Recruitment of human cyclin T1 to nuclear bodies through direct interaction with the PML protein

**Alessandro Marcello^{1,2,3}, Aldo Ferrari²,
Vittorio Pellegrini², Gianluca Pegoraro^{1,2},
Marina Lusic¹, Fabio Beltram² and
Mauro Giacca^{1,2}**

¹Molecular Medicine Laboratory, International Centre for Genetic Engineering and Biotechnology (ICGEB), Area Science Park, Padriciano 99, 34012 Trieste and ²NEST-INFM and Scuola Normale Superiore, Piazza dei Cavalieri 7, 56126 Pisa, Italy

³Corresponding author
e-mail: marcello@icgeb.org

Human cyclin T1, the cyclin partner of Cdk9 kinase in the positive transcription elongation factor b (P-TEFb), is an essential cellular cofactor that is recruited by the human immunodeficiency virus type 1 (HIV-1) Tat transactivator to promote transcriptional elongation from the HIV-1 long terminal repeat (LTR). Here we exploit fluorescence resonance energy transfer (FRET) to demonstrate that cyclin T1 physically interacts *in vivo* with the promyelocytic leukaemia (PML) protein within specific subnuclear compartments that are coincident with PML nuclear bodies. Deletion mutants at the C-terminal region of cyclin T1 are negative for FRET with PML and fail to localize to nuclear bodies. Cyclin T1 and PML are also found associated outside of nuclear bodies, and both proteins are present at the chromatinized HIV-1 LTR promoter upon Tat transactivation. Taken together these results suggest that PML proteins regulate Tat-mediated transcriptional activation by modulating the availability of cyclin T1 and other essential cofactors to the transcription machinery.

Keywords: cyclin T1/FRET/HIV-1/PML/P-TEFb/Tat

Introduction

P-TEFb (positive transcription elongation factor b) is a general elongation cofactor for RNA polymerase II (RNAP II)-directed transcription (see Price, 2000; and references therein). The factor is composed of the cyclin-dependent kinase Cdk9 associated with cyclin T1, or with one of the other related members of the cyclin T family, including cyclin T2a, T2b or cyclin K (Peng *et al.*, 1998); its transcriptionally inactive form also associates with the regulatory small nuclear RNA 7SK (Nguyen *et al.*, 2001; Yang *et al.*, 2001). Both *in vitro* and *in vivo*, P-TEFb is able to phosphorylate the C-terminal domain (CTD) of RNAP II, a molecular event associated with increased transcriptional processivity (Price, 2000). P-TEFb was identified originally in *Drosophila melanogaster* as a kinase required for the transcription of several genes (Marshall and Price, 1992). In mammalian cells, its role was highlighted initially by the association of cyclin T1

with the human immunodeficiency virus type 1 (HIV-1) Tat transactivator (Wei *et al.*, 1998). A tripartite complex is formed between Tat, cyclin T1 and the *cis*-acting transactivation-responsive region (TAR) present at the 5' end of each viral mRNA. The formation of the P-TEFb–Tat complex is an essential step towards the assembly of the processive RNAP II machinery at the long terminal repeat (LTR) promoter (Bieniasz *et al.*, 1998; Fujinaga *et al.*, 1998; Garber *et al.*, 1998; Zhou *et al.*, 1998), probably through the release of negative elongation factors from the RNAP II CTD (see Garber and Jones, 1999, and references therein). Beside its functional interaction with P-TEFb, Tat associates with the transcriptional co-activator and acetyltransferase p300 and with the highly homologous cAMP-responsive element binding protein (CREB)-binding protein (CBP) as well as with different basal transcription factors (Marcello *et al.*, 2001b).

Transcriptional regulation in mammalian cells is a highly dynamic process, requiring temporal and spatial coordination of functional protein complex assembly (Stein *et al.*, 2000; Carmo-Fonseca, 2002). In addition, transcription overlaps extensively with downstream processes such as capping, termination and splicing of nascent mRNA (Hirose and Manley, 2000). Several of the factors participating in these events are found localized in specific subnuclear compartments. Among these, nuclear speckles are irregularly shaped regions of the nucleoplasm rich in splicing factors. It has been proposed that nuclear speckles are storage/assembly sites for pre-mRNA splicing factors and that splicing factors are recruited from these compartments to sites of active transcription (Misteli, 2001). Other subnuclear compartments of increasing interest are those defined by the accumulation of the promyelocytic leukaemia protein (PML) and are alternatively called PML nuclear bodies, ND10 or PODs (for PML oncogenic domains). In addition to the PML protein, the nuclear bodies contain other different proteins, among which several are known to participate in transcriptional regulation, including p300/CBP, p53, pRb, HTLV-1 Tax and Sp1 (recently reviewed in Zhong *et al.*, 2000; Negorev and Maul, 2001); increasing evidence suggests that some of these proteins shuttle between nuclear bodies and the nucleoplasm (Phair and Misteli, 2000; Boisvert *et al.*, 2001). In this scenario, nuclear bodies represent the compartment where post-transcriptional modification of these proteins, such as acetylation/deacetylation or sumoylation, or proteasome-dependent degradation might occur (Khan *et al.*, 2001; Lallemand-Breitenbach *et al.*, 2001; Seeler and Dejean, 2001).

One of the most powerful emerging techniques to study formation of specific protein complexes inside the cell nucleus is based on fluorescence resonant energy transfer (FRET) between two interacting proteins (Selvin, 2000;

Day *et al.*, 2001; van Roessel and Brand, 2002). Recently, we have used FRET to study the interaction of human cyclin T1 with HIV-1 Tat. We observed, also in agreement with another study (Herrmann and Mancini, 2001), that cyclin T1 resides in specific foci inside the nucleus. We also noticed that these cyclin T1 foci are often juxtaposed, but not exactly coincident, with nuclear speckles, and that overexpression of Tat determines recruitment of cyclin T1 outside of these compartments (Marcello *et al.*, 2001a).

Here we demonstrate, biochemically and by FRET analysis, that cyclin T1 interacts with the PML protein *in vivo* within specific subnuclear compartments that are coincident with PML nuclear bodies. The results obtained suggest a model by which Tat-mediated assembly of an active transcription complex is regulated by the nuclear bodies through the modulation of the availability of cyclin T1 as well as of other transcriptional cofactors at the site of transcription.

