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CREB-binding Protein (CBP)/p300 and RNA Polymerase II Colocalize in Transcriptionally Active Domains in the Nucleus

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Abstract. The spatial organization of transcription-associated proteins is an important control mechanism of eukaryotic gene expression. Here we analyzed the nuclear distribution of the transcriptional coactivators CREB-binding protein (CBP)/p300 in situ by confocal laser scanning microscopy, and in vivo complex formation by coimmunoprecipitation. A subpopulation of CBP and p300 is targeted to active sites of transcription and partially colocalizes with hyper- and hypophosphorylated RNA polymerase II (pol II) in discrete regions of variable size throughout the nucleus. However, the coactivators were found in tight association with hypophosphorylated, but not hyperphosphorylated pol II. Transcriptional inhibition induced a relocation of CBP/p300 and pol II into speckles. Moreover, double and triple immunofluorescence analyses revealed the presence of CBP, p300, and pol II in a subset of promyelocytic leukemia (PML) bodies. Our results provide evidence for a dynamic spacial link between coactivators of transcription and the basal transcription machinery in discrete nuclear domains dependent upon the transcriptional activity of the cell. The identification of pol II in CBP/PML-containing nuclear bodies supports the idea that transcription takes place at PML bodies.

Key words: transcription • coactivators • nuclear body • RNA polymerase II • promyelocytic leukemia

Introduction

The eukaryotic nucleus is dynamically organized with respect to particular activities such as transcription, RNA processing, and replication (Singer and Green, 1997; Lamond and Earnshaw, 1998; Schul et al., 1998a). The cellular organization of transcription-related factors seems to be particularly important in facilitating or regulating gene expression (Stenoien et al., 1998; Kornberg, 1999). In actively transcribing cells, nascent pre-mRNA transcripts localize in a nucleoplasm-wide meshwork pattern and in discrete foci of variable size, as visualized by immunofluorescence after nucleotide incorporation (Jackson et al., 1993; Wansink et al., 1993). RNA polymerase II (pol II)[1] localization coincides with these transcription sites to a high degree during periods of active transcription (Iborra et al., 1996; Grande et al., 1997; Zeng et al., 1997; Schul et al., 1998b). In transcriptionally inactive cells pre-mRNA transcription is no longer detectable and pol II undergoes a dynamic relocation into a network of speckles (Zeng et al., 1997). Mammalian cell nuclei commonly contain 20–40 speckles (Fu and Maniatis, 1990; Spector, 1993). Although transcription of some genes can occur in or near the periphery of speckles (Xing et al., 1993; Zhang et al., 1994; Misteli and Spector, 1998), these structures have been suggested to function primarily as temporary storage sites or recycling centers for multiple factors required for mRNA biogenesis (Spector, 1993; Zeng et al., 1997; Lamond and Earnshaw, 1998; Misteli and Spector, 1998).

Promyelocytic leukemia (PML) protein–containing nuclear bodies (PML oncogenic domains; PODs, nuclear domain 10; ND10, or Kr bodies) also have been functionally linked to transcription, e.g., of viral genes (Maul, 1998), and other nuclear processes such as RNA processing, transport, and RNP assembly (Doucas and Evans, 1996; Sternsdorf et al., 1997; Maul 1998; Matera, 1999). PML bodies appear as 5–30 discrete punctate regions distinct from speckles after immunofluorescence staining (Brasch and Ochs, 1992). Direct evidence for a role of PML bodies in transcription comes from the demonstration of nascent pol II transcripts within this nuclear body (LaMorte et al., 1998). In contrast, other groups detected nascent RNA in the periphery, indicating that the surroundings of the PML

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[1]*Abbreviations used in this paper:* α-am, α-Amanitin; BrU, Bromouridine triphosphate, CBP, CREB-binding protein; pol II, RNA polymerase II; PML, promyelocytic leukemia.

nuclear bodies are the sites of transcriptional activity (Grande et al., 1996; Boisvert et al., 2000). Thus, it remains controversial, whether transcription is occurring in or near PML bodies.

CREB-binding protein (CBP) and p300 are structurally and functionally related transcriptional coactivator proteins that operate at the end points of a variety of signal transduction pathways, thereby modulating specific gene expression programs involved in cell growth, differentiation, homeostasis, and viral pathogenesis (for review see Janknecht and Hunter, 1996; Shikama et al., 1997). The ability of CBP and p300 to interact with multiple, signal-dependent transcription factors suggested that these coactivators function as signal integrators by coordinating complex signal transduction events at the transcriptional level (Chrivia et al., 1993; Arany et al., 1994; Arias et al., 1994; Kwok et al., 1994; Eckner et al., 1994; Lundblad et al., 1995). CBP and p300 have been proposed to mediate transcription induction via intrinsic and associated histone-acetylase activities, which may facilitate binding of nuclear factors to their target sites by destabilizing promoter-bound nucleosomes (Bannister and Kouzarides, 1996; Ogryzko et al., 1996; Yang et al., 1996). Additionally, CBP/p300 may also mediate target gene activation through association with active pol II complexes, thereby bridging upstream transcription factors with the general transcription machinery (Kee et al., 1996; Nakajima et al., 1997a,b; Neish et al., 1998; Cho et al., 1998).

To investigate step(s) in gene activity modulation in which CBP/p300 operate, we have compared the spatial distribution and the characteristics of the association of the coactivators with RNA polymerase II and active transcription in mammalian nuclei.

Materials and Methods

Cell Culture

HEp-2, HeLa, MCF-7, and T-24 cells were obtained from the American Tissue Culture Collection and grown as recommended. Transcriptional inhibition was induced by adding α-Amanitin (α-am; 10 μg/ml working concentration; Sigma-Aldrich) for 8 h.

