated. Therefore, we tested whether the recruitment of NDH II into PML

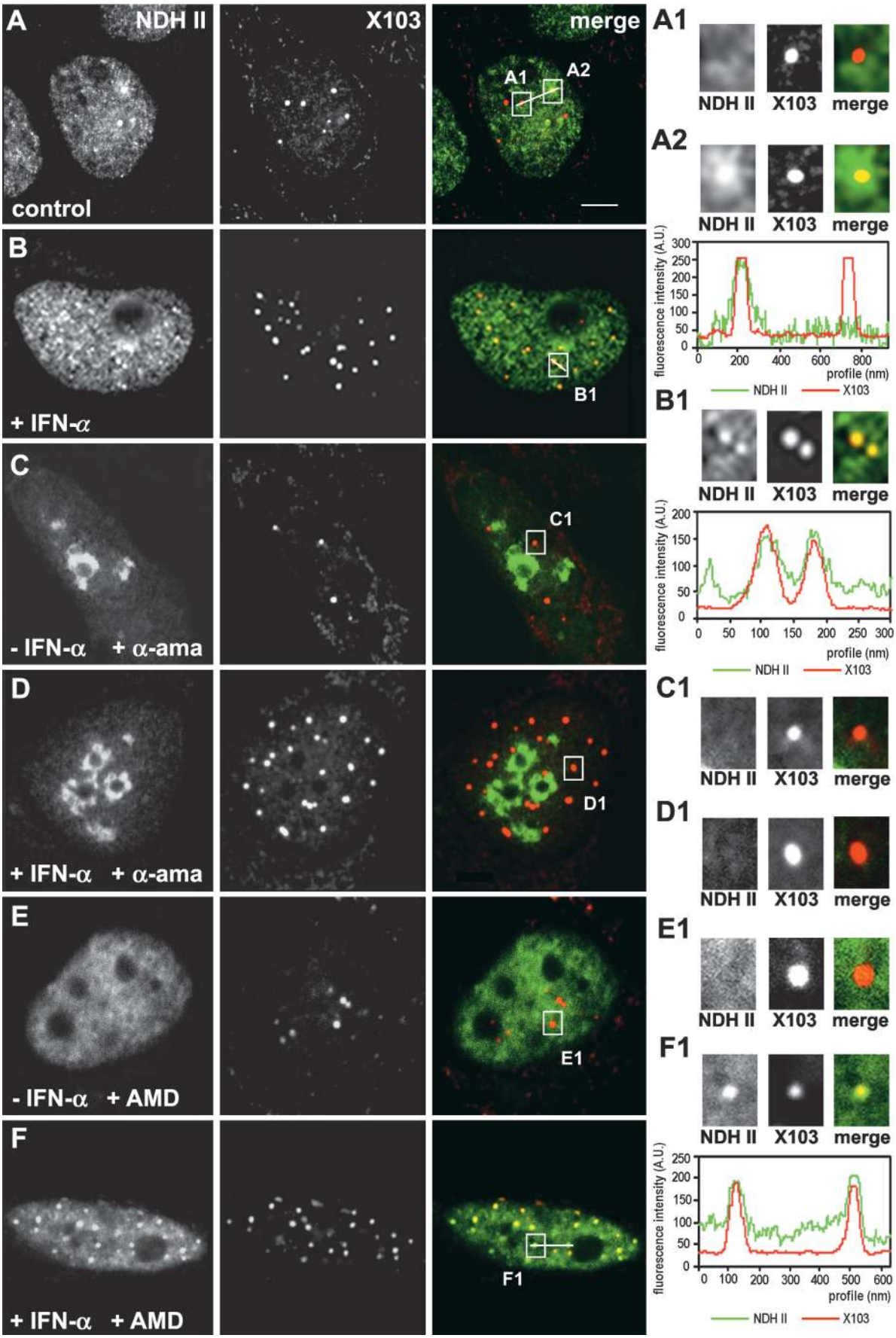

Figure 2. **Confocal micrographs of HeLa cells double labeled with the anti–NDH II antibody and anti-Sp100 antibody (X103).** (A) control cells; only some PML NBs colocalize with NDH II. (A1 and A2) Magnified views of indicated PML NBs show no colocalization (A1) or complete

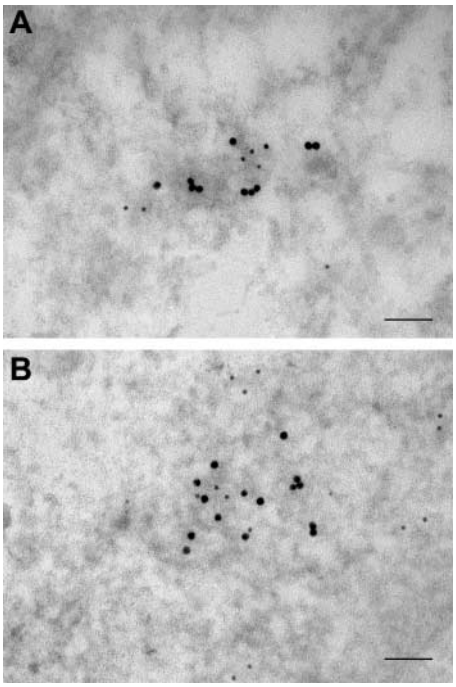

Figure 3. **Electron micrographs of IFN-α–treated HeLa cells (1,000 U/ml for 24 h).** (A) Double immunogold labeling using anti–NDH II (5-nm particles) and anti-Sp100 antibody (X103; 10-nm particles); NDH II localizes to PML NBs. (B) Double immunogold labeling using anti–POL II (5-nm particles) and anti-Sp100 antibody (X103; 10-nm particles). POL II localizes to PML NBs. Bars, 50 nm.