Results

Co-localization of human cyclin T1 and PML in nuclear bodies

Several nuclear proteins are known to be present in specific subnuclear compartments. We and others (Herrmann and Mancini, 2001; Marcello *et al.*, 2001a) recently have observed that human cyclin T1 is found in the nucleus in a speckled pattern. These cyclin T1 foci are coincident with those defined by the localization of the non-RNP protein SC35, the hallmark of nuclear speckles. However, cyclin T1 subnuclear domains appear distinct from nuclear speckles, being, in most cases, spatially juxtaposed (Marcello *et al.*, 2001a). Another important subnuclear compartment is the one defined by the localization of the PML protein. To understand what is the relationship between PML nuclear bodies and cyclin T1 foci, we tested the co-localization of PML and enhanced green fluorescent protein (EGFP)–cyclin T1, a protein that retains the same functional activity and localization as the wild-type protein (Marcello *et al.*, 2001a). We found that the two proteins overlapped extensively inside the nucleus of both HL3T1 and U2OS cells, showing the formation of large nuclear bodies, a characteristic that is peculiar to overexpressed PML (Figure 1, rows A and B). Localization of cyclin T1 in nuclear bodies was not a consequence of PML transfection, since endogenous PML was also found to co-localize with EGFP–cyclin T1 in the same domains (Figure 1, row C). Additionally, endogenous cyclin T1 was also observed to reside in specific nuclear compartments, some of which clearly co-localize with those containing endogenous PML (row D). Finally, haemagglutinin (HA)-tagged cyclin T1, probed with an anti-HA antibody, also showed the same nuclear pattern as PML (Figure 1, row E), ruling out a possible unspecific effect of EGFP. Co-localization in nuclear bodies appears specific for this protein pair, since the localization of other proteins that normally reside in the nucleoli (fibrillarin, Figure 1, row F) or in nuclear speckles (SF2/ASF, Figure 1, row G) was not altered by the concomitant overexpression of PML-IV.

We have shown previously that cyclin T1 interacts inside the cells with the HIV-1 Tat protein, and that this interaction brings Tat to cyclin T1 foci (Marcello *et al.*,

2001a). We therefore tested the effect of PML transfection on Tat localization. In both HL3T1 (not shown) and U2OS cells, Tat-EGFP (the localization of which is typically nuclear with a prominent nucleolar accumulation; Marcello *et al.*, 2001a) instead was coincident with PML bodies upon overexpression of PML-IV (Figure 1, row H).

Taken together, these results suggest that specific protein interactions might be responsible for PML-mediated cyclin T1 and Tat recruitment to nuclear bodies. Visualization of their interactions is beyond the lower limits (~0.5 μ m) of light microscopy co-localization studies. Hence, other means are necessary to map the protein–protein bindings that occur *in vivo* in the cell nucleus.

Visualization of direct cyclin T1–PML interaction in human cells by FRET

FRET experiments were performed by transfection of human HL3T1 cells with plasmids expressing the EGFP-conjugated proteins together with blue variant of EGFP (BFP)–PML-IV. As a positive control we used the EGFP–cyclin T1:Tat–BFP protein pair that we previously have shown to be positive for FRET in the same experimental conditions (Marcello *et al.*, 2001a). FRET image analysis of individual transfected cells is shown in Figure 2A. Panels in row b show the intracellular distribution of fluorescence at 520 nm (the peak wavelength of EGFP emission) under excitation at 480 nm. In these conditions, most cells transfected with EGFP–cyclin T1:BFP–PML-IV (panel b1) showed the characteristic pattern already observed in Figure 1 and consisting of large nuclear foci of PML-IV and cyclin T1. FRET analysis was performed by comparing EGFP emission at 520 nm, following BFP excitation at 350 nm (Figure 2A, panels in row c), with that following excitation at 480 nm of the same cells (Figure 2A, panels in row b). In these conditions, samples expressing both EGFP–cyclin T1 and BFP–PML-IV scored positive for FRET, indicating direct interaction between the two proteins. In contrast, no FRET was observed between Tat and PML, despite localization of the latter protein in nuclear bodies. We also extended these experiments to probe the interaction of p300/CBP with PML. It is known that p300/CBP binds PML and resides in nuclear bodies (Doucas *et al.*, 1999; von Mikecz *et al.*, 2000). As shown in panel c4 of Figure 2A, EGFP–p300 was also found to produce FRET with BFP–PML *in vivo*. In contrast, control cells expressing fibrillarin–EGFP or EGFP–SF-2 were consistently negative for direct interactions. The detailed, quantitative analysis of at least 10 cells expressing each of the analysed protein pairs is presented in Figure 2B, showing the percentile distribution of FRET values.

Taken together, the FRET results indicate that physical interaction can be visualized in the cells between PML and cyclin T1, as well as between PML and p300. They also reinforce the notion that simple co-localization of two proteins is not indicative of direct interaction, as is the case for Tat and PML. To explain localization of Tat in nuclear bodies upon PML overexpression, it is conceivable that Tat is recruited to this subnuclear compartment through its interaction with cyclin T1. Alternatively, Tat could be recruited to nuclear bodies through its well-characterized

Fig. 1. Co-localization of human cyclin T1 and PML in nuclear bodies. In rows (A) and (B), HL3T1 and U2OS cells (as indicated) were transfected with pcDNA3-EGFP-cyclin T1 (EGFP, green) and pcDNA3-PML-IV (TRITC, red). In row (C), U2OS cells were transfected only with pcDNA3-EGFP-cyclin T1 (EGFP, green) to visualize endogenous PML (TRITC, red). In row (D), co-localization of endogenous cyclin T1 (Alexa fluor 488, green) and endogenous PML (Alexa fluor 594, red) is shown in HL3T1 cells. The inset shows an enlargement of two PML bodies, one of which also contains cyclin T1 (yellow dot). In row (E), U2OS cells were transfected with pcDNA3-HA-cyclin T1 (TRITC, red) and pcDNA3-PML (FITC, green). In row (F), control plasmid fibrillar-EGFP (green), which localizes into nucleoli, was co-transfected with pcDNA3-PML-IV (red). In row (G), another control plasmid expressing pEGFP-SF2/AFP (green) was co-transfected with pcDNA3-PML-IV (red). In row (H), U2OS cells were transfected with pcDNA3-Tat86-EGFP (green) and pcDNA3-PML-IV (red).

interaction with p300/CBP (Benkirane *et al.*, 1998; Hottiger and Nabel, 1998; Marzio *et al.*, 1998).

The C-terminus of cyclin T1 mediates the interaction with PML

The FRET data indicating specific association between cyclin T1 and PML were reinforced further by the results of a co-immunoprecipitation experiment. In Figure 3A, an antiserum against PML was able to immunoprecipitate at least a fraction of endogenous cyclin T1. Furthermore, cell transfection with c-Myc-tagged cyclin T1 and EGFP-tagged PML-IV followed by immunoprecipitation with anti-EGFP antibody resulted in specific co-immunoprecipitation of cyclin T1 (Figure 3B).