Antibodies

Polyclonal rabbit antibodies CBP-C20, CBP-A22 (both from Santa Cruz Biotechnology, Inc.), CBP-5614, CBP-5729, and CBP-254-256 have been described previously (Arias et al., 1994; Kee et al., 1996; Bex et al., 1998; Doucas et al., 1999). Antibody 254-256 is directed against aa 634–648 of CBP (Arias et al., 1994), and was affinity purified from a peptide column for immunohistochemistry and immunoblots. mAb p300-CT (clone RW128; Upstate Biotechnology) has been described previously (Bex et al., 1998). Polyclonal rabbit antibodies p300-N15 and p300-C20 were purchased from Santa Cruz Biotechnology, Inc. To detect pol II, monoclonal antibodies mara3 (Patturajan et al., 1998; kindly donated by Bart Sefton, Salk Institute, La Jolla, CA), and 8WG16 (Thompson et al., 1989; kindly provided by Nancy Thompson, University of Madison, WI), were used. PML bodies were visualized in immunofluorescence studies with rat antibodies directed against the PML protein or the SP100 protein (kindly provided by Thomas Sternsdorf, Heinrich-Pette-Institute, Hamburg, Germany). The nuclear mitotic apparatus protein NuMa, coiled bodies, nucleoli, and centromeres were stained with human autoimmune sera obtained from the reference serum bank of the W.M. Keck Autoimmune Disease Center, The Scripps Research Institute.

Immunocytochemistry

Cells grown on coverslips were fixed by treatment with methanol at −20°C for 5 min followed by acetone (prechilled to −20°C) for 2 min, or by incubation in 2% paraformaldehyde for 20 min at room temperature followed by acetone treatment (prechilled to −20°C) for 2 min. Immunofluorescence was performed as previously described (von Mikecz et al., 1997). For dual or triple immunofluorescence staining, primary antibodies from different sources (mouse, rabbit, human, or rat) were used simultaneously and detected with species-specific secondary antibodies linked to fluorescein, rhodamine, or Cy5 (Jackson ImmunoResearch Laboratories).

Confocal Microscopy and Scatter Diagrams

Samples were scanned with a Zeiss LSM 510 laser scanning confocal device attached to an Axioplan 2 microscope using a 63× Plan-Apochromat oil objective (Carl Zeiss, Inc.), or on a Fluoview laser scanning system from Olympus. Fluorescein, rhodamine, or Cy5 dyes were excited by laser light at a 488-, 543-, or 633-nm wavelength, respectively. To avoid bleed-through effects in double or triple staining experiments, each dye was scanned independently using the multitracking function of the LSM 510 unit. Single optical sections were selected either by eye-scanning the sample in z axis for optimal fluorescence signals, or taken from stack projections. Images were electronically merged using the LSM 510 (Carl Zeiss, Inc.) software and stored as TIFF files. Figures were assembled from the TIFF files using Adobe Photoshop software. Colocalization scatter diagrams were generated with the LSM 510 software. A scatter diagram represents a comparison of pixel coincidence between two images (i.e., red and green). All pixels having the same position in both images are considered a pair. Of every pair of pixels (P1, P2) from the two source images, the brightness level of pixel P1 is interpreted as the x coordinate, and that of pixel P2 as the y coordinate of the scatter diagram. The value of the pixel thus addressed is increased by one every time, up to the maximum number of pixels used. This way, each pixel of the scatter diagram is a value that shows how often a particular pair of pixels has occurred. Two completely overlapping images would result in a straight, diagonal line from bottom left to top right of the scatter diagram. Accordingly, two proteins displaying a high degree of colocalization would produce diagonal club-like patterns from bottom right to top left.

In Situ Immunolabeling of Nascent RNA Transcripts

Bromouridine triphosphate (BrU) was used for detecting ongoing RNA synthesis in permeabilized HEp-2 cells by immunodetection with antibody against bromodeoxyuridine (Boehringer) as previously described (Zhang et al., 1999), according to protocols developed by Jackson et al. (1993) and Wansink et al. (1993).

Immunoprecipitation and Immunoblotting

Immunoprecipitations were performed with HEp-2 whole cell lysates according to protocols described by Kee et al. (1996). A rabbit anti–mouse bridging antibody (Jackson ImmunoResearch Laboratories) was added in precipitations using mAbs. Immunoprecipitates were resuspended in SDS loading buffer and analyzed by SDS-PAGE. For immunoblotting, gels were transferred onto nitrocellulose and specific proteins were visualized using enhanced chemiluminescence according to the manufacturer's instructions (Amersham Pharmacia Biotech).

Results

CBP and p300 Localize to Distinct Nuclear Domains

To study the spatial relationship between CBP/p300 and pol II, several antibodies directed against different peptides from CBP, p300, and pol II were analyzed by immunofluorescence experiments on HEp-2 cells followed by confocal laser scanning microscopy and by immunoblotting (Fig. 1). The epitopes recognized by antibodies used in this study are schematically depicted in Fig. 1 (left). Three antibodies (A22, C20, and 254-256) identified CBP in a meshwork staining pattern throughout the nucleoplasm overlaid with several brighter foci of variable size (Fig. 1, micrographs, top), and with dot-like structures (Fig. 1, A22, inset). In addition, affinity-purified antibody