NBs is related to transcriptional levels in a cell. The effect of transcriptional inhibition on NDH II localization relative to PML NBs was assessed by double labeling of control and IFN-α–treated cells (1,000 U/ml for 24 h) using the anti-Sp100 antibody (X103) and the anti–NDH II antibody.

Treatment of cells with α-amanitin (100 μg/ml for 3 h) at doses that block mRNA synthesis (Nguyen et al., 1996) induced rapid repositioning of NDH II, and the nuclear pattern of NDH II localization was changed whether the cells were (Fig. 2 D) or were not stimulated by IFN-α (Fig. 2 C). In both cases, the nucleoplasm is almost cleared of NDH II, and NDH II completely disassociates from PML NBs. Instead, it associates with nucleolar periphery. The results were identical in cells treated with another transcriptional inhibitor actinomycin D (AMD) at doses that inhibit POL II transcription (0.5 μg/ml for 3 h; Perry and Kelley, 1970; data not shown).

In contrast, when cells were treated with AMD at doses that inhibit POL I but not POL II transcription (0.02 μg/ml for 3 h), no changes in the nuclear pattern of NDH II staining were observed in both nonstimulated (Fig. 2 E) and IFN-α–stimulated cells (Fig. 2 F). Again, the effect of POL I inhibition on NDH II nuclear distribution and its association with PML NBs was quantified by measuring the fluorescence intensities overlap along profiles spanning the PML NBs. The frequency of the colocalization between PML NBs and NDH II in cells treated with both IFN-α and AMD (0.02 μg/ml; 3 h) is similar to that of the cells treated only with IFN-α (Fig. 5). NDH II remains in the association with PML NBs and colocalizes with >75% of nuclear bodies. Thus, the IFN-α–stimulated recruitment of NDH II into the PML NBs is dependent on active transcription by POL II, but not by POL I.

DNA transcription takes place at a subset of PML NBs

If transcription takes place at PML NBs, one would expect the presence of POL II in these bodies. Indeed, POL II was found to coimmunoprecipitate with the protein complex of PML NBs from both control and IFN-α–treated cells (Fig. 1 F, lanes 3 and 6). Furthermore, POL II was present in PML NBs in IFN-α–stimulated cells as shown by immunoelectron microscopy (Fig. 3 B).

To demonstrate that the recruitment of NDH II into the PML NBs is connected with transcription, the colocalization of transcription foci with NDH II and PML NBs was studied by confocal microscopy. Nascent transcripts were labeled in vivo with bromouridine (BrU; 20 mM for 10 min) in control and in IFN-α–stimulated (1,000 U/ml IFN-α for 24 h) HeLa cells. The sites containing nascent BrRNA were then labeled with anti-BrdU antibody. For triple immunofluorescence labeling, the cells were in addition stained with the anti-Sp100 antibody (X103) and anti–NDH II antibody. In Fig. 6 (A and B), optical sections through the center of nuclei show distribution of BrRNA in relationship to PML NBs and NDH II. In the absence of IFN-α stimulation in control cells, most PML NBs do not overlap with transcription sites (Fig. 6, A and C). After IFN-α treatment (Fig. 6 B), some of the transcription sites containing BrRNA enlarged, and they frequently overlap with PML NBs as well as with NDH II foci. PML NBs that are associated with discrete sites of transcription containing BrRNA clearly colocalize with NDH II (Fig. 6, B, E, and F). Identical results were obtained when nascent transcripts were labeled using bromouridine triphosphate (BrUTP) in gently permeabilized cells. This method gives an advantage of known transcription rate (Jackson et al., 1993), so we were sure that the localization of nascent transcripts was observed (Fig. 7). The

colocalization with NDH II (A2). The graph below shows the overlap of fluorescence intensity peaks along profiles spanning the PML NBs as indicated in the merged micrograph. (B) IFN-α–treated cells (1,000 U/ml for 24 h; +IFN-α); NDH II is recruited to PML NBs. (B1) A magnified view of indicated PML NBs shows complete colocalization with NDH II foci. The graph below shows the overlap of fluorescence intensity peaks along profiles spanning the PML NBs as indicated in the merged micrograph. (C and D) After inhibition of POL II transcription with α-amanitin (100 μg/ml for 3 h); (C) cells not stimulated with IFN-α (−IFN-α/+α-ama); (D) IFN-α–treated cells (1,000 U/ml for 24 h; +IFN-α/+α-ama). NDH II dissociates from PML NBs after inhibition of POL II transcription and is translocated to nucleolar periphery. (C1 and D1) Magnified views of indicated PML NBs show no colocalization with NDH II. (E and F) After treatment with AMD at concentration inhibiting only POL I transcription (0.02 μg/ml for 3 h); (E) cells not stimulated with IFN-α (−IFN-α/+AMD 0.02 μg/ml); (F) IFN-α–treated cells (1,000 U/ml for 24 h; +IFN-α/+AMD 0.02 μg/ml). Inhibition of POL I transcription does not cause NDH II dissociation from PML NBs and its translocation to nucleolar periphery. (E1 and F1) Magnified views of indicated PML NBs show no colocalization with NDH II in IFN-α–nonstimulated cells (E1), but complete colocalization in IFN-α–stimulated cells (F1). The graph below shows the overlap of fluorescence intensity peaks along profiles spanning the PML NBs as indicated in the merged micrograph. Bar, 2 μm.