Cyclin T1 can be subdivided roughly into two major domains (Figure 4A). The N-terminus of the protein (amino acids 1–300) shares partial homology with other members of the cyclin T family (cyclin box) (Wei *et al.*, 1998). At its C-terminal boundary, it contains the Tat recognition motif (TRM) that includes a critical cysteine residue at position 261 present in human cyclin T1, but absent in its rodent homologue (Bieniasz *et al.*, 1998; Garber *et al.*, 1998). The N-terminal domain of cyclin T1 is also important for the protein–protein associations of the protein with Cdk9, NF- κ B, CIITA and c-Myc (Garber *et al.*, 1998; Kanazawa *et al.*, 2000; Barboric *et al.*, 2001; Eberhardy and Farnham, 2001). The C-terminus of cyclin T1 is less well characterized and contains a putative coiled-coil region (amino acids 379–430), a histidine-rich

Fig. 2. FRET analysis of protein–protein interactions *in vivo*. **(A)** Visualization of FRET. The plasmid constructs indicated on top of each column were transfected in HL3T1 cells; transfected cells were visualized by transmitted light in Nomarski configuration (panels in row a), by excitation at 480 nm and collection at 520 nm, showing EGFP fluorescence after direct EGFP excitation (panels in row b), and by excitation at 350 nm and collection at 520 nm, showing EGFP fluorescence after BFP excitation, indicating FRET (panels in row c). **(B)** Quantification of FRET. Fluorescent emission at 520 nm from individual cells transfected with the indicated constructs was recorded after excitation at 350 or 480 nm, and integrated intensities over the whole cell were evaluated. Plotted values (indicated by dots) represent the ratio between these two measurements; higher values indicate more efficient resonant energy transfer between BFP and EGFP. Ten consecutively analysed cells were considered for each transfection; both their individual fluorescence ratio and their percentile box-plot distribution are shown. Horizontal lines of the percentile box plot distribution of FRET values, from top to bottom, mark the 10th, 25th, 50th, 75th and 90th percentile, respectively.

domain (amino acids 506–530) and a C-terminal PEST sequence (amino acids 700–726). It has been shown recently that the C-terminus of cyclin T1 is involved in the association with the CTD of RNAP II (Fong and Zhou, 2000; Taube *et al.*, 2002). To define which regions of cyclin T1 interact with PML, the human cyclin T1 cDNA was used to construct both EGFP- and GST-tagged mutants, carrying progressive deletions from the C-terminus of the protein that specifically remove the above-mentioned domains one by one. As shown in Figure 4B, only the wild-type protein was fully competent for *in vitro* binding to PML-IV in GST pull-down assays, while the N-terminal region (amino acids 1–300), as well as a longer fragment containing the coiled-coil region (amino acids 1–495), had a markedly reduced binding capacity. The construct also containing the histidine-rich motif (amino acids 1–595) retained ~30–40% of binding capacity to PML-IV. However, the histidine-rich domain alone was not able to associate with PML-IV, since the

fragment containing amino acids 482–551 fused to GST was negative for binding (data not shown). These results indicate that binding to PML requires an intact C-terminal region of cyclin T1.

Cyclin T1 binds all PML isoforms

The PML gene was identified originally through its fusion with the RAR α gene in acute promyelocytic leukaemia. The PML protein contains three cysteine-rich zinc-binding domains, a RING-finger, two B-boxes (B1 and B2) and a putative coiled-coil domain that together form the RBCC motif (or tripartite motif, TRIM) (Jensen *et al.*, 2001). The RBCC motif is present in all PML isoforms and in the PML–RAR α oncogenic fusion protein (Figure 4C). In order to investigate whether the interaction between cyclin T1 and PML is isoform specific or common to all PML proteins, we analysed the interaction of cyclin T1 with the PML-IV isoform [previously PML3 (Jensen *et al.*, 2001)], with the PML-III isoform (previously PML-L) and with

Fig. 3. Co-immunoprecipitation of cyclin T1 with PML. (A) Endogenous PML and cyclin T1. Total cell lysates from 293 cells were used for immunoprecipitation with rabbit antisera against PML, cyclin T1 or with a control antiserum against human VEGF, as indicated. Western blot analysis was performed to reveal immunoprecipitated cyclin T1. (B) Transfected PML and cyclin T1. 293 cells were transfected with c-Myc-tagged cyclin T1 and EGFP-tagged PML-IV expression plasmids, as indicated. The top panel shows immunoprecipitation with an anti-EGFP antiserum followed by western blotting with an anti-c-Myc antibody to reveal immunoprecipitated c-Myc-cyclin T1. The bottom two panels are western blottings on total cell lysates from the same cells reacted with anti-c-Myc or anti-EGFP antibodies to reveal expression of the transfected plasmids.

the oncogenic form PML-RAR α . These three PML variants, which differ in their C-termini but maintain an intact RBCC motif, were all capable of binding cyclin T1 (Figure 4D), suggesting that the interaction domain lies within the RBCC region. In fact, the RBCC motif (amino acids 1–381) alone was able to associate with cyclin T1 *in vitro* (Figure 4D). We also assayed a deletion mutant of PML-III, PML Δ (216–333), lacking the coiled-coil domain, and another mutant, PML A3 RING, with a non-functional RING domain. While the latter behaved like the wild-type protein, the mutant in the coiled-coil domain lost its ability to associate with cyclin T1 (Figure 4D).

This experiment shows that the association of cyclin T1 with PML is not limited to a subset of PML isoforms and requires an intact coiled-coil domain.

Effect of deletions in cyclin T1 on protein localization and PML binding *in vivo*

To test whether localization of cyclin T1 to nuclear bodies is the direct consequence of its interaction with PML, we

tagged the above-described deletion mutants with EGFP and visualized subcellular protein localization in HL3T1 cells. In agreement with the previous *in vitro* observations, progressive deletions from the C-terminus of cyclin T1 dramatically modified localization of the protein *in vivo*. Unlike full-length cyclin T1, localization of mutants 1–495 and 1–300 was no longer speckled. These mutant proteins were found diffused in the nucleoplasm and, especially for the latter, the protein was also visible in the cytoplasm. Conversely, full-length EGFP-cyclin T1 localization in mouse embryonic fibroblasts lacking PML (PML $^{-/-}$ MEFs) appeared diffused in the nucleoplasm (Figure 5A).

We also analysed the presence of FRET between PML and the different cyclin T1 mutants. Consistent with the *in vitro* biochemical data, FRET was detected with the wild-type protein, but not with the mutants (shown for individual cells in Figure 5B and quantified in Figure 5C). It should be noted that mutant cyclin T1 proteins, dispersed in the nucleoplasm, also become speckled upon PML overexpression (row b in Figure 5B), even in the absence of FRET with PML. This result suggests that recruitment of cyclin T1 to nuclear bodies also occurs through other mechanisms in addition to direct PML binding.

We also took advantage of the possibility of quantifying FRET in different nuclear compartments (Marcello *et al.*, 2001a), to understand the precise localization of the sites of interaction of cyclin T1 with PML. Surprisingly, we found that the full-length protein showed FRET not only in the nuclear bodies, as expected, but also in the nucleoplasm (Figure 5D), demonstrating that interacting pairs of PML-cyclin T1 proteins are also found outside of the bodies, and in a region characterized by a much lower level of expression of the two proteins.