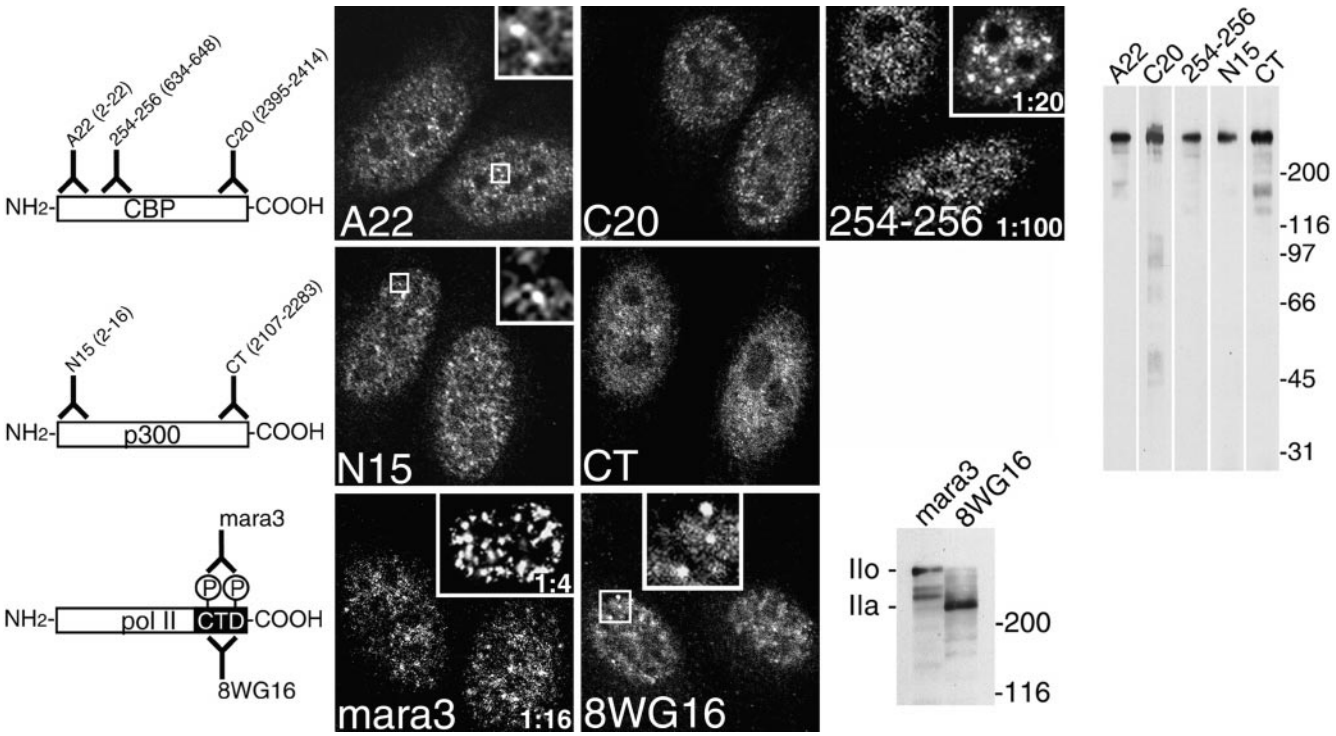

Figure 1. Specificity of anti-CBP/p300 and anti–pol II antibodies. (Left) Schematic representation of CBP, p300, and pol II primary structure (CTD, COOH-terminal domain; *P* denotes the phosphorylated CTD). The epitopes recognized by antibodies used in this study are indicated. (Center) For the micrographs, HEp-2 cells grown on coverslips were fixed and labeled with anti-CBP–specific antibodies A-22, C-20, and 254-256, p300-specific antibodies N15 and CT, or anti–pol II antibodies mara3 and 8WG16. Immunofluorescence staining was analyzed by confocal laser scanning microscopy. Single confocal sections are shown for each antibody as indicated. Areas marked by a rectangle are shown enlarged as insets in the respective images. Antibodies 254-256 against CBP and mara3 against phosphorylated pol II revealed speckle-like staining in a subpopulation of HEp-2 cell nuclei when used at higher antibody concentrations (insets; numbers indicate dilution factor). (Right) In immunoblots prepared from HEp-2 cell nuclear extracts, anti-CBP and anti-p300 antibodies recognized a single band migrating at ~250 kD. Mara3 predominantly recognizes hyperphosphorylated forms of pol II (IIo), whereas antibody 8WG16 predominantly recognizes hypophosphorylated pol II (IIa) in immunoblots of nuclear extracts.

254-256 detected CBP in a speckle-like pattern when used at higher working concentration (Fig. 1, top, 254-256, inset). The latter observation can be explained by the dependence of immunofluorescence staining on the working concentration of the primary antibody, as has previously been described in detail for other anti-speckle antibodies (Neugebauer and Roth, 1997). However, A22 and C20 did not decorate speckles when used at higher working concentration (data not shown). Antibodies N15 and CT revealed nuclear staining patterns of p300 that were very similar to those obtained for CBP (Fig. 1, micrographs, middle). Similar localization patterns of CBP and p300 staining were observed in different mammalian cell lines, including HeLa, MCF-7, and T24, and using different cell fixation protocols (von Mikecz, A., and P. Hemmerich, unpublished results). The specificity of anti-CBP and anti-p300 antibodies was confirmed by immunoblotting on nuclear extracts from HEp-2 cells (Fig. 1, immunoblot, right). All antibodies recognized a single protein band at ~250 kD corresponding to the molecular weight of CBP and p300 (Chrivia et al., 1993; Eckner et al., 1994).

