The Cdk9 and cyclin T subunits of TAK/P-TEFb localize to splicing factor-rich nuclear speckle regions

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SUMMARY

TAK/P-TEFb is an elongation factor for RNA polymerase II-directed transcription that is thought to function by phosphorylating the C-terminal domain of the largest subunit of RNA polymerase II. TAK/P-TEFb is composed of Cdk9 and cyclin T and serves as the cellular cofactor for the human immunodeficiency virus transactivator Tat protein. In this study, we examined the subcellular distribution of Cdk9 and cyclin T1 using high resolution immunofluorescence microscopy and found that Cdk9 and cyclin T1 localized throughout the non-nucleolar nucleoplasm, with increased signal present at numerous foci. Both Cdk9 and cyclin T1 showed only limited colocalization with different phosphorylated forms of RNA polymerase II. However, significant colocalization with antibodies to several splicing factors that identify nuclear 'speckles' was observed for Cdk9 and especially for cyclin T1. The pattern of Cdk9 and cyclin T1 distribution was altered in cells treated with transcription inhibitors. Transient expression of cyclin T1 deletion mutants indicated that a region in the central portion of cyclin T1 is important for accumulation at speckles. Furthermore, cyclin T1 proteins that accumulated at speckles were capable of recruiting Cdk9 and the HIV Tat protein to this compartment in overexpression experiments. These results suggest that cyclin T1 functions to recruit its binding partners to nuclear speckles and raises the possibility that nuclear speckles are a site of TAK/P-TEFb function.

Key words: TAK, P-TEFb, Cdk9, Cyclin T, Speckles, RNA polymerase II, CTD, Transcription, Immunofluorescence, Deconvolution microscopy

INTRODUCTION

The generation of functional messenger RNA (mRNA) in eukaryotic cells is a highly regulated multistep process that involves transcription initiation, elongation, capping, splicing, polyadenylation and transport to the cytoplasm. The spatial organization of the nucleus to accommodate these varied processes is a subject of active research. Distinct subnuclear structures, such as 'speckles' and Cajal (coiled) bodies, can be visualized microscopically and are associated with increased concentrations of factors involved in transcription and pre-mRNA splicing (Spector, 1993; Lamond and Earnshaw, 1998; Matera, 1999). Whether these structures serve as active areas of mRNA synthesis or as storage/assembly areas for necessary factors has not been definitely resolved, but recent evidence indicates that they are highly dynamic and dependent on the transcriptional and RNA processing activity of the cell (reviewed by Misteli, 2000).

There is increasing evidence that the processes of transcription and mRNA processing are coordinated. Although much remains to be learned about the underlying spatial organization, it is becoming clear that the C-terminal domain (CTD) of the largest subunit of RNA polymerase II (Pol II) is important in effectively linking these events (Corden and Patturajan, 1997; Bentley, 1999; Hirose and Manley, 2000). The CTD is comprised of tandem heptapeptide repeats with the consensus sequence YSPTSPS, and is unique to RNA Pol II. The CTD appears to serve as a platform for the binding of cellular factors that may associate in a temporal manner dependent on the phosphorylation state of the CTD.

Multiple protein kinases have been identified that can phosphorylate the CTD, but the precise functions of many of these kinases during transcription are unclear, as are their spatial relationships to nuclear substructures. Two of the most highly investigated CTD kinase complexes are Cdk7-cyclin H and Cdk9-cyclin T. Both of these kinases are members of the cyclin-dependent kinase family, which require a cyclin partner for catalytic function. Cdk7-cyclin H is a component of the basal transcription factor TFIIH and functions at an early step in transcription (Zawel et al., 1995). The Cdk9-cyclin T complex is known as P-TEFb and was originally identified as a positive transcription elongation factor in *Drosophila* transcription extracts (Marshall and Price, 1995). It is thought that P-TEFb stimulates elongation by phosphorylating the CTD with the consequent dissociation of negative elongation factors from the CTD (Price, 2000).