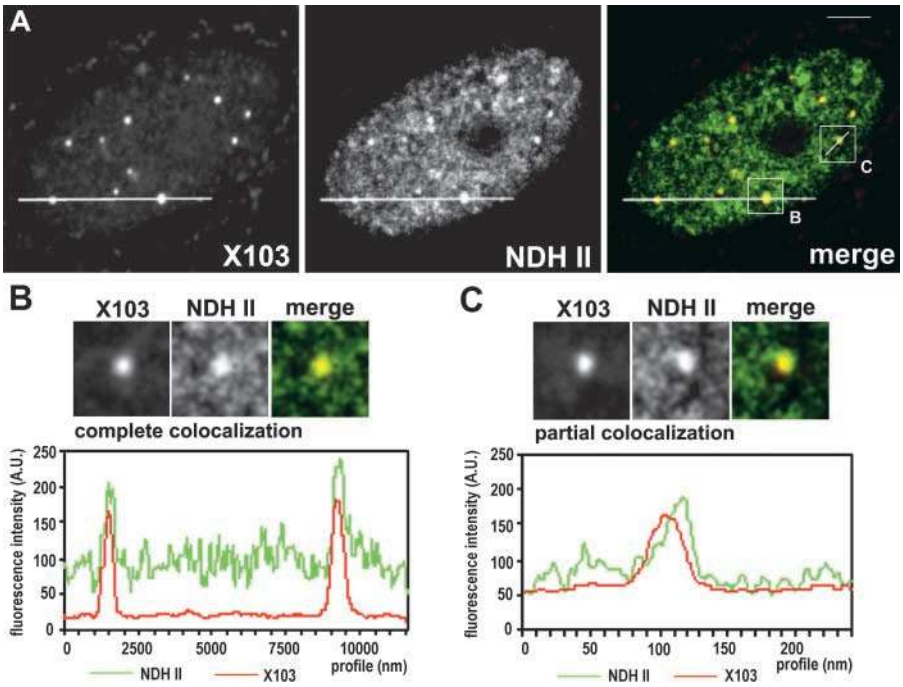

Figure 4. **Complete and partial colocalization of PML NBs with NDH II.** (A) The ratio of colocalization was quantified by measuring the fluorescence intensities overlap along profiles spanning the PML NBs using confocal software. (B and C) Magnified views of indicated PML NBs show complete colocalization (B) and partial colocalization (C) with NDH II foci. The colocalization was considered as complete when two peaks were overlapping and the maxima were shifted <20 nm (B; see graph). A side overlap of two peaks (maxima shifted >20 nm) was taken as a partial colocalization (C; see graph). Bar, 2 μm.

mutual relationship between PML NBs, NDH II, and the sites containing nascent RNA was assessed by measuring the three fluorescence intensities overlap along profiles spanning PML NBs using the Leica confocal software (Fig. 8). In control cells, ~9% of PML NBs contain both NDH II and transcription sites, and 75% of bodies do not contain either NDH II or nascent transcripts. About 16% of bodies contain only NDH II but, most importantly, no bodies contain only transcription sites. After IFN-α stimulation, the number of PML NBs that colocalize with transcription sites and NDH II increased up to 51% and the number of PML NBs not containing either NDH II or nascent transcripts decreased. These data show that in addition to recruitment of NDH II into the PML NBs, some prominent transcription foci are found associated with PML NBs in IFN-α–treated cells, particularly with those containing NDH II. These results strongly imply the involvement of the PML NBs in transcription processes triggered by IFN stimulation.

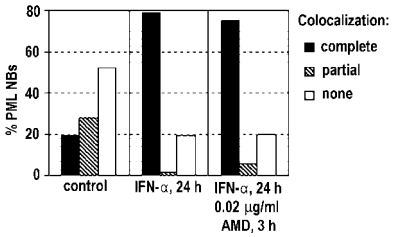

Figure 5. **Quantification of the colocalization of PML NBs and NDH II in control cells, in IFN-α–stimulated cells, and after inhibition of POL I transcription (0.02 μg/ml AMD; 3 h) performed in confocal images described in Fig. 2 (n = 20).** Because there is no colocalization after inhibition of POL II transcription (100 μg/ml α-amanitin for 3 h; Fig. 2, C and D) the quantification was not performed in this case. In control cells, NDH II colocalizes completely with 19% of PML NBs, 28% of PML NBs colocalize partially, and ~53% do not colocalize with NDH II foci. In IFN-α–treated cells, NDH II colocalizes with 79% of PML NBs, ~2% colocalize partially, and 19% of PML NBs do not colocalize with NDH II. After inhibition of POL I transcription, NDH II remains in the association with PML NBs and colocalizes with >75% of nuclear dots. Less than 5% of PML NBs colocalize partially, and 20% do not colocalize with NDH II.