Effects of cyclin T1 localization on its transcriptional activity

Expression of cyclin T1 has been shown to increase the transcriptional activation of the HIV-1 LTR exerted by the Tat protein (Wei *et al.*, 1998). Figure 6A and B shows the effects of transfection of different concentrations of an expression vector for cyclin T1 on Tat transactivation in human HL3T1 cells and in CHO cells. The former cell line carries an integrated HIV-1 LTR and expresses endogenous levels of human cyclin T1. In the latter cell line, in which the HIV-1 LTR reporter was co-transfected, hamster cyclin T1 bears a mutation in the Tat-binding domain that impairs Tat transactivation (Bieniasz *et al.*, 1998; Garber *et al.*, 1998). Thus, rodent cells represent a valuable tool to assess the effects of co-expressed human cyclin T1 with limited interference from the endogenous protein. In both experimental settings, transfection of increasing amounts of cyclin T1, together with a limiting amount of Tat, generated a peculiar transcriptional response, consisting of a remarkable increase of transcription at low concentrations of cyclin T1 followed by a progressive decrease at higher amounts of the protein (Figures 6A and 5B). The slopes of the two transcriptional response curves were slightly different in the two cell lines, consistent with the presence of an endogenous level of cyclin T1 in HL3T1 cells.

Fig. 4. *In vitro* mapping of the cyclin T1-PML association. (A) Schematic representation of human cyclin T1 and deletion mutants used in this work. Positions of the Tat recognition motif (TRM) in cyclin T1 and of Cys261 critical for Tat binding are indicated. Several other potential functional domains in cyclin T1 have been described, including the cyclin box, a coiled-coil region (coil), a histidine-rich domain (his) and a C-terminal PEST sequence (PEST). (B) PML-IV binds the C-terminus of cyclin T1. Each binding reaction contained 2–5 μ g of GST fusions immobilized to glutathione-CL4 beads and *in vitro* translated, 35 S-labelled PML-IV in NETN buffer. After binding at 4°C, beads were washed extensively prior to loading onto a 10% SDS-polyacrylamide gel. Bound material is indicated as a percentage of input material. (C) Schematic representation of human PML-IV, PML-III and PML-RAR α . R, RING finger domain; RBCC motif (RING + B-boxes + coiled-coil). Alterations of PML-III present in the mutants used for this experiment are also indicated. (D) Cyclin T1 binds within the coiled-coil domain of the RBCC motif of PML. Binding reactions were conducted as described in (B).

One possible explanation for the bell-shaped effect of cyclin T1 on Tat transactivation might be related to the subcellular localization of cyclin T1, and in particular to its propensity to accumulate in nuclear bodies upon overexpression (Marcello *et al.*, 2001a). Consistent with this interpretation, we found that in cells lacking PML (PML $^{-/-}$ MEFs), transcriptional activation by full-length cyclin T1 (the localization of which is diffuse in the nucleus of these cells, see Figure 5A) increased at concentrations at which transactivation by the wild-type protein began to decrease (Figure 6C). Cyclin T1 mutant 1–300 behaved similarly in wild-type cells (Figure 6D), as expected by its pattern of localization (Figure 5A).

These results indicate that the subcellular localization of cyclin T1 contributes to the regulation of LTR transcription.

Both cyclin T1 and PML associate with the HIV-1 LTR following Tat transactivation

FRET quantification of cyclin T1 and PML interaction indicates that these two proteins are in equilibrium between the nucleoplasm and nuclear bodies (Figure 5D).

As far as the cyclin T1 interaction with Tat is concerned, we have already observed that this interaction is stronger outside of nuclear bodies (Marcello *et al.*, 2001a). These observations suggest that Tat might recruit cyclin T1 outside of nuclear bodies for transcriptional activation, and that PML itself, through its interaction with cyclin T1, might participate in the transcriptional process. Exploiting chromatin immunoprecipitation (ChIP), we have already observed that Tat-mediated transactivation determines recruitment of p300/CBP to the LTR (Marzio *et al.*, 1998), yet another protein that has been shown to localize in nuclear bodies (Bieniasz *et al.*, 1998; Garber *et al.*, 1998; Marcello *et al.*, 2001b). Three different genomic sites were investigated in HL3T1 cells. One region mapped on the HIV-1 LTR DNA (region LTR, encompassing the basal promoter and enhancer), and two regions in the lamin B2 gene domain (B48, close to a human origin of DNA replication and B13, 5 kb away from that origin; Giacca *et al.*, 1994) (Figure 7A). The competitive PCR quantifications, shown in Figure 7C–E, were carried out by the addition of increasing amounts of the multi-competitor to a fixed volume of immunoprecipitated DNA, followed

Fig. 5. *In vivo* analysis of the cyclin T1 C-terminal deletion mutants. (A) Subcellular localization of EGFP-cyclin T1 in human HL3T1 cells and PML^{-/-} MEFs. The same mutants depicted in Figure 4A were also fused to EGFP and transfected in HL3T1 cells. Cells were fixed in paraformaldehyde and analysed by confocal microscopy. (B) Visualization of the interaction between cyclin T1 deletion mutants and PML *in vivo* by FRET. HL3T1 cells were transfected with the cyclin T1 deletion mutants together with BFP-PML-IV. FRET was measured as indicated in Figure 2A. Despite PML-driven re-localization of the mutants into nuclear bodies (panels in row b, EGFP fluorescence), no FRET is observed between the deleted cyclin T1 proteins and PML-IV (panels in row c, FRET). (C) Quantification of FRET for the interaction between cyclin T1 deletion mutants and PML. Data were analysed and plotted as indicated for Figure 2B. (D) Quantification of FRET in different subnuclear compartments. NP, nucleoplasm; NB, nuclear bodies. Data were analysed and plotted as indicated for Figure 2B.

by PCR amplification of aliquots of the mixture with the appropriate primer pairs.

Analysis of protein interactions at the selected regions was performed in mock- and GST-Tat86-treated HL3T1 cells, by taking advantage of the property of this protein of being rapidly internalized by living cells in a transcriptionally active form (Tyagi *et al.*, 2001). Consistent with our previous results, immunoprecipitation with an antibody against cellular transcription factor USF resulted in 10-fold enrichment for the DNA segments encompassing both the LTR and B48 regions (not shown) (Giacca *et al.*, 1994; Marzio *et al.*, 1998; Zentilin *et al.*, 2001). In the absence of Tat, the anti-cyclin T1 and anti-PML antibodies failed to immunoprecipitate the LTR DNA segment as well as the other segments. After Tat treatment, a 5.8- and a 3.3-fold enrichment for this genomic region (but not for lamin B2) was observed using the anti-cyclin T1 and anti-PML antibody, respectively (shown in Figure 7F).