P-TEFb activity is contained within several distinct complexes that differ by their cyclin regulatory subunit. Cdk9 has been shown to associate with three related members of the cyclin T family, T1, T2a and T2b, in vivo in mammalian cells (Wei et al., 1998; Peng et al., 1998), and with cyclin K in the yeast two-hybrid system (Fu et al., 1999). All of these complexes possess CTD kinase activity and stimulate transcription elongation in transcription systems in vitro. The

Cdk9-cyclin T1 complex, also known as TAK (Tat-associated kinase), is unique in that it acts as a cellular coactivator for human immunodeficiency virus (HIV) transcription (reviewed by Taube et al., 1999).

TAK was originally identified as a CTD kinase activity that specifically interacts with the activation domain of the HIV transactivator Tat protein (Herrmann and Rice, 1993; Herrmann and Rice, 1995). Tat interacts with a region of cyclin T1 known as the Tat recognition motif, which contains a critical cysteine residue not present in the cyclin T2 proteins (Garber et al., 1998; Bieniasz et al., 1998; Wimmer et al., 1999). Tat is essential in the HIV replication cycle for promoting the production of full-length viral transcripts (Jones and Peterlin, 1994; Karn, 1999). Tat recruits the TAK complex, via its interaction with cyclin T1, to its cis-acting RNA target, TAR, which is located at the 5′ end of nascent transcripts. Once positioned in proximity of the viral promoter, TAK is thought to hyperphosphorylate the CTD, thus permitting processive elongation of HIV transcription.

In addition to its critical role during HIV replication as the cellular cofactor for Tat, TAK/P-TEFb has been shown to function as a coactivator of transcription factor CIITA for the activation of the class II major histocompatibility complex (Kanazawa et al., 2000). Although TAK/P-TEFb was shown to be required for the transcription of a number of *Drosophila* genes in an in vitro system and to be recruited to the *Drosophila* heat shock locus (Marshall and Price, 1992; Lis et al., 2000), it is not clear if TAK/P-TEFb function is required for transcription of all mammalian genes or only a subset. Information is also lacking regarding the subcellular distribution of the TAK/P-TEFb subunits. As understanding of the spatial organization of the nucleus increases, determination of subnuclear localization can provide insights into functions of specific proteins.

To determine the spatial organization of TAK/P-TEFb, we analyzed the subcellular localization of the two known subunits, Cdk9 and cyclin T1, by immunofluorescence and deconvolution microscopy. We found that Cdk9 and cyclin T1 were present throughout the nucleoplasm, with the exception of the nucleolus, and clustered in speckle-like regions where their colocalization was most significant. Cdk9 and cyclin T1 showed only limited colocalization with RNA Pol II but showed significant colocalization with a number of splicing factors that produce a speckled labeling pattern. The distribution of Cdk9 and cyclin T1 was altered by inhibitors of RNA Pol II transcription. Transient expression of C-terminally truncated cyclin T1 mutants plasmids indicated that a region within the central portion of the protein is important for the association with speckles and, furthermore, that cyclin T1 proteins that accumulated at speckles could recruit Cdk9 and the HIV Tat protein to speckles. These results suggest that cyclin T1 functions to recruit its binding partners to nuclear speckles, and raise the possibility that nuclear speckles are a site of TAK/P-TEFb function.