Discussion

There has been considerable effort in recent years to define a function for PML NBs, and more specifically, their involvement in transcription. In this paper, we provided several lines of evidence that NDH II is a component of PML NBs, and that its association with PML NBs is interrupted by inhibition of POL II transcription. Furthermore, we have localized nascent RNA to the PML NBs in IFN-α–treated cells, and these transcripts were found to be associated with the subset of PML NBs recruiting NDH II. These data show that a fraction of NDH II is compartmentalized to the PML NBs and strongly support an idea that the nuclear bodies contribute to transcriptional regulation.

We have shown that the human serum X103 recognizes Sp100 protein, and that it can be used to immunoprecipitate the protein complex of PML NBs as confirmed by presence of typical PML NBs proteins Sp100, PML, and BLM in the immunoprecipitates. Immunofluorescence analysis revealed differential distribution of NDH II in the nucleus. It is homogeneously distributed through the nucleus, which corresponds to the published data (Zhang et al., 1999), or compartmentalized into foci that colocalize with a subset of PML NBs. This colocalization is dramatically increased upon the IFN-α treatment. Thus, we show here that a portion of NDH II is present in PML NBs under certain conditions. Another component of PML NBs, CBP, also was shown to be a dynamic component of the bodies (Boisvert et al., 2001). Hence, some components that are transient occu-