Nuclear distribution of pol II was detected with mAb mara3, which specifically targets phosphorylated epitopes in the COOH-terminal domain of pol II (pol IIo, Patturajan et al., 1998), and 8WG16, which detects hypophosphorylated forms of pol II (pol IIa, Thompson et al., 1989; Bregman et al., 1995). Both pol II isoforms are believed to have distinct functional roles in transcriptional initiation (pol IIa) and elongation (pol IIo; Dahmus, 1995). The transition of pol II from initiation to elongation is associated with phosphorylation of the COOH-terminal domain of subunit IIa (Payne et al., 1989). Mara3 and 8WG16 produced a broad staining of the nonnucleolar portion of the nucleus of HEp-2 cells overlaid with discrete sites of preferred staining (Fig. 1, micrographs, bottom). In addition, at higher antibody concentrations, mara3 stained speckle-like structures (Fig. 1, micrograph, bottom, mara3, inset). We did not observe speckle-like staining of HEp-2 cells using 8WG16 (data not shown). In addition to the broad nucleoplasmic staining, 8WG16 revealed ~3–10 bright dot-like foci in >50% of HEp-2 cells (Fig. 1, micrograph, bottom row, 8WG16 and inset). Immunoblot analyses confirmed the specificity of the anti–pol II antibodies. Mara3 preferentially reacted with the hyperphosphorylated form of pol II (IIo), whereas 8WG16 predominantly recognized hypophosphorylated forms of pol II (IIa) (Fig. 1, immunoblot, bottom).

CBP/p300 Are Targeted to Active Sites of Transcription

Since CBP/p300 are believed to function as bridging molecules between transcription factors and the basal transcription machinery, we investigated the spatial relationship of the coactivators with the largest subunit of pol II and active sites of transcription. The staining patterns of pol IIo and pol IIa were compared with nucleoplasmic CBP distribution in double-labeling experiments, followed by confocal microscopy (Fig. 2, a and b). The overlay images show that the nucleoplasmic meshwork staining patterns of CBP partially overlapped with pol IIo and pol IIa (Fig. 2, a and b). CBP and pol II did not occupy the same discrete domains, rather they appeared to be present in individual irregularly shaped nucleoplasmic structures that partially overlapped (Fig. 2; see magnified areas in insets). The merged images also revealed multiple regions and dot-like structures of CBP and pol II that did not colocalize, indicating the existence of nuclear domains of exclusive localization of either CBP or pol II. We also analyzed the nuclear distribution of CBP with respect to transcription sites by in situ transcriptional run-on experiments (Fig. 2 c). Nascent transcripts in HEp-2 nuclei were labeled with BrU and detected with anti-bromodeoxyuridine antibodies. Double labeling revealed partial overlap between CBP staining and sites of transcription (Fig. 2 c). However, many domains exclusively contain CBP and no or little nascent RNA, and vice versa. Similar results were obtained when p300 labeling was compared with pol II staining or BrU incorporation (data not shown). No colocalization could be detected between CBP or p300 and NuMa, a 200-kD transcription-unrelated nuclear protein (Cleveland, 1995) that served as a control (Fig. 2 d and data not shown, respectively), indicating that the regions of overlap between CBP/p300 and pol II, or transcription, are not fortuitous.

Colocalization scatter diagrams were generated to assess the degree of colocalization between CBP and pol II, BrU incorporation, or NuMa. A scatter diagram repre-