MATERIALS AND METHODS

Antibodies

The Cdk9 antibody used in this study was produced in rabbits (Santa Cruz Biotechnology) and used at dilutions of 1:600 (0.17 μg/ml) and 1:5000 (0.02 μg/ml) for immunofluorescence and immunoblotting, respectively. Goat cyclin T1 and cyclin T2b antibodies (Santa Cruz Biotechnology) were used at dilutions of 1:100 (2 μg/ml) and 1:1000 (0.2 μg/ml) for immunofluorescence and immunoblotting, respectively. RNA polymerase antibody 8WG16 was purchased from Promega, H5 from Covance, and H14 was obtained from Steve Warren (Gilead). Splicing factor antibody SC35 (Sigma) was used at a dilution of 1:20,000 (0.23 μg/ml). Antibody to SRm160 (B1C8) (Wan et al., 1994) was generously provided by Jeff Nickerson (MIT), and antibodies Y12 (Lerner et al., 1981) and mAb104 (Roth et al., 1990) were kindly provided by Sue Berget (Baylor College of Medicine). Anti-HA antibody (Roche Molecular Biochemicals) was used at a dilution of 1:500 (0.8 μg/ml) and 1:5000 (0.08 μg/ml) for immunofluorescence and immunoblotting, respectively. Primary antibodies were detected using appropriate secondary antibodies conjugated to FITC or Alexa 488 (green), Texas Red or Alexa 594 (red), or Cy5. Secondary antibodies were purchased from Molecular Probes, Jackson Immunochemicals, and Roche Molecular Biochemicals and were generally used at a dilution of 1:600. Secondary antibodies for immunoblotting were purchased from Southern Biotechnology.

Plasmids and transfections

HA-cyclin T1 expression plasmids were kindly provided by Kathy Jones (Salk Institute) (Garber, et al., 1998) and Qiang Zhou (UC Berkeley) (Fong and Zhou, 2000). The GFP-Cdk9, GFP-Tat, and GFP-Pro18 mutants were constructed by standard techniques. We have confirmed that GFP-Cdk9 retains kinase activity (not shown). GFP-Tat encodes the 86 residue HIV type 1 Tat protein and GFP-Pro18 contains a 2 amino acid insertion between amino acids 18 and 19 of the Tat protein. This mutation has been shown to abrogate Tat function in Tat transactivation assays (Rice and Carlotti, 1990). We have confirmed that the GFP-Tat fusion protein, but not the GFP-Pro fusion protein, is functional in a Tat transactivation assay using an HIV-LTR luciferase reporter plasmid (not shown).

HeLa cells grown on coverslips in six-well dishes were transfected with 200 ng of the HA-cyclin T1 plasmids using Lipofectamine (Life Tech), following the manufacturer's protocol. Cells were fixed for immunofluorescence at 20-24 hours post-transfection. Expression of HA-cyclin T1 proteins was confirmed by immunoblot analysis using the HA antibody (not shown).

Preparation of cell extracts, immunoprecipitations and immunoblots

Cell extracts were prepared by EBC lysis as previously described (Herrmann and Rice, 1995) except that the EBC buffer (50 mM Tris-HCl pH 8.0, 250 mM NaCl, 0.5% Nonidet P-40, 5 mM dithiothreitol, aprotinin, leupeptin and phenylmethylsulfonyl fluoride) contained a higher salt concentration and cell extracts were frozen and thawed three times prior to clarification. Immunoprecipitations and immunoblots were performed by standard procedures as described previously (Herrmann et al., 1998).

Preparation of core nuclear matrix

Extraction of soluble proteins to reveal the residual nuclear matrix core was performed essentially as described previously (Mancini et al., 1994; Stenoien et al., 2000). Briefly, HeLa cells grown on coverslips on six-well dishes were washed in cold PBS and then incubated with CSK buffer (10 mM PIPES, pH 6.8, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100, aprotinin, leupeptin, phenylmethylsulfonyl fluoride and vanadyl ribonucleoside complex) for 3 minutes on ice. Chromatin was removed by digestion with RNAse-free DNase I (400U/ml) for 50 minutes at 30°C. Proteins were further extracted by the sequential dropwise addition of ammonium sulfate to a final concentration of 0.25 M, followed by addition of NaCl to a final concentration of 2 M. CSK-washed cells were then either prepared for immunofluorescence or lysed in SDS-PAGE sample buffer for immunoblot analysis.