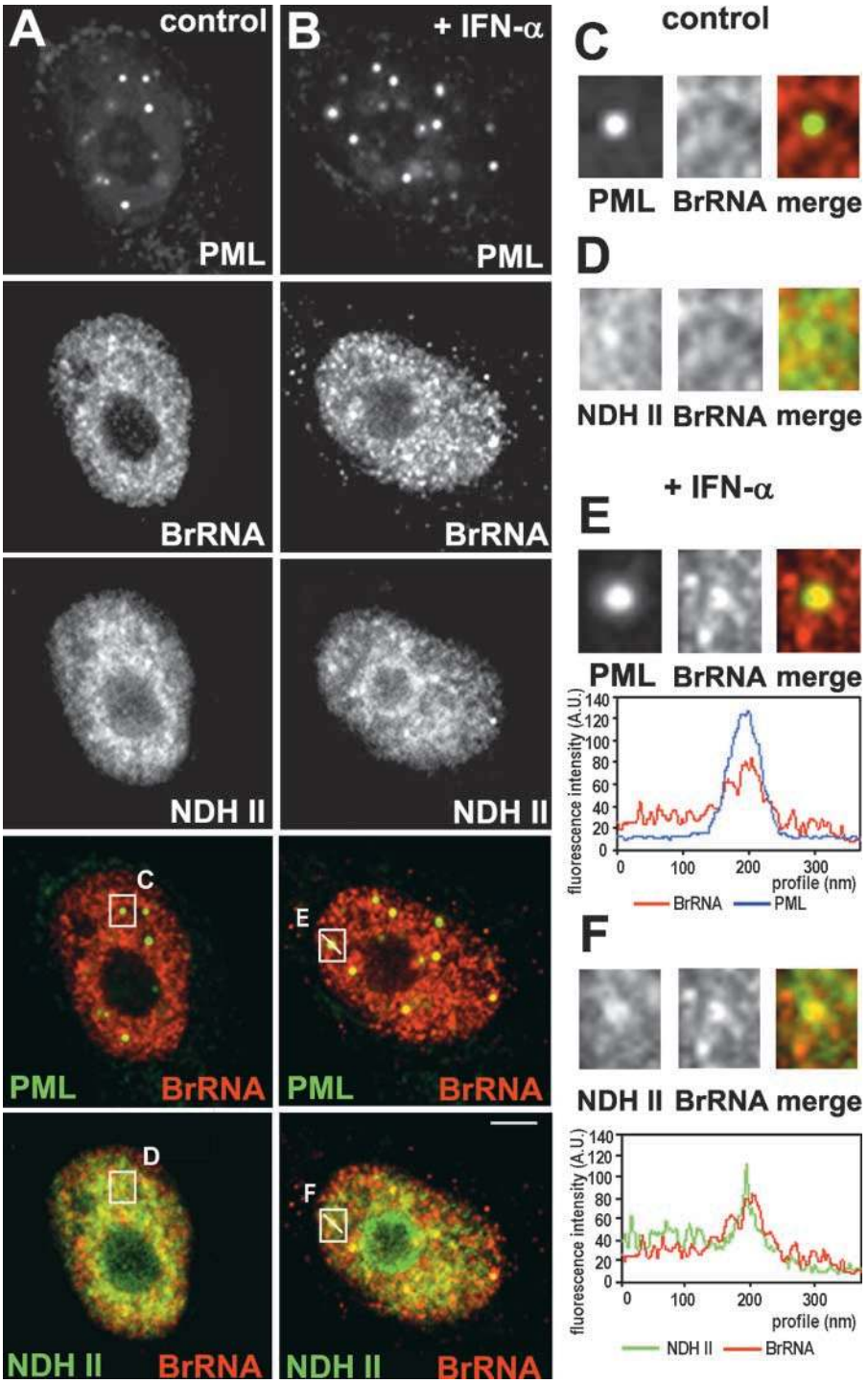

Figure 6. **Transcription sites imaged by confocal microscopy of HeLa cells.** Nascent transcripts were labeled using BrU; triple immunostaining was performed using the anti-BrdU antibody, anti-Sp100 antibody (X103), and anti–NDH II antibody. (A) Cells not stimulated with IFN-α (control). BrRNA foci do not overlap with PML NBs. (C and D) Magnified views of indicated PML NBs. (B) IFN-α–treated cells; BrRNA foci are found in association with a subset of PML NBs and NDH II foci. (E and F) Magnified views of indicated PML NBs show complete colocalization with BrRNA (E) and also with NDH II (F). The graphs below show the overlap of fluorescence intensity peaks along profiles spanning the PML NBs as indicated in the micrographs. For quantification of the results, see Fig. 8. Bar, 2 μm.

pants of PML NBs may additionally function at multiple sites throughout the nucleoplasm, and they can be recruited to PML NBs under certain conditions, for instance, when the transcriptional level of target genes is stimulated by IFN.

We showed that after transcriptional inhibition of POL II, NDH II completely dissociates from PML NBs and associates with the nucleolar periphery. However, inhibition of POL I does not cause spatial repositioning of NDH II and its dissociation from PML NBs. Therefore, we conclude that IFN-α–stimulated recruitment of NDH II into the PML NBs is dependent on an active POL II transcription. Moreover, we have observed that after IFN-α treatment, transcription foci are also found in association with PML NBs containing NDH II. Thus, not only is the association of NDH II with PML NBs transcriptionally dependent, but PML NBs associated with NDH II also colocalize with discrete sites of transcription.

Similar observation has been made by LaMorte et al. (1998). These authors localized nascent RNA to PML NBs. In agreement with our results, not all of the PML NBs within a cell accumulated nascent RNA, suggesting more than one functional state of PML NBs. In their approach, nascent RNA was labeled by incorporation of a fluorescein-labeled nucleotide (FITC-UTP) microinjected into living Hep-2 cells as well as at the ultrastructural level by EDTA-