To prove that PML is also recruited to the viral LTR upon transcriptional activation of virus-infected cells, we applied the ChIP method to U1 cells, a cell line chronically infected with HIV-1 and bearing two copies of the HIV provirus that are transcriptionally silent in the absence of

stimulation (Folks *et al.*, 1988). After treatment of these cells with 1×10^{-7} M 12-*o*-tetradecanoylphorbol-13-acetate (TPA) for 5 h, a treatment that results in transcriptional activation and viral replication (Demarchi *et al.*, 1993), followed by ChIP with an anti-PML antibody, we observed a 2.3-fold enrichment for the DNA segment encompassing the LTR, but not for the control B13 region (the competitive PCR results are shown in Figure 7G and H, and are summarized in I).

Taken together, these results indicate that transcriptional activation of the integrated LTR *in vivo* is concomitant with the specific recruitment of both cyclin T1 and PML to the promoter region.

Discussion

Regulation of HIV-1 transcription is a complex event of remarkable pathological relevance that recapitulates general concepts of cellular transcription, with some peculiarities. The viral Tat transactivator functions as a highly unusual transcription factor that interacts with the stem-loop RNA structure (TAR) found at the 5' end of all viral transcripts. From there, on one side, Tat induces a

modification of chromatin at the HIV-1 LTR promoter, while on the other side it stimulates the formation of elongation-competent RNAP II complexes capable of processive transcription. Increased transcriptional elongation is the consequence of the interaction of Tat with P-TEFb, while Tat-induced chromatin remodelling is concomitant with the recruitment of p300/CBP (see Marcello *et al.*, 2001b, and references therein). Both of these activities of Tat are probably exerted by the transient formation of large subnuclear complexes at the site of viral transcription and must therefore be spatially and temporally regulated.

It has been demonstrated that p300/CBP localize to nuclear bodies, particularly when the PML protein is overexpressed (Doucas *et al.*, 1999; von Mikecz *et al.*, 2000). As shown in Figure 1, analogously to what was observed for p300, cyclin T1 is also recruited to PML nuclear bodies. This effect appears to be specific since other nuclear proteins such as fibrillarin and SF-2/ASF retain their nucleolar and speckled localization, respectively. By exploiting FRET with EGFP:BFP pairs, we demonstrate that co-localization of cyclin T1 and p300 with PML reflects a direct interaction between these proteins. In particular, the cyclin T1–PML interaction occurs via the C-terminal portion of cyclin T1 and involves the RBCC motif of PML. Accordingly, cyclin T1 deletion mutants unable to associate with PML *in vitro* are also less prone than the wild-type protein to form nuclear foci *in vivo*. More importantly, such mutants are negative for FRET with PML.

What is the functional consequence of the cyclin T1 localization to nuclear bodies on LTR transcription? We and others have observed that the cyclin T1-mediated up-regulation of transcription from the HIV LTR decreases by increasing the concentration of cyclin T1 in the assay (Figure 6A and B; see also Wei *et al.*, 1998). This correlates with the formation of subnuclear aggregates rich in cyclin T1 (Marcello *et al.*, 2001a). However, a cyclin T1 mutant unable to bind PML, but still retaining the Tat-binding domain as well as the Cdk9-binding domain present in the cyclin box, is able to transactivate the HIV LTR at doses that instead were inhibitory for the wild-type protein. This experiment suggests that aggregation of cyclin T1 into PML nuclear bodies is inhibitory for transcription.

FRET between PML and cyclin T1, as well as between PML and p300, is not restricted to nuclear bodies but also occurs in the nucleoplasm. This is in agreement with a

similar conclusion from data about the interaction between Tat and cyclin T1, which showed FRET values lower in nuclear bodies and higher in the nucleoplasm (Marcello *et al.*, 2001a). In agreement with other studies, which have

Fig. 6. Transcriptional activity of cyclin T1 on Tat-mediated LTR transactivation. **(A)** Titration of HA-tagged cyclin T1 on the HIV-1 LTR in HL3T1 cells, a HeLa derivative carrying an integrated LTR-CAT cassette. The experiments were performed by calcium phosphate transfection of a fixed limiting amount of pcDNA3-Tat86 (50 ng) and increasing amounts of pCMV-HA-CycT1. **(B)** Titration of HA-tagged cyclin T1 on the HIV-1 LTR in CHO cells. Cells were co-transfected with 500 ng of pU3R-III (LTR-CAT reporter), pcDNA3-Tat86 (100 ng) and increasing amounts of pCMV-HA-CycT1, as indicated. **(C)** Titration of EGFP-tagged cyclin T1 on the HIV-1 LTR in PML^{-/-}MEFs. Cells were co-transfected as in **(B)** using an LTR-luciferase reporter (500 ng). **(D)** Transcriptional activity of full-length cyclin T1 and of the 1–300 deletion mutant on the HIV LTR promoter. CHO cells were co-transfected as in **(B)**. Expression of the two constructs was monitored by immunoblot (anti-EGFP).

Fig. 7. Recruitment of cyclin T1 and PML to the HIV-1 LTR upon transcriptional activation. (A) Chromosomal regions analysed by quantitative ChIP. These include the HIV LTR and the B13 and B48 DNA segments in the lamin B2 gene domain, a single-copy region of the human genome. For each of these regions, specific primers were selected (small, converging arrows). The diagrams schematically indicate the location of relevant genomic elements (transcription start site and transcription factor-binding sites in the HIV LTR, lamin B2 gene 3' end and *ppv1* gene) with respect to primer localization. The localizations of transcription factor USF-binding sites in both the LTR and B48 are indicated. (B) Schematic representation of the multicompetitor DNA used for DNA quantification by competitive PCR. This DNA fragment contains all primer recognition sites arranged to generate PCR amplification products of sizes different from, but comparable with those obtained from the amplification of genomic DNA. (C–E) Results of quantitative ChIP in HL3T1 cells. Cells were treated by incubation with GST–Tat86 for 5 h and cross-linked with formaldehyde; chromatin was then sonicated and immunoprecipitated with specific antibodies against cyclin T1 (D) and PML (E). A fixed amount of total sonicated DNA (C) and immunoprecipitated DNA (D and E) was mixed with increasing concentrations of the multicompetitor, as indicated on top of each panel, and PCR-amplified with primers in the LTR, B13 and B48 regions. After amplification, products were resolved by PAGE, stained with ethidium bromide and photographed. Specific bands were quantified by densitometric analysis. The number of DNA molecules corresponding to each genomic region (shown in the tables on the right sides of each panel, together with the LTR/B13 and B48/B13 ratios) were calculated according to the principles of competitive PCR by evaluating the slope of the curve fitting the values corresponding to competitor:genomic ratios (Diviacco *et al.*, 1992). In total genomic DNA from HL3T1 cells, the LTR/B13 ratio is ~5, indicating that more than one copy of the LTR–CAT cassette is integrated in these cells. M, molecular weight markers; molec., number of DNA molecules; comp., competitor PCR product. (F) The graph reports the results obtained in the experiment shown in (C–E). Enrichment of the B48 (control) and LTR regions over B13 was normalized by dividing the experimental values by those obtained in total input DNA. Consistent results have been observed in at least three independent experiments. (G and H) Results of quantitative ChIP in HIV-infected U1 cells stimulated with TPA. U1 cells were treated with 1×10^{-7} M TPA for 5 h and then processed as outlined above. After ChIP with an anti-PML antibody, quantification of input and immunoprecipitated DNA was performed as described for HL3T1 cells. (I) The graph reports the results obtained in the experiment shown in (G) and (H). Enrichment of the LTR region after ChIP with anti-PML antibody was calculated after normalization for the DNA quantification values in total input DNA.

shown that the PML protein associates with active genes *in vivo* (Tsukamoto *et al.*, 2000; Shiels *et al.*, 2001), we also found that anti-PML antibodies specifically immunoprecipitate the LTR DNA in ChIP experiments after promoter activation. Since ChIP allows the quantitative recovery of direct and indirect protein–DNA and protein–protein complexes at a given genomic region, these experiments suggest that PML itself, in addition to cyclin T1, p300/CBP (Marzio *et al.*, 1998) and other cellular transcription factors, associates with the LTR upon transcriptional activation.