Indirect immunofluorescence

HeLa cells were grown on coverslips in 6- or 24-well culture dishes. Cells were washed in ice-cold PBS and fixed in 4% formaldehyde in PEM buffer (80 mM PIPES, pH 6.8, 5 mM EGTA, and 2 mM $MgCl_2$) for 30 minutes on ice. Cells were permeabilized in PEM buffer containing 0.5% Triton X-100 for 30 minutes at room temperature and blocked in TBST buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.1% Tween) containing 5% nonfat dry milk for 1 hour at room temperature. Cells were incubated with primary antibodies at appropriate dilutions overnight at 4°C, followed by incubation with appropriate secondary antibodies for 40 minutes at room temperature. Cells were then fixed in 4% formaldehyde in PEM buffer, quenched in 1 mg/ml $NaBH_4$ in PEM, counterstained with 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI) in TBST for 1 minute, and mounted onto slides using ProLong Antifade (Molecular Probes) mounting media. Appropriate controls were conducted to demonstrate specificity of primary and secondary antibody staining. Peptides used to generate the rabbit Cdk9 and goat cyclin T1 antibodies completely blocked staining of the cognate antibody (data not shown).

Microscopy and image processing

Deconvolution microscopy was performed on a Carl Zeiss AxioVert S100 TV microscope and a DeltaVision Restoration Microscopy System. A Z-series of focal planes were digitally imaged and deconvolved with the DeltaVision constrained iterative algorithm (Agard, 1984;Swedlow et al., 1997). This process removes out-of-focus light and reassigns it to its point of origin to generate high resolution images. Multiple images containing several cells were collected and representative cells are shown. All images were digitally processed for presentation using Adobe Photoshop. Images shown are deconvolved and represent single Z-sections.

RESULTS

Cdk9 and cyclin T1 accumulate in speckle-like regions of the nucleus

Although TAK/P-TEFb function has been studied in transcription systems in vitro and transactivation assays in vivo (Marshall and Price, 1992; Zhu et al., 1997; Mancebo et al., 1997; Gold et al., 1998), the subcellular distribution of the TAK/P-TEFb subunits and their association with components of the transcription machinery at the single cell level has not been investigated. To approach this issue, we used immunofluorescence and deconvolution-based microscopy to link the increasingly appreciated importance of subnuclear compartmentalization to TAK/P-TEFb function. HeLa cells were fixed in formaldehyde, permeabilized and simultaneously immunolabeled with a rabbit Cdk9 antibody and a goat cyclin T1 antibody. Cdk9 and cyclin T1 were detected using appropriate secondary antibodies conjugated to green or red fluorochromes, respectively. A merge of the individual images shows the overlap between Cdk9 and cyclin T1 in yellow.

In interphase cells, Cdk9 and cyclin T1 were excluded from the nucleolus but distributed throughout the remainder of the nucleoplasm with increased signal at numerous foci (Fig. 1A, upper panels). Cdk9 and cyclin T1 exhibited significant colocalization. For Cdk9, in addition to nuclear staining, we observed a striking pattern of cytoplasmic labeling. However, cytoplasmic staining was not detected using another antibody that is directed at a different epitope of Cdk9 and which produced a similar nuclear pattern (data not shown). Furthermore, only nuclear localization was observed in cells transiently transfected with low amounts of Cdk9 expression plasmids (see below). The nuclear speckled pattern of cyclin T1 staining was also seen using another antibody directed at a different epitope of cyclin T1 (data not shown), and in cells transiently transfected with wild-type cyclin T1 expression vectors (see below). Therefore, both Cdk9 and cyclin T1 localize to the nucleus of interphase cells, concentrating in speckle-like structures.