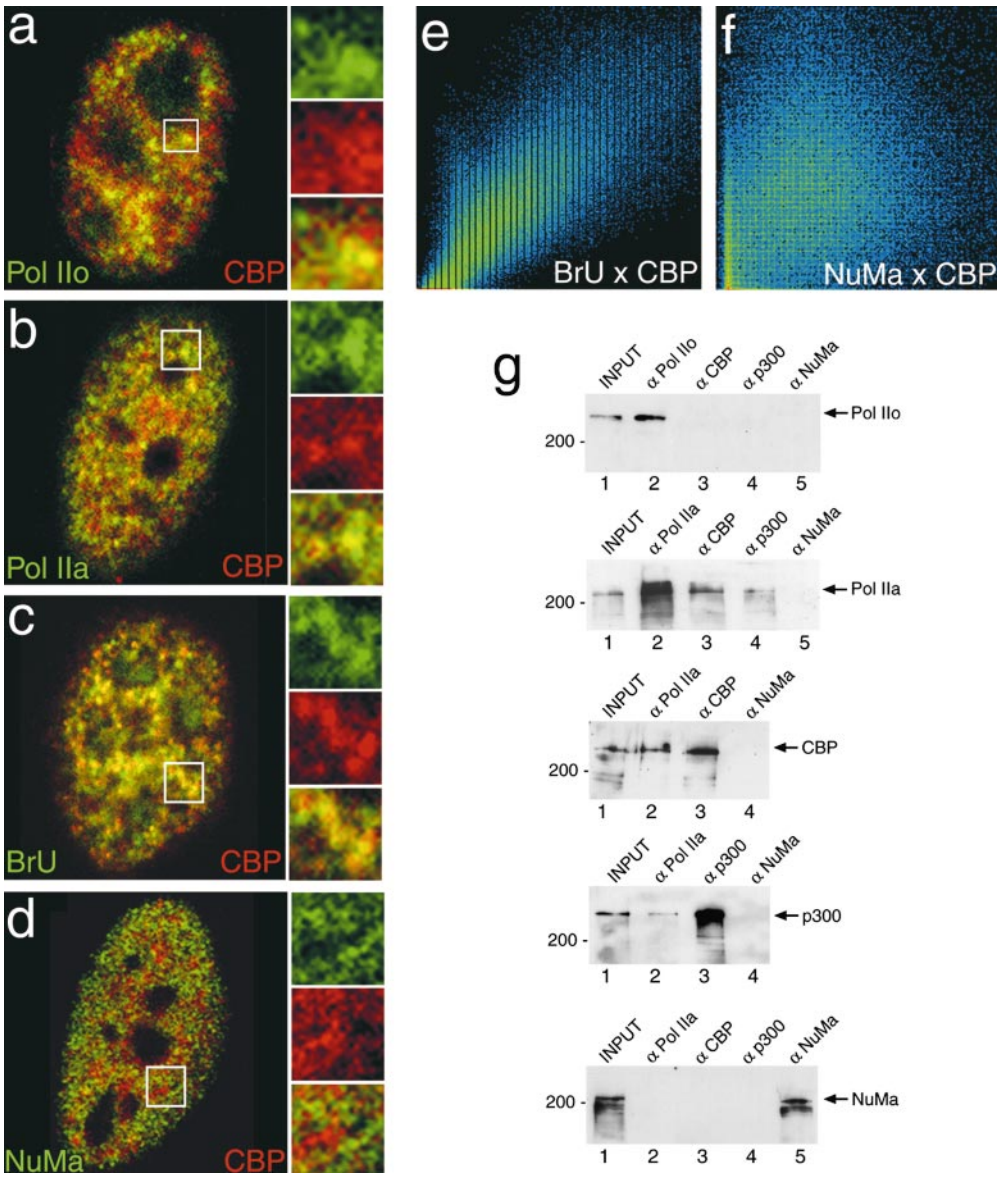

Figure 2. CBP/p300 is targeted to active sites of transcription. Fixed HEp-2 cells were double labeled with anti-CBP antibodies C20 (a–d, red) and mara3 against pol IIo (a, green), 8WG16 against pol IIa (b, green), anti-BrU antibody detecting nascent RNA after in situ incorporation of BrU (c, green), or antinuclear mitotic apparatus protein (NuMa) antibody (d, green). Single optical sections of each fluorochrome were recorded in independent scans. Rectangle-marked areas of each image are shown enlarged as green signal only (upper insets), red signal only (middle insets), or as the overlay image (bottom insets). The nucleolar BrU staining in c is presumably due to rRNA transcription by RNA polymerase I. (e, f), Colocalization scatter diagrams were generated from the cells shown in c and d, respectively. (g) Western blot analysis of pol IIo, pol IIa, CBP, p300, and NuMa recovered from immunoprecipitates of HEp-2 cell protein lysates using mara3 (α-Pol IIo), 8WG16 (α-Pol IIa), anti-CBP (α-CBP), anti-p300 (α-p300), or anti-NuMa (α-Numa) antibody. In each case 20% of input extract (INPUT) is shown.

sents a comparison of pixel coincidence between two images (see Materials and Methods). The scatter diagram of BrU/CBP staining shows a club-shaped distribution from bottom left to upper right, indicative of a considerable spatial coincidence between CBP and BrU staining (Fig. 2 e). In contrast, the scatter diagram application of NuMa/CBP was very diffuse, indicating little overlap between NuMa and CBP distribution (Fig. 2 f). Scatter diagrams generated from pol IIo/CBP and pol IIa/CBP comparisons also showed club-like distributions (data not shown).

To test whether the colocalization between CBP/p300 and pol II correlates with in vivo complex formation of these proteins we performed coimmunoprecipitation studies on protein lysates from HEp-2 cell nuclei. This analysis revealed that pol IIo is not present in immunoprecipitates from CBP or p300 antibodies (Fig. 2 g, row 1, lanes 3 and 4). In contrast, ~10–20% of pol IIa was recovered from CBP or p300 immunoprecipitates (Fig. 2 g, row 2, compare lane 2 with lanes 3 and 4, respectively). A similar fraction of endogenous CBP or p300 was recovered from pol IIa immunoprecipitates (Fig. 2 g, rows 3 and 4). As a control, anti-NuMa immunoprecipitation complexes did not contain pol II, CBP, or p300 protein (Fig. 2 g, rows 1–4, lane 5). NuMa was not detected in immunoprecipitates of pol IIa, CBP, or p300 (Fig. 2 g, row 5, lanes 2–4). Taken together, these results suggest that a subpopulation of CBP and p300 is in close vicinity to sites of mRNA transcription and in tight association with hypo-, but not hyperphosphorylated pol II. Thus, although CBP/p300 colocalized with pol IIo and pol IIa in situ, the coactivators appear to participate only in pol II a–containing complexes in vivo.

Dynamic Relocation of CBP/p300 into Speckles

In transcriptionally inhibited cells, factors involved in mRNA biogenesis including pol II redistribute to a classical speckle distribution pattern (Spector, 1993; Bregman et al., 1995). A speckled pattern of pol II localization has been shown to be characteristic of poorly transcribing or transcriptionally inactive cells because they fail to show incorporation of BrU into nascent mRNA in nuclear run-on experiments (Zeng et al., 1997). α-am, a compound that completely blocks pol II–mediated transcription (Lindell et al., 1970), was used to experimentally induce the redistribution of pol II into speckle domains (Fig. 3). In HEp-2 cells treated with α-am (10 μg/ml), CBP (detected by antibody 254-256) redistributed into speckle structures where it strongly colocalized with pol IIo and splicing factor SC35 (Fig. 3, top and middle). A subpopulation of p300 exhibited a similar redistribution into pol II–containing speckles in transcriptionally inhibited cells (Fig. 3, bottom). However, it should be noted that CBP and p300 were not detected in all pol II–containing speckles and vice versa, thus indicating a heterogeneity of the molecular composition of speckles with respect to the coactivators. Pol II could not be detected in speckle structures of transcriptionally inhibited cells using antibody 8WG16 (Fig. 4 h). This result indicates that speckle-bound pol II is hy-