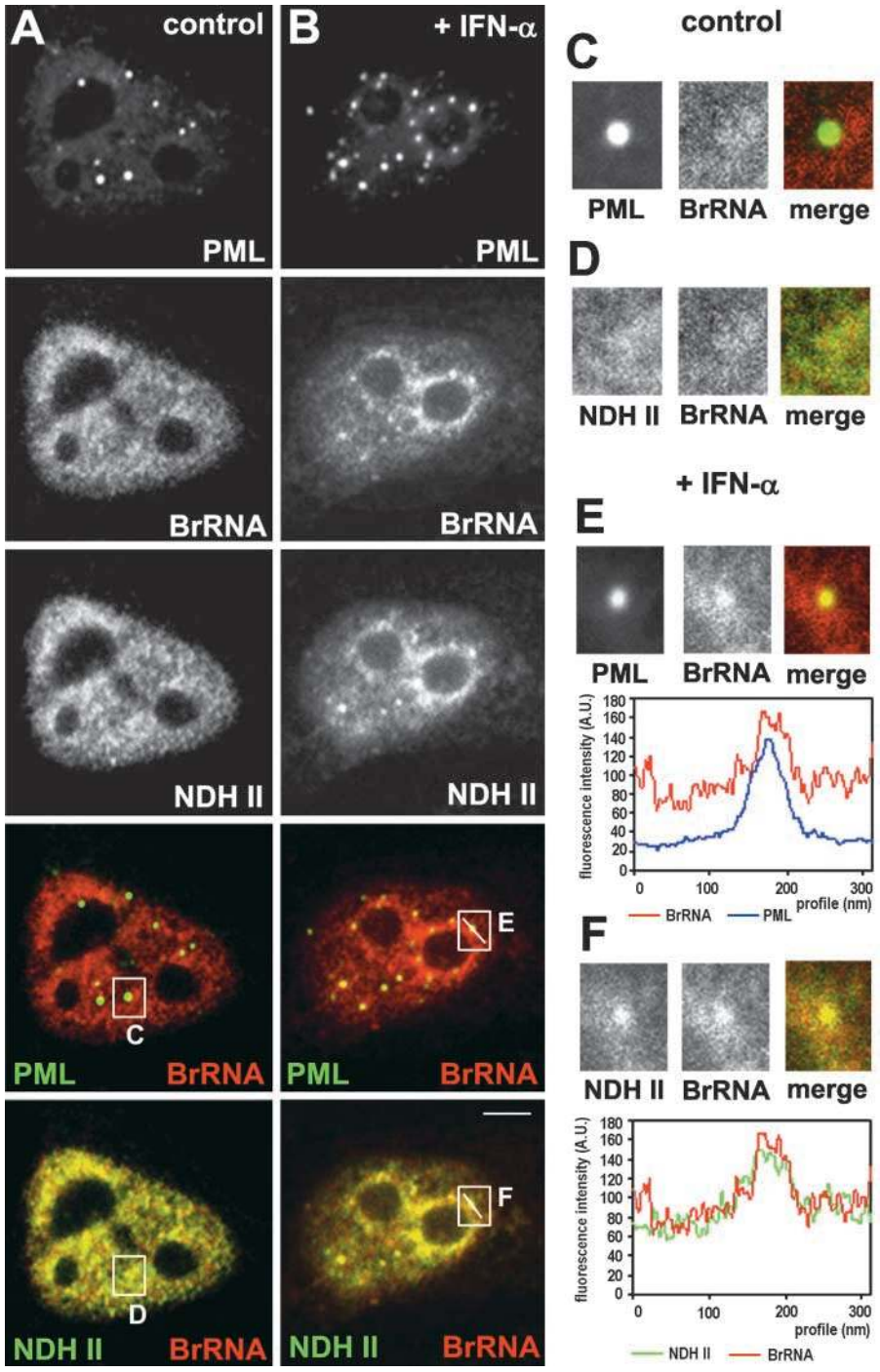

Figure 7. **Transcription sites imaged by confocal microscopy of HeLa cells.** Nascent transcripts were labeled using BrUTP in gently permeabilized cells. Triple immunostaining was performed using the anti-BrdU antibody, anti-Sp100 antibody (X103), and anti–NDH II antibody. (A) Cells not stimulated with IFN-α (control). BrRNA foci do not overlap with PML NBs. (C and D) Magnified views of indicated PML NBs. (B) IFN-α–treated cells; BrRNA foci are found in association with a subset of PML NBs and NDH II foci. (E and F) Magnified views of indicated PML NBs show complete colocalization with BrRNA (E) and also with NDH II (F). The graphs below show the overlap of fluorescence intensity peaks along profiles spanning the PML NBs as indicated in the merged micrographs. Bars, 2 μm.

regressive staining. In contrast, Boisvert et al. (2000) used phosphorus mapping by electron spectroscopic imaging and fluorine-substituted uridine incorporation detected by fluorescence microscopy to show the presence of nascent RNA at the periphery of this structure, but not in the protein core of the PML NBs. In both cases, the observations were made on cells not stimulated by IFN-α. Here, we show using two labeling approaches (BrU and BrUTP incorporation) that after IFN-α treatment the association of transcription foci with PML NBs increases from 9% of PML NBs in control cells up to 51%. Thus, our work is able to accommodate both sites of results and imply the involvement of the PML NBs in transcription processes triggered by IFN stimulation. Furthermore, we found POL II to locate in PML NBs and coimmunoprecipitate with PML NBs proteins from both control and IFN-α–treated cells. This is consistent with the results by von Mikecz et al. (2000) showing the presence of POL II and CBP in a subset of PML NBs. Therefore, we suggest that NDH II recruitment to PML NBs is connected with transcriptional regulation of genes attached to PML NBs.

When considered in the light of recent studies on the function of NDH II, our results suggest that NDH II may represent a link between PML NBs and the transcription processes. It has been shown that NDH II is a component of the POL II holoenzyme that binds directly to CBP (Nakajima et al.,

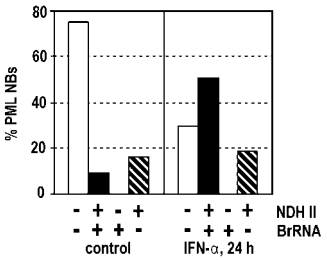

Figure 8. **Significant number of PML NBs associate with transcription sites after IFN-α induction.** Quantification of the colocalization of PML NBs with NDH II and transcription sites in control and IFN-α–stimulated cells performed in confocal images described in Fig. 6 ($n = 20$). In control cells, 75% of PML NBs do not contain either NDH II or nascent transcripts, ~9% of bodies contain both NDH II and transcription sites, and 16% of bodies contain only NDH II; no bodies contain only transcription sites. After IFN-α treatment, the number of PML NBs colocalizing with transcription sites and NDH II increases up to 51%, whereas the number of PML NBs not containing either NDH II or nascent transcripts decreases to 30%.

1997). It functions as a bridging element for the attraction of the transcriptional coactivator CBP to POL II complex. This CBP–NDH II interaction is essential for CREB-dependent transcriptional activation of target genes. Our data are also in agreement with the fact that a fraction of CBP was shown to be compartmentalized to PML NBs through its direct association with PML, although the precise mechanism whereby PML modulates CBP function is yet unknown (Doucas et al., 1999). These results raise the intriguing possibility that upon transcriptional activation, for instance by IFN-α, components of the transcriptional regulatory complexes are transiently recruited to the nuclear bodies, to take part in transcription of genes associated with PML NBs. Because the complex formation between CBP and POL II requires NDH II, we can speculate that NDH II recruited into PML NBs links the transcriptional coactivator CBP modulated by PML to POL II.

Interferon-stimulated gene factor-3 (ISGF-3), a multiprotein, IFN-α–activated transcription factor consisting of a 48-kD DNA binding protein and two proteins termed Stat1 and Stat2 (for signal transducers and activators of transcription), binds to IFN-α signal response elements and activates the transcription of cellular antiviral defense genes. Stat2 was shown to interact specifically with CBP, indicating that Stat2-containing ISGF-3 complex may function in transcription activation partly through the action of CBP (Bhattacharya et al., 1996). The fact that NDH II binds CBP to POL II complex and our observation that NDH II recruitment by PML NBs is sensitive to the IFN-α stimulation speaks in favor of involvement of PML NBs in transcription of the IFN-α–stimulated genes. This is consistent with the data showing a cooperation of CBP and Stat2 in signaling induced by IFN-α.