Taken together, these results are consistent with the notion that transcription itself might occur at the periphery of nuclear bodies (Boisvert *et al.*, 2000), and that different transcription cofactors are dynamically recruited from the bodies upon transcriptional activation. Consistent with this interpretation is also the recent observation that p300/CBP dynamically shuttles in and out of the bodies (Boisvert *et al.*, 2001). Forced expression of PML or of other proteins residing in nuclear bodies, e.g. cyclin T1 (Marcello *et al.*, 2001a; this study) or p300 (Doucas *et al.*, 1999; Marcello *et al.*, 2001b), might shift this

dynamic equilibrium toward the formation of larger bodies which do not participate in transcription.

In this framework, what is the actual role of nuclear bodies themselves? A wide variety of functions have been suggested so far for these structures, including transcriptional activation and repression, protein storage and interferon-induced antiviral defence (Zhong *et al.*, 2000; Everett, 2001; Negorev and Maul, 2001; Regad and Chelbi-Alix, 2001; and references therein). Recently, it has been suggested that PML nuclear bodies are mobile structures that recognize the assembly of protein–protein and protein–DNA complexes which appear ‘foreign’ or ‘suspect’ to the nucleus (Tsukamoto *et al.*, 2000; Muratani *et al.*, 2001) and drive unwanted proteins to a proteasome-dependent degradation pathway (Everett *et al.*, 1997; Lallemand-Breitenbach *et al.*, 2001; Mattsson *et al.*, 2001). This concept of regulating the availability of nucleoplasmic factors seems perfectly adaptable also for a role for nuclear bodies in the regulation of gene promoter activity. In this case, it is conceivable that nuclear bodies could ‘sense’ the assembly of factors on promoters and control the availability of certain key factors through direct protein–protein interactions.

Materials and methods

Plasmids and cells

pcDNA3-Tat-EGFP and pcDNA3-EGFP-cyclin T1 plasmids have been described previously (Marcello *et al.*, 2001a). pCMV-HA-CycT1 and pSF-1, containing full-length human cyclin T1 fused to GST, were a kind gift of K.A.Jones, San Diego. c-Myc-cyclin T1 was kindly provided by B.M.Peterlin, San Francisco. pEGFP-SF2/ASF and pFibrillarin-EGFP were generously provided by T.Misteli, Washington. pcDNA3-PML-IV and pcDNA3-PMLRAR α were kindly provided by S.Minucci, Milan. PSG5-PML-III isoform and mutants PMLstop381, PML Δ (216–333) and PML-A3-RING were kindly provided by H.de Thé, Paris. pGEX-PML-IV was obtained by PCR amplification of pcDNA3-PML-IV and cloning as an *Bam*HI–*Eco*RI fragment into pGEX-2T. pEGFP/BFP-PML-IV was obtained by subcloning the *Bam*HI–*Eco*RI fragments into the *Bgl*III–*Eco*RI sites of pEGFP/BFP-C1 (Clontech). CycT1 deletion mutants were obtained by PCR amplification of human cyclin T1 with primers specific for the 3′-deleted versions. The p300–EGFP construct, with EGFP fused at the C-terminus of the protein, was made by subcloning a *NorI*–*NheI* fragment, containing the whole p300 cDNA, into pQBI25fN1 (Qbiogene).

Cell lines used in this study were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. PML–/–MEFs were generously provided by P.P.Pandolfi, New York.

Immunofluorescence

Following paraformaldehyde fixation, cells were washed with 100 mM glycine and permeabilized with 0.1% Triton X-100 for 5 min. Primary antibodies against HA (Roche), PML and cyclin T1 (Santa Cruz) were incubated at 37°C for 1 h in a humidified chamber in phosphate-buffered saline with the addition of 1% bovine serum albumin and 0.1% Tween-20. Slides were mounted in Vectashield (Vector) and observed with a Zeiss Axiovert X100 confocal microscope. Images were acquired with LSM510 software.

Fluorescence resonance energy transfer

FRET experiments were conducted as previously described in detail (Marcello *et al.*, 2001a). FRET measurements were performed in two steps. First, EGFP emission was collected by integrating the fluorescence signal around 520 nm under EGFP excitation at 480 nm. Secondly, EGFP emission in the same frequency range was measured after excitation at 350 nm. Background was detected out of the cell under study for each frame and subtracted from the relevant fluorescent signal. Following this procedure, the ratio between the two measured EGFP emissions (data taken following excitation at 350 nm divided by those at 480 nm) provides the FRET signal. Data acquisition and analysis were performed

with Metamorph software. When evaluating FRET ratios, emission intensities were scaled to take into account the different detection times.

Recombinant GST fusion proteins, in vitro binding assays and co-immunoprecipitations

³⁵S-Labelled proteins were produced *in vitro* by using a coupled transcription–translation system (Promega) according to the manufacturer’s instructions. Pull-down experiments using GST-tagged proteins and co-immunoprecipitation experiments were performed as already described (Marzio *et al.*, 1998; Marcello *et al.*, 2000). Cyclin T1 and PML were immunoprecipitated with polyclonal antibodies (Santa Cruz) and cyclin T1 revealed by western blotting against cyclin T1. A control antiserum against human vascular endothelial growth factor (VEGF; Santa Cruz) was used as control for the immunoprecipitation reactions.

Chromatin immunoprecipitation

ChIP was performed essentially as previously described (Marzio *et al.*, 1998; Zentilin *et al.*, 2001). Briefly, upon 5 h treatment with recombinant GST–Tat protein (2 μ g/ml), HL3T1 cells were cross-linked with formaldehyde. U1 cells, chronically infected with HIV-1, were stimulated for 5 h with 10^{–7} M TPA and then treated with formaldehyde. After extensive washings, cross-linked DNA was sonicated down to fragments of ~500 bp and the protein–DNA complexes were immunoprecipitated with different antibodies (all from Santa Cruz). Quantification of the immunoprecipitated material was performed by competitive PCR on the eluted DNA after thermal reversion of protein–DNA cross-links. Primer sequences and amplification conditions for the B48 and B13 DNA segments in the lamin B2 genomic region have already been described (Giacca *et al.*, 1994). Primers designed as LTRs amplify the HIV-1 LTR region (from position –164 to +84). A multicompetitor DNA containing all the utilized primer sets (LTRdx/LTRsx, B13dx/B13sx and B48dx/B48sx) was constructed according to established recombinant PCR procedures (Diviacco *et al.*, 1992; Marzio *et al.*, 1998).