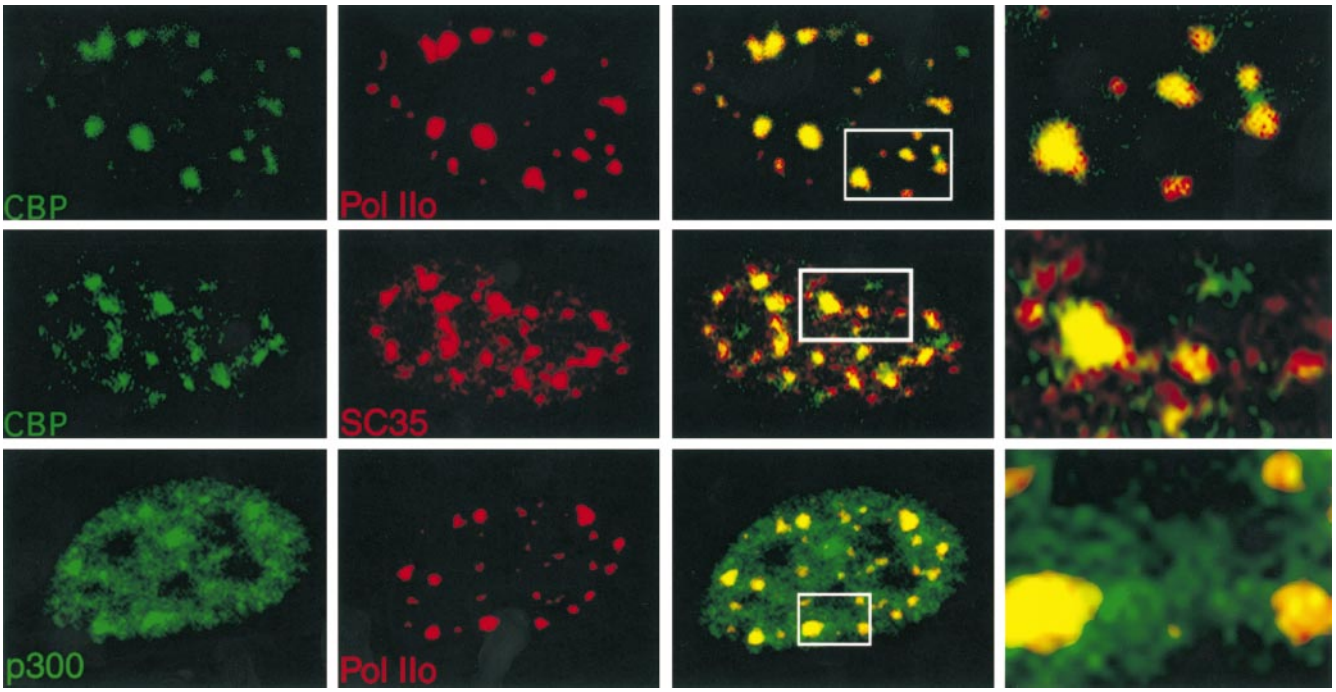

Figure 3. Transcriptional inhibition causes a redistribution of CBP and p300 in HEp-2 cells. HEp-2 cells were α-am–treated (10 μg/ml) to arrest transcription and processed for double labeling using the following antibody combinations: top row, 254-256 against CBP (green) and mara3 against pol II (red); middle row: 254-256 against CBP (green) and anti-SC35 antibody (red); bottom row: N-15 against p300 (green) and mara3 against pol II (red). Single confocal sections are shown. Areas marked by a rectangle are shown enlarged on the right side of each overlay image. Note that CBP was stained with antibody 254-256 which preferentially recognizes CBP in speckles (see Fig. 1).

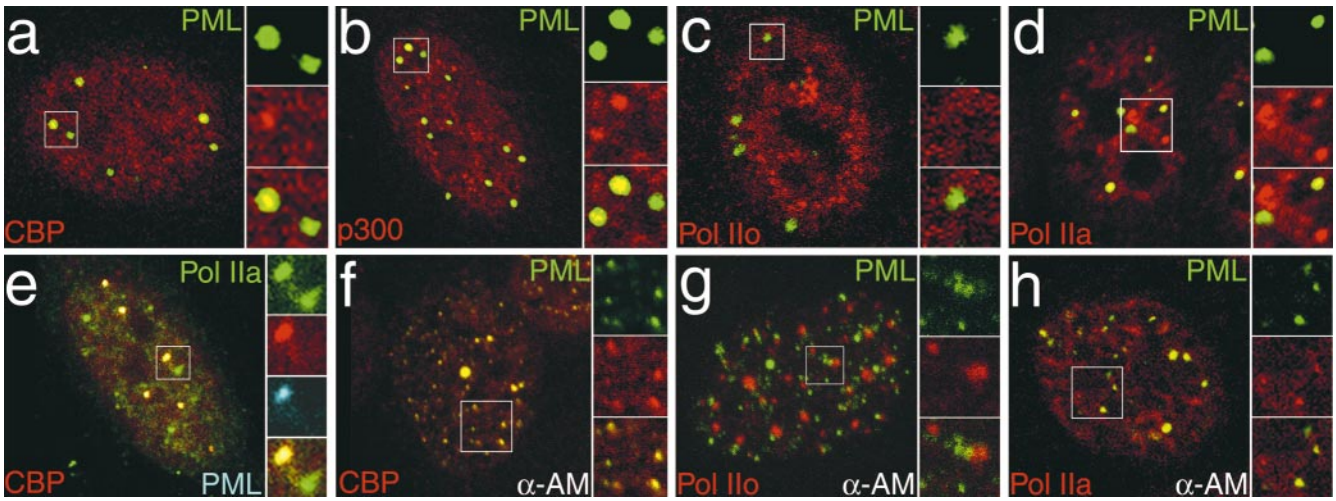

Figure 4. PML bodies contain CBP, p300 and pol II. HEp-2 cells were double labeled with a rat antibody against the PML protein (a–d and f–h, green; e, blue), and antibodies CBP-A22 (a, e, and f, red), p300-N15 (b, red), mara3 against pol IIo (c and g, red), and 8WG16 against pol IIa (d and h, red). In e, triple fluorescence is shown with the following antibody combination: 8WG16 against pol IIa (as detected with fluorescein-labeled anti–mouse antibody, green), A22 against CBP (as detected with rhodamine-labeled anti–rabbit antibody, red), and anti-PML antibody (as detected with Cy5-labeled anti–rat antibody, blue pseudo-colored). In f–h, cells were treated with α-AM (10 μg/ml). Areas marked by a rectangle are shown enlarged on the right side of each overlay image as insets. Each inset represents one dye only, except for the bottom insets, which represent the merged image. Note that CBP was stained with antibody A22, which preferentially recognizes CBP in PML bodies (see Fig. 1).

perphosphorylated, which is in agreement with prior observations (Bregman et al., 1995). Taken together, these results suggest that a fraction of CBP and p300 is dynamically redistributed together with pol IIo into speckled domains in transcriptionally inactive cells.