Nonuniform appearance of PML NBs may lead to a new consideration as to the subclasses of PML NBs that differ in their composition and function. Presented data showed that not all of PML NBs are equivalent, and that they may have more than one functional state. This is consistent with the earlier observation that not all of PML NBs within a cell accumulate nascent RNA (LaMorte et al., 1998). Speculation could be made that the recruitment of the respective PML NBs–associated protein into the nuclear bodies under certain conditions may modulate their cellular function. Indeed, a dynamic mechanism for protein recruitment to these nuclear domains controlled by the SUMO-1 modification state of PML has already been suggested, although in this case a role of PML NBs as nuclear depots for a number of proteins was postulated (Ishov et al., 1999). However, the differential recruitment of CBP to PML NBs through its direct association with PML was also demonstrated, showing that PML can function as a CBP cofactor in CBP-mediated activation of transcription (Doucas et al., 1999). We can speculate that PML NBs may represent storage sites of NDH II and other components of transcriptional complexes used for associated genes transcribed at PML NBs, similarly to nuclear speckles containing a reservoir of splicing factors. On the other hand, rather than storage sites they can actively participate in transcriptional regulation. Our data speak in favor of the latter. Even though further analysis will be necessary to elucidate the role of PML NBs, we suggest that the recruitment of NDH II into PML NBs is functionally linked with the contribution of these structures to the transcription of specific genes, possibly such as antiviral defense genes.

To understand the role of PML NBs in the control of transcription, it is critical to clarify how individual PML NBs components participate in these processes. Identification of NDH II in PML NBs, which is a component of POL II holoenzyme, bridging POL II and CBP, reveals an exciting direct link between transcriptional machinery and PML NBs. It will be critical to determine whether the association of NDH II with PML NBs can serve as the regulatory element. The identification of the genes expressed at/in PML NBs and the use of $PML^{-/-}$ cells to test whether there is an accumulation of NDH II and POL II with Sp100 at IFN up-regulated gene clusters could further shed a light on the mechanisms by which PML NBs could regulate transcription.

Materials and methods

Cells, growth conditions, and transcription inhibitors

HeLa cells in monolayer cultures were grown in DME (Sevapharma) containing 5% (vol/vol) FBS and antibiotics at 37°C in a humidified 5% CO_2 atmosphere. Suspension cultures of HeLa cells were grown in S-MEM (Sigma-Aldrich) supplemented with 5% FBS (vol/vol), 1 mM sodium pyruvate, nonessential amino acids, and antibiotics. For IFN-α induction, exponentially growing cells were exposed for 24 h to 1,000 U/ml IFN-α (interferonum α-2b; Schering-Plough Corporation). For inhibition of POL II transcription, α-amanitin (Sigma-Aldrich) was added to the culture medium at a final concentration of 100 μg/ml for 3 h (Nguyen et al., 1996). AMD (Sigma-Aldrich) was added for 3 h at final concentrations of either 0.02 μg/ml for inhibition of POL I transcription or 0.5 μg/ml for inhibition of POL II transcription (Perry and Kelley, 1970).

Antibodies

Human autoimmune serum X103, positive for PML NBs, was obtained from the Institute of Rheumatology with the patient's consent and according to the ethical guidelines of the state. The following antibodies were used: mouse mAb 5E10 against PML protein (a gift from Dr. R. van Driel, E.C. Slater Institute for Biochemical Research, University of Amsterdam, Netherlands; Stuurman et al., 1992), the rabbit anti-Sp100 (Sp-26) antiserum (obtained from Dr. T. Sternsdorf, Max Planck Institute for Biochemistry, Martinsried, Germany; Szostecki et al., 1990), the rabbit polyclonal anti–NDH II antibody (Zhang et al., 1995) donated by Dr. S. Zhang (Institute of Molecular Biotechnology, Jena, Germany), the rabbit polyclonal anti-BLM antibody (Neff et al., 1999), and the mouse mAb against POL II (a gift from Dr. M. Vigneron, Institute of Genetics and Molecular and Cell Biology, Illkirch, France). BrRNA was immunolabeled with anti-BrdU mAb (6 μg/ml; clone

BMC9318; Boehringer). The secondary antibodies were donkey anti–mouse, anti–rabbit, or anti–human IgG conjugated with FITC, Cy3, LRSC, or Cy5 (1:100 dilution; Jackson ImmunoResearch Laboratories).

Immunofluorescent microscopy

Cells grown as a monolayer were fixed in acetone at −20°C (3 min) followed by 20 min in 4% PFA in PBS. After washing with PBS, cells were incubated with primary antibody (1 h), washed with PBS, and incubated with secondary antibody (45 min) at RT. DNA was stained with DAPI (0.2 μg/ml in PBS for 10 min; Boehringer). Immunofluorescence was visualized using either a conventional fluorescence microscope (Vanox-S; Olympus) or a confocal laser scanning microscope (TCS SP; Leica). In the first case, pictures were captured by a chilled CCD camera (model C5985; Hamamatsu Corporation). The systems were carefully tested for the overlap of the three optical channels. This is documented also in Fig. 6 (C–F), where no leakage between optical channels is present. Image files were processed with Adobe Photoshop®. The ratio of colocalization was quantified by measuring the fluorescence intensities overlap along profiles spanning the PML NBs using Leica confocal software (Fig. 4). The colocalization was considered complete (Fig. 4 B) when two peaks were overlapping and the maxima were shifted <20 nm. A side overlap of two peaks (maxima shifted >20 nm) was taken as a partial colocalization (Fig. 4 C).