In HeLa cells undergoing mitosis, both Cdk9 and cyclin T1 were dispersed uniformly throughout the cell, with the exception of the chromosomal regions, and showed some but incomplete overlap (Fig. 1A, lower panels). This pattern has been observed for other speckle-associated proteins in mitotic cells (Spector et al., 1991)

Although Cdk9 and cyclin T1 showed significant overlap, particularly in the speckle regions, they clearly did not colocalize at all sites. Cdk9 is known to be present in several other complexes that do not contain cyclin T1 (Peng et al., 1998; O'Keeffe et al., 2000), but we were somewhat surprised that not all the cyclin T1 appeared complexed with Cdk9. Using a biochemical approach, a previous study found no evidence for free P-TEFb subunits in HeLa cells (Peng et al., 1998). To address this issue, we depleted HeLa cell extracts of Cdk9 by successive immunoprecipitations using the rabbit Cdk9 antibody, and then performed immunoblots using either the Cdk9 or cyclin T1 antibodies. As shown in Fig. 1B (left panel, lanes 5,6), we did not detect Cdk9 or cyclin T1 in immunoprecipitates using the Cdk9 antibody from Cdk9-depleted extracts. However, we detected cyclin T1 in the Cdk9-depleted extracts and in cyclin T1 immunoprecipitates from the Cdk9-depleted extract (Fig. 1B, right panel, lanes 4,6), suggesting that at least a portion of cyclin T1 exists free of Cdk9.

The presence of Cdk9 and cyclin T1 in diffuse and speckled areas of the nucleus suggested to us that these proteins are present in two subpopulations that might differ in their solubility properties. When the nucleus is subjected to various extraction methods, an insoluble residue remains that has been termed the nuclear matrix or nucleoskeleton. Although a thorough understanding of this structure remains incomplete, the nuclear matrix has been proposed to function in organizing various activities that occur within the mammalian nucleus by serving as an attachment scaffold for proteins involved in DNA replication, transcription, splicing and mRNA transport (reviewed by Nickerson et al., 1995). When HeLa cells were treated to reveal this insoluble core structure (He et al., 1990; Stenoien et al., 2000), we found that although the majority of Cdk9 and cyclin T1 is released from the nucleus, a significant portion remains associated with the core (Fig. 2A, compare with Fig. 1A). The remaining Cdk9 and cyclin T1 colocalize at a significant number of foci. Partitioning with the insoluble fraction was also examined biochemically by incubating cells successively with Triton X-100-containing CSK buffer, DNase I and high salt buffers. The supernatants at each step and the remaining 'core' pellet were analyzed by immunoblotting probing with the Cdk9 and cyclin T1 antibodies (Fig. 2B). Consistent with the immunofluorescence results, a percentage of Cdk9 and cyclin T1 was present in the core fraction. This suggests that a subpopulation of Cdk9 and cyclin T1 associates with the insoluble nuclear compartment.