PML Bodies Contain CBP, p300, and pol II

CBP has been shown recently to be recruited by PML protein to nuclear bodies in which both proteins may directly participate in modulation of nuclear receptor–induced transcriptional activation (Doucas et al., 1999). In addition, nascent RNA has been detected within LaMorte et al. (1998), or in the periphery of PML bodies (Grande et al., 1996; Boisvert et al., 2000). These results and our observation that a subfraction of CBP and p300 colocalizes with pol II in discrete nuclear domains prompted us to test whether p300 and pol II are also present in PML bodies. Colocalization of CBP with the PML bodies was analyzed in double-labeling experiments using antibody A22 to detect dot-localized CBP. The merged image and the color-separated enlargements shown in Fig. 4 a confirmed the presence of CBP in PML bodies of HEp-2 cell nuclei, but also identified PML bodies that contained little or no CBP (Fig. 4 a, insets). Using antibody against an NH_2-terminal peptide of p300 we were also able to identify the p300 coactivator in PML protein–containing nuclear bodies (Fig. 4 b). Similar to CBP, p300 was present in only a subset of PML bodies (Fig. 4 b, merged image and insets). These results indicate that the molecular composition of PML bodies can vary with respect to the transcriptional coactivators CBP/p300. We did not observe colocalization of CBP or p300 with coiled bodies, the nucleolus, or centromeres (data not shown), indicating that nuclear substructure localization of the coactivators is restricted to PML protein–containing domains.

The spatial relationship of PML bodies and pol II was also analyzed in double-labeling experiments. Cells exhibiting the broad nucleoplasmic meshwork staining of pol IIo overlaid with sites of preferred localization produced by mara3 did not reveal any colocalizing structures (Fig. 4 c). In contrast, some of the dot structures labeled by antibody 8WG16 colocalized with PML protein–containing nuclear bodies (Fig. 4 d). However, some PML bodies do not contain pol IIa and some of the pol IIa–containing dot structures do not contain PML protein (Fig. 4 d, insets). Pol-IIa–containing nuclear dots, as detected with mAB 8WG16, also colocalized with some of the p300 dot-shaped foci (data not shown). The finding that only a subset of PML bodies colocalized with CBP/p300 or pol IIa raised the question whether the same nuclear bodies contain both the coactivators and the enzyme. We addressed this question by performing triple-labeling immunofluorescence experiments. This analysis demonstrated that CBP, pol IIa, and the PML protein can be detected in the same nuclear bodies (Fig. 4 e). Taken together these results show that the CBP/p300 coactivators are associated with pol IIa and PML protein in a subset of nuclear bodies and suggest that they may function as modulators of active transcription in or near these structures.

When transcription was arrested by α-am (10 μg/ml), PML protein dissociated into numerous smaller domains scattered throughout the nucleoplasm (Fig. 4, f–h). Dual labeling of such cells revealed that CBP, as detected with antibody A22, colocalized with these domains (Fig. 4 f). In drug-treated cells, speckle-associated RNA polymerase, as stained with mara3, did not colocalize with the scattered PML domains, although a few speckles can be seen to

overlap with PML protein-containing domains (Fig. 4 g). In contrast, a large number of PML foci appeared to contain pol IIa, as revealed with antibody 8WG16 (Fig. 4 h). These observations confirm the association of CBP, pol IIa, and PML protein in discrete nuclear domains and suggest that a subfraction of CBP and pol IIa is dynamically reorganized into many small PML protein–containing nuclear foci in transcriptionally inactive cells.

Discussion

The transcriptional coactivators CBP/p300 are believed to connect DNA-bound transcription factors to the transcription machinery (Kwok et al., 1994; Kee et al., 1996; Nakajima et al., 1997a,b; Neish et al., 1998; Cho et al., 1998). In addition, CBP/p300 have been proposed to induce transcription activation via intrinsic (Bannister and Kouzarides, 1996; Ogryzko et al., 1996) and associated (Yang et al., 1996) histone acetylase activities that may facilitate binding of nuclear factors to their target sites by destabilizing promoter-bound nucleosomes (Montminy, 1997). Thus, the coactivators may function as a transcriptional scaffold that enables the holoenzyme and regulatory molecules to be recruited to and assembled at relevant sites of transcriptional activity (Shikama et al., 1997; Parvin and Young, 1998; Torchia et al., 1998).