Acknowledgements

We thank B.Bozigrav and P.Faraci for excellent technical assistance, and C.Rubiolo and R.Cinelli for help in some experiments. This work was supported by grants from the AIDS Programme of the ISS to M.G. and A.M., from MIUR to M.G., A.M. and F.B., from INFN to V.P., and from HFSP to A.M. and V.P.

References

- Barboric,M., Nissen,R.M., Kanazawa,S., Jabrane-Ferrat,N. and Peterlin, B.M. (2001) NF- κ B binds P-TEFb to stimulate transcriptional elongation by RNA polymerase II. *Mol. Cell*, **8**, 327–337.
- Benkirane,M., Chun,R.F., Xiao,H., Ogryzko,V.V., Howard,B.H., Nakatani,Y. and Jeang,K.T. (1998) Activation of integrated provirus requires histone acetyltransferase. p300 and P/CAF are coactivators for HIV-1 Tat. *J. Biol. Chem.*, **273**, 24898–24905.
- Bieniasz,P.D., Grdina,T.A., Bogerd,H.P. and Cullen,B.R. (1998) Recruitment of a protein complex containing Tat and cyclin T1 to TAR governs the species specificity of HIV-1 Tat. *EMBO J.*, **17**, 7056–7065.
- Boisvert,F.M., Hendzel,M.J. and Bazett-Jones,D.P. (2000) Promyelocytic leukemia (PML) nuclear bodies are protein structures that do not accumulate RNA. *J. Cell Biol.*, **148**, 283–292.
- Boisvert,F.M., Kruhlak,M.J., Box,A.K., Hendzel,M.J. and Bazett-Jones,D.P. (2001) The transcription coactivator CBP is a dynamic component of the promyelocytic leukemia nuclear body. *J. Cell Biol.*, **152**, 1099–1106.
- Carmo-Fonseca,M. (2002) The contribution of nuclear compartmentalization to gene regulation. *Cell*, **108**, 513–521.
- Day,R.N., Periasamy,A. and Schaufele,F. (2001) Fluorescence resonance energy transfer microscopy of localized protein interactions in the living cell nucleus. *Methods*, **25**, 4–18.
- Demarchi,F., D’Agaro,P., Falaschi,A. and Giacca,M. (1993) *In vivo* footprinting analysis of constitutive and inducible protein–DNA interactions at the long terminal repeat of human immunodeficiency virus type 1. *J. Virol.*, **67**, 7450–7460.
- Diviacco,S., Norio,P., Zentilin,L., Menzo,S., Clementi,M., Biamonti,G., Riva,S., Falaschi,A. and Giacca,M. (1992) A novel procedure for