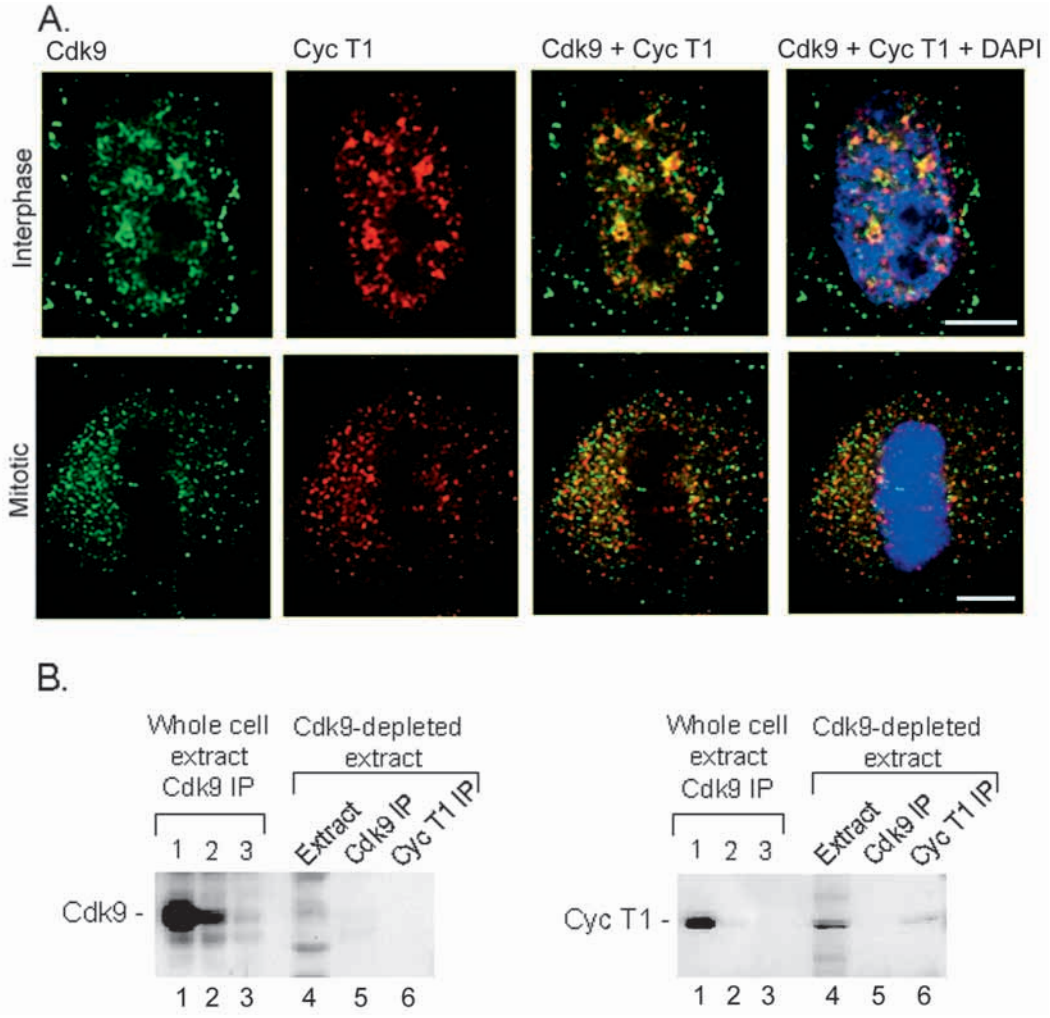

Fig. 1. Subcellular distribution of Cdk9 and cyclin T1 in interphase and mitotic cells. (A) Fixed and permeabilized HeLa cells were dual labeled with a rabbit antibody directed against Cdk9 and a goat antibody against cyclin T1. Cdk9 was detected using fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit secondary antibody and cyclin T1 was detected using an Alexa 594 (red)-conjugated donkey anti-goat secondary antibody. The panels marked Cdk9 + Cyc T depict the merge of the green (Cdk9) and red (cyclin T1) images. The areas of overlap appear as yellow. As shown in the upper panels, in interphase cells Cdk9 and cyclin T1 are present in numerous foci throughout the nucleoplasm but their overlap is greatest in areas with the highest signal. Signal was effectively competed using cognate antibodies including cytoplasmic labeling of Cdk9. The lower row shows Cdk9 and cyclin T1 present fairly uniformly throughout the non-chromosomal regions of mitotic cells. DNA was counterstained using DAPI. Images are deconvolved and represent a single Z-section. Bar, 5 μm. (B) To investigate whether cyclin T1 exists in cells free of Cdk9, HeLa whole cell extracts were depleted by three rounds of immunoprecipitation using the Cdk9 antibody. Products of each of these sequential immunoprecipitations are shown in first three lanes of both panels. The right three lanes (lanes 4-6) represent depleted extracts loaded directly on the gel (Extract, lane 4) or Cdk9-depleted extracts subjected to immunoprecipitation with either the Cdk9 (lane 5) or cyclin T1 (lane 6) antibody as indicated. Proteins were transferred to nitrocellulose and immunoblotted for Cdk9 (left panel) or cyclin T1 (right panel). Cyclin T1 was detected in Cdk9-depleted extracts.