Electron microscopy

HeLa cells grown in suspension were treated with IFN-α as described in the first paragraph of Materials and methods. Cells were pelleted, fixed, and embedded in LR White™ resin (Polysciences, Inc; Hozak et al., 1994). The ultrathin sections were double immunolabeled with the anti-Sp100 antibody (X103) and the rabbit anti–NDH II antibody or the mouse anti–POL II mAb. The primary antibodies were visualized using 5-nm gold-conjugated antibodies to rabbit or mouse IgG and 10-nm gold-conjugated antibodies to human IgG (dilution 1:30; Jackson ImmunoResearch Laboratories). Sections were contrasted with a saturated solution of uranyl acetate and examined using an electron microscope (Philips Morgagni, FEI) equipped with a CCD MegaView II camera (Soft Imaging System).

Western blot analysis

Protein samples were separated on 10% SDS-PAGE gels according to Laemmli (1970). Proteins were transferred to nitrocellulose membranes as described by Towbin et al. (1979). The blots were blocked with 5% nonfat dried milk and 0.5% BSA in TBS (0.02 M Tris-HCl and 0.5 M NaCl, pH 7.5) for 2 h at RT. After an overnight incubation with primary antibodies, nitrocellulose membranes were treated with alkaline phosphatase–conjugated secondary antibodies recognizing mouse, human, or rabbit IgG (Sigma-Aldrich) diluted 1:3,000 in TBS supplemented with 2.5% nonfat dried milk and 0.25% BSA for 1 h at RT. After extensive washes with TBS containing 0.05% Tween 20, proteins were visualized by color development with 5-bromo-4-chloro-3-indolyl phosphate/nitrobluetetrazolium (Sigma-Aldrich).

Immunoprecipitation of proteins under nondenaturing conditions

Immunoprecipitation was performed as described previously (Lukas and Bartek, 1998), except that after binding the antibodies to the protein G–Sepharose beads, they were cross-linked with the protein G via a bifunctional coupling reagent dimethylpimelimidate (Sigma-Aldrich) as described by Harlow and Lane (1988). All steps were performed on ice or at 4°C with antiproteases (1 mM PMSF, 1 μg/ml leupeptin, and 0.5 μg/ml pepstatin). The immunoprecipitates were analyzed either by Western blotting or by SDS-PAGE followed by mass spectrometry.

Mass spectrometry

Coomassie brilliant blue R-250–stained protein bands were digested by sequencing grade trypsin (50 ng/μl; Promega) in a cleavage buffer containing 0.01% 2-mercaptoethanol, 0.1 M 4-ethylmorpholine acetate, pH 8.1, 10% ACN, and 1 mM $CaCl_2$. Digestion was performed overnight at 37°C, and the resulting peptides were extracted with 30% ACN/1% TFA and subjected to mass spectrometric analysis.

Mass spectra were measured on a mass spectrometer (BIFLEX MALDI-TOF; Bruker Daltonics) equipped with a nitrogen laser (337 nm) and gridless delayed extraction ion source. Ion acceleration voltage was 19 kV, and the reflectron voltage was set to 20 kV. Spectra were calibrated externally using the monoisotopic $[M + H]^+$ ion of peptide standard somatostatin (Sigma-Aldrich). A saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50% ACN/0.2% TFA was used as a MALDI matrix. 1 μl of matrix solution was mixed with 1 μl of the sample on the target, and the droplet was allowed to dry at ambient temperature.

Detection of transcription sites

For labeling of nascent transcripts with BrU, HeLa cells were grown as monolayers in DME containing 5% (vol/vol) FBS and BrU (20 mM; Sigma-Aldrich) for 10 min to allow incorporation, rinsed in ice-cold medium and PBS, and immediately fixed. The labeling of nascent transcripts with BrUTP was performed as described previously (Pombo et al., 1999). In brief, cells were lysed by addition of saponin (final concentration, 1 mg/ml; S-7900; Sigma-Aldrich) in PB-BSA (physiological buffer [mM]: 100 potassium acetate, 30 KCl, 10 Na_2HPO_4, 1 $MgCl_2$, 1 Na_2ATP, 1 DTT, and 0.2 PMSF, pH 7.4 [Jackson et al., 1993] supplemented with 100 mg/ml BSA and 10 U/ml human placental RNase inhibitor) for 5 min, preincubated in PB-BSA (5 min, 35°C), and transcription was initiated by adding a transcription mixture to give final concentrations of 100 μM of CTP, GTP, BrUTP, and 0.3 mM $MgCl_2$ (10 min, 35°C). Cells were fixed (20 min, 4°C) in 4% PFA in Sörensen buffer (0.1 M sodium/potassium phosphate buffer, pH 7.3) supplemented with 0.5% Triton X-100. BrRNA was immunolabeled as described above. Images were collected using a confocal laser scanning microscope (TCS SP; Leica) and processed as described in the first paragraph of Materials and methods.

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