- quantitative polymerase chain reaction by coamplification of competitive templates. *Gene*, **122**, 313–320.
- Doucas, V., Tini, M., Egan, D.A. and Evans, R.M. (1999) Modulation of CREB binding protein function by the promyelocytic (PML) oncoprotein suggests a role for nuclear bodies in hormone signaling. *Proc. Natl Acad. Sci. USA*, **96**, 2627–2632.
- Eberhardy, S.R. and Farnham, P.J. (2001) c-Myc mediates activation of the cad promoter via a post-RNA polymerase II recruitment mechanism. *J. Biol. Chem.*, **276**, 48562–48571.
- Everett, R.D. (2001) DNA viruses and viral proteins that interact with PML nuclear bodies. *Oncogene*, **20**, 7266–7273.
- Everett, R.D., Meredith, M., Orr, A., Cross, A., Kathoria, M. and Parkinson, J. (1997) A novel ubiquitin-specific protease is dynamically associated with the PML nuclear domain and binds to a herpesvirus regulatory protein. *EMBO J.*, **16**, 566–577.
- Folks, T.M., Justement, J., Kinter, A., Schnittman, S., Orenstein, J., Poli, G. and Fauci, A.S. (1988) Characterization of a promonocyte clone chronically infected with HIV and inducible by 13-phorbol-12-myristate acetate. *J. Immunol.*, **140**, 1117–1122.
- Fong, Y.W. and Zhou, Q. (2000) Relief of two built-in autoinhibitory mechanisms in P-TEFb is required for assembly of a multicomponent transcription elongation complex at the human immunodeficiency virus type 1 promoter. *Mol. Cell. Biol.*, **20**, 5897–5907.
- Fujinaga, K., Cujec, T.P., Peng, J., Garriga, J., Price, D.H., Grana, X. and Peterlin, B.M. (1998) The ability of positive transcription elongation factor B to transactivate human immunodeficiency virus transcription depends on a functional kinase domain, cyclin T1 and Tat. *J. Virol.*, **72**, 7154–7159.
- Garber, M.E. and Jones, K.A. (1999) HIV-1 Tat: coping with negative elongation factors. *Curr. Opin. Immunol.*, **11**, 460–465.
- Garber, M.E., Wei, P., KewalRamani, V.N., Mayall, T.P., Herrmann, C.H., Rice, A.P., Littman, D.R. and Jones, K.A. (1998) The interaction between HIV-1 Tat and human cyclin T1 requires zinc and a critical cysteine residue that is not conserved in the murine CycT1 protein. *Genes Dev.*, **12**, 3512–3527.
- Giacca, M. et al. (1994) Fine mapping of a replication origin of human DNA. *Proc. Natl Acad. Sci. USA*, **91**, 7119–7123.
- Herrmann, C.H. and Mancini, M.A. (2001) The Cdk9 and cyclin T subunits of TAK/P-TEFb localize to splicing factor-rich nuclear speckle regions. *J. Cell Sci.*, **114**, 1491–1503.
- Hirose, Y. and Manley, J.L. (2000) RNA polymerase II and the integration of nuclear events. *Genes Dev.*, **14**, 1415–1429.
- Hottiger, M.O. and Nabel, G.J. (1998) Interaction of human immunodeficiency virus type 1 Tat with the transcriptional coactivators p300 and CREB binding protein. *J. Virol.*, **72**, 8252–8256.
- Jensen, K., Shiels, C. and Freemont, P.S. (2001) PML protein isoforms and the RBCC/TRIM motif. *Oncogene*, **20**, 7223–7233.
- Kanazawa, S., Okamoto, T. and Peterlin, B.M. (2000) Tat competes with CIITA for the binding to P-TEFb and blocks the expression of MHC class II genes in HIV infection. *Immunity*, **12**, 61–70.
- Khan, M.M. et al. (2001) Role of PML and PML-RAR α in Mad-mediated transcriptional repression. *Mol. Cell*, **7**, 1233–1243.
- Lallemant-Breitenbach, V. et al. (2001) Role of promyelocytic leukemia (PML) sumolation in nuclear body formation, 11S proteasome recruitment and As₂O₃-induced PML or PML/retinoic acid receptor α degradation. *J. Exp. Med.*, **193**, 1361–1371.
- Marcello, A., Massimi, P., Banks, L. and Giacca, M. (2000) Adeno-associated virus type 2 rep protein inhibits human papillomavirus type 16 E2 recruitment of the transcriptional coactivator p300. *J. Virol.*, **74**, 9090–9098.
- Marcello, A., Cinelli, R.A., Ferrari, A., Signorelli, A., Tyagi, M., Pellegrini, V., Beltram, F. and Giacca, M. (2001a) Visualization of *in vivo* direct interaction between HIV-1 TAT and human cyclin T1 in specific subcellular compartments by fluorescence resonance energy transfer. *J. Biol. Chem.*, **276**, 39220–39225.
- Marcello, A., Zoppe, M. and Giacca, M. (2001b) Multiple modes of transcriptional regulation by the HIV-1 Tat transactivator. *IUBMB Life*, **51**, 175–181.
- Marshall, N.F. and Price, D.H. (1992) Control of formation of two distinct classes of RNA polymerase II elongation complexes. *Mol. Cell. Biol.*, **12**, 2078–2090.
- Marzio, G., Tyagi, M., Gutierrez, M.I. and Giacca, M. (1998) HIV-1 tat transactivator recruits p300 and CREB-binding protein histone acetyltransferases to the viral promoter. *Proc. Natl Acad. Sci. USA*, **95**, 13519–13524.
- Mattsson, K., Pokrovskaja, K., Kiss, C., Klein, G. and Szekely, L. (2001) Proteins associated with the promyelocytic leukemia gene product (PML)-containing nuclear body move to the nucleolus upon inhibition of proteasome-dependent protein degradation. *Proc. Natl Acad. Sci. USA*, **98**, 1012–1017.
- Misteli, T. (2001) Protein dynamics: implications for nuclear architecture and gene expression. *Science*, **291**, 843–847.
- Muratani, M., Gerlich, D., Janicki, S.M., Gebhard, M., Eils, R. and Spector, D.L. (2001) Metabolic-energy-dependent movement of PML bodies within the mammalian cell nucleus. *Nat. Cell Biol.*, **4**, 106–110.
- Negorev, D. and Maul, G.G. (2001) Cellular proteins localized at and interacting within ND10/PML nuclear bodies/PODs suggest functions of a nuclear depot. *Oncogene*, **20**, 7234–7242.
- Nguyen, V.T., Kiss, T., Michels, A.A. and Bensaude, O. (2001) 7SK small nuclear RNA binds to and inhibits the activity of CDK9/cyclin T complexes. *Nature*, **414**, 322–325.
- Peng, J., Zhu, Y., Milton, J.T. and Price, D.H. (1998) Identification of multiple cyclin subunits of human P-TEFb. *Genes Dev.*, **12**, 755–762.
- Phair, R.D. and Misteli, T. (2000) High mobility of proteins in the mammalian cell nucleus. *Nature*, **404**, 604–609.
- Price, D.H. (2000) P-TEFb, a cyclin-dependent kinase controlling elongation by RNA polymerase II. *Mol. Cell. Biol.*, **20**, 2629–2634.
- Regad, T. and Chelbi-Alix, M.K. (2001) Role and fate of PML nuclear bodies in response to interferon and viral infections. *Oncogene*, **20**, 7274–7286.
- Seeler, J.S. and Dejean, A. (2001) SUMO: of branched proteins and nuclear bodies. *Oncogene*, **20**, 7243–7249.
- Selvin, P.R. (2000) The renaissance of fluorescence resonance energy transfer. *Nat. Struct. Biol.*, **7**, 730–734.
- Shiels, C., Islam, S.A., Vatcheva, R., Sasieni, P., Sternberg, M.J., Freemont, P.S. and Sheer, D. (2001) PML bodies associate specifically with the MHC gene cluster in interphase nuclei. *J. Cell Sci.*, **114**, 3705–3716.
- Stein, G.S., van Wijnen, A.J., Stein, J.L., Lian, J.B., Montecino, M., Choi, J., Zaidi, K. and Javed, A. (2000) Intracellular trafficking of transcription factors: implications for biological control. *J. Cell Sci.*, **113**, 2527–2533.
- Taube, R., Lin, X., Irwin, D., Fujinaga, K. and Peterlin, B.M. (2002) Interaction between P-TEFb and the C-terminal domain of RNA polymerase II activates transcriptional elongation from sites upstream or downstream of target genes. *Mol. Cell. Biol.*, **22**, 321–331.
- Tsakamoto, T., Hashiguchi, N., Janicki, S.M., Tumber, T., Belmont, A.S. and Spector, D.L. (2000) Visualization of gene activity in living cells. *Nat. Cell Biol.*, **2**, 871–878.
- Tyagi, M., Rusnati, M., Presta, M. and Giacca, M. (2001) Internalization of HIV-1 tat requires cell surface heparan sulfate proteoglycans. *J. Biol. Chem.*, **276**, 3254–3261.
- van Roessel, P. and Brand, A.H. (2002) Imaging into the future: visualizing gene expression and protein interactions with fluorescent proteins. *Nat. Cell Biol.*, **4**, 15–20.
- von Mikecz, A., Zhang, S., Montminy, M., Tan, E.M. and Hemmerich, P. (2000) CREB-binding protein (CBP)/p300 and RNA polymerase II colocalize in transcriptionally active domains in the nucleus. *J. Cell Biol.*, **150**, 265–273.
- Wei, P., Garber, M.E., Fang, S.M., Fischer, W.H. and Jones, K.A. (1998) A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. *Cell*, **92**, 451–462.
- Yang, Z., Zhu, Q., Luo, K. and Zhou, Q. (2001) The 7SK small nuclear RNA inhibits the CDK9/cyclin T1 kinase to control transcription. *Nature*, **414**, 317–322.
- Zentilin, L., Marcello, A. and Giacca, M. (2001) Involvement of cellular double-stranded DNA break binding proteins in processing of the recombinant adeno-associated virus genome. *J. Virol.*, **75**, 12279–12287.
- Zhong, S., Salomoni, P. and Pandolfi, P.P. (2000) The transcriptional role of PML and the nuclear body. *Nat. Cell Biol.*, **2**, 85–90.
- Zhou, Q., Chen, D., Pierstorff, E. and Luo, K. (1998) Transcription elongation factor P-TEFb mediates Tat activation of HIV-1 transcription at multiple stages. *EMBO J.*, **17**, 3681–3691.

Received November 8, 2002; revised February 7, 2003;
accepted March 6, 2003