Here, we show by confocal microscopy that the nuclear distribution of CBP/p300 partially coincides with that of hyper- and hypophosphorylated pol II and active sites of transcription. The pattern and degree of colocalization between CBP/p300 and pol II throughout the nucleoplasm was very similar to the colocalization characteristics of CBP/p300 and sites of transcription. Colocalization studies of transcription factors with sites of BrU incorporation or pol II domains frequently reveal a partial overlap (van Steensel et al., 1995; Grande et al., 1997; Schul et al., 1998b; Stenoien et al., 1998). Obviously, CBP/p300 coactivators share this property with some of these transcription factors (Grande et al., 1997), and also other chromatin remodeling coactivator complexes, such as human SWI/SNF (Reyes et al., 1997). There is a high degree of colocalization between hyper- and hypophosphorylated pol II with sites of nascent RNA, indicating that both forms of RNA polymerases are in close vicinity to ongoing transcription (Grande et al., 1997; Zeng et al., 1997; Schul et al., 1998b). These observations and the finding that CBP/p300 (and other transcription-related factors) are associated with only a limited number of BrU foci (and/or pol II domains) suggest that the coactivators are dynamically recruited to a subfraction of nuclear sites with transcriptional activity at a given time. In such subfractions of nuclear sites, CBP/p300 might also associate with specific transcription-related factors. For example, BRCA1, a tumor suppressor protein with properties of a transcription factor, associates functionally and colocalizes partially with p300 in interphase nuclei of HeLa cells (Pao et al., 2000). Moreover, a subfraction of BRCA1 has been found to copurify with pol II in immunoprecipitation analyses (Scully et al., 1997). CBP/p300 domains that are not spatially related to active transcription may serve as storage, or supply sites, or be involved in other transcription-related events such as initiation complex regeneration.

It is believed that pol IIa participates in initiation-competent forms of pol II complexes and pol IIo in elongation-competent forms (Payne et al., 1989; Dahmus, 1995). Although both pol IIo and pol IIa colocalized with CBP/p300 in the nucleoplasm, only pol IIa was found in complex with the coactivators in vivo (Fig. 2). This observation confirms the finding that p300 specifically interacts with the nonphosphorylated form of purified pol II complexes (Cho et al., 1998), and that CBP copurifies with pol II complexes precipitated with anti–pol IIa antibody (Kee et al., 1996). Taken together, CBP may serve as a bridging/coactivating/chromatin-remodeling factor during transcriptional initiation, but may be separated from phosphorylated pol II during elongation. In vivo, the interaction between CBP/p300 and pol IIa seems to be mediated either by RNA helicase A (Nakajima et al., 1997a,b), or by direct binding of the COOH-terminal half of CBP/p300 (Cho et al., 1998). The recent identification of "zinc bundles" as putative protein–protein interaction modules in the COOH-terminal CH3 domain of CBP/p300 (Newton et al., 2000) suggests that this domain may contribute to the interaction between the coactivators and pol IIa.

When transcription was arrested by specific inhibitors, CBP/p300 redistributed to pol IIo–containing minimal domains corresponding to speckles (Fig. 4). Speckles are characterized by an accumulation of factors involved in mRNA biogenesis, mainly splicing factors (Spector, 1993). Our results and other studies indeed revealed colocalization of CBP with splicing factor Sm and SC-35 in speckle structures, but not in the more diffusely stained compartment of the nucleoplasm (Bex et al., 1998; Fig. 3, and data not shown, respectively). However, we were not able to detect Sm protein or other splicing factors in immunoprecipitates of CBP, suggesting that there is no functional relationship between splicing and CBP (von Mikecz, A., and M. Montminy, unpublished results). It has been proposed that speckles may function as storage places supplying active gene loci with factors needed for mRNA biogenesis (Antoniou et al., 1993; Gui et al., 1994; Misteli et al., 1997; Zeng et al., 1997; Lamond and Earnshaw, 1998; Misteli and Spector, 1998). If speckles serve as depots for CBP/p300 in transcriptionally inactive cells, it is conceivable that the "stored" coactivators are dynamically recruited together with pol II from speckles to active loci of transcription (or other sites) throughout the nucleoplasm in transcriptionally active cells.

We also demonstrated that CBP/p300 colocalize with pol IIa in a subset of PML bodies. Although a variety of nuclear processes have been suggested to take place at or near PML bodies, the function of these structures is unknown (Doucas and Evans, 1996; Sternsdorf et al., 1997; Maul, 1998; Matera, 1999). They do not seem to be major sites of splicing factor localization (Grande et al., 1996); instead, proteins associated with these nuclear domains show properties of transcriptional modulators (Doucas and Evans, 1996; Bloch et al., 1999; Doucas et al., 1999). Most importantly, LaMorte et al. have identified nascent RNA within nuclear bodies, including PML bodies, by in vivo nucleic acid labeling (1998). However, it was shown by analytical transmission microscopy that PML nuclear bodies do not accumulate RNA (Boisvert et al., 2000), but that newly synthesized RNA is associated with the periph-

ery of the PML bodies (Grande et al., 1996; Boisvert et al., 2000). The contradictory results might be due to (a) usage of different techniques and (b) different cell lines, since PML body number, structure, composition, and thus function can vary considerably depending on the cell type (Maul, 1998). The presence of PML protein, CBP, and pol IIa in the same PML bodies (Fig. 4) suggests that they may serve as supply or recycling sites of preformed initiation complexes for transcription of gene loci that are localized adjacent to PML bodies. Such complexes may be recruited from the core to PML body–associated genes to facilitate and regulate the expression of these genes. A similar model has recently been proposed for coiled bodies (Schul et al., 1998b).

Several viral transforming factors, including the adenoviral E1A protein, the SV40 T antigen, and Tax from HTLV-1 interact with CBP/p300 to modulate transcriptional activity of target genes (for review see Shikama et al., 1997). Some of these proteins target PML bodies, causing a dramatic redistribution of PML body proteins, including PML itself, to transcription sites scattered throughout the nucleoplasm (Doucas et al., 1996; Maul, 1998). Viral proteins may disrupt transcription of PML body–specific genes and/or target transcription (initiation) complexes containing PML, CBP/p300, and pol II within the PML bodies in order to recruit such complexes to genes whose expression is important for efficient virus propagation. We are confident that identification of the genes expressed at PML bodies will provide further insight into PML nuclear body function and molecular mechanisms of virus propagation and evasion of the host immune defense as well.

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