Colocalization of Cdk9 and cyclin T1 with various phospho-forms of RNA Pol II

Current evidence points to the CTD of RNA Pol II as being a major physiological substrate of TAK/P-TEFb. Pol II exists in cells in different states of phosphorylation with the different forms being functionally distinct. The hypophosphorylated Pol II assembles into the pre-initiation complex, whereas the hyperphosphorylated form is associated with elongation complexes. Antibodies are available to distinguish between the different forms: 8WG16 binds preferentially to hypophosphorylated Pol II (Thompson et al., 1989), H14 recognizes multiple intermediately phosphorylated forms as well as the hyperphosphorylated form (Bregman et al., 1995), whereas H5 recognizes only the hyperphosphorylated Pol II protein (Bregman et al., 1995). When we analyzed the colocalization of Cdk9 and cyclin T1 with these various forms of RNA Pol II, we found that both Cdk9 and cyclin T1 were present at relatively few sites per focal plane with all three of the Pol II antibodies tested (Fig. 3). An enlarged image revealed that, in most cases, the labeling was not superimposed but rather the Cdk9 or cyclin T1 proteins existed in overlapping regions with Pol II. This result indicates that, although there is a limited degree of co-association of Cdk9 or cyclin T1 with RNA Pol II, the

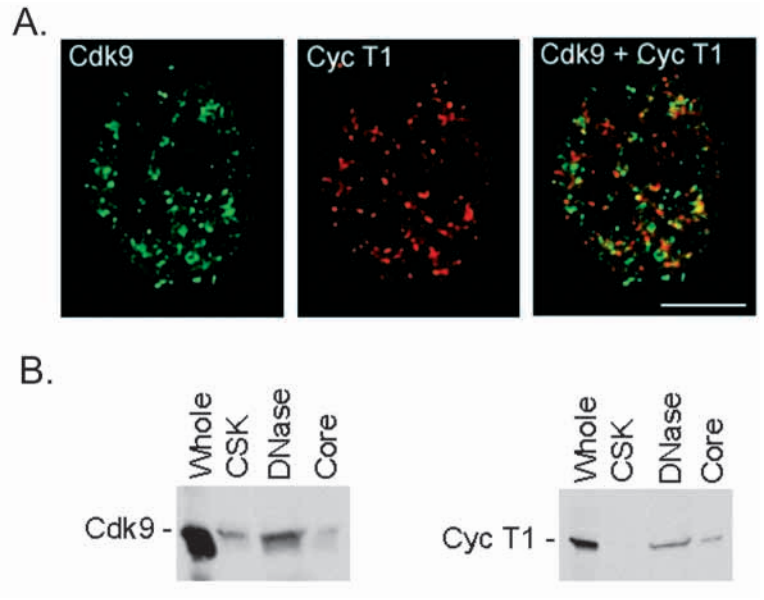

Fig. 2. Subpopulation of Cdk9 and cyclin T1 associates with the insoluble nuclear matrix. HeLa cells were incubated with CSK buffer containing 0.5% Triton X-100, treated with DNase I, and then subjected to sequential high salt extractions to reveal the insoluble nuclear matrix core structure. (A) Fully extracted cells were visualized by dual immunolabeling of Cdk9 (green) and cyclin T1 (red). This shows that a portion of Cdk9 and cyclin T1 associate with the nuclear matrix. Cells were counterstained with DAPI to verify removal of the vast majority of DNA (not shown). Bar, 5 μm. (B) Equal aliquots of supernatants following the CSK or DNase extraction steps were analyzed by immunoblotting to Cdk9 (left panel) or cyclin T1 (right panel). The lane marked core represents the DNA-depleted and RNA-rich pellet remaining after the final salt extraction. Consistent with the immunofluorescence images, a portion of Cdk9 and cyclin T1 was found in the core fraction.

majority of Cdk9 and cyclin T1 is not present at sites containing RNA Pol II.

Colocalization of Cdk9 and cyclin T1 with splicing factors

The speckled pattern of Cdk9 and cyclin T1 distribution was highly reminiscent of that seen using antibodies to a number of splicing factors. The term 'speckle' has been used to describe the 20 to 50 irregularly shaped domains seen in mammalian nuclei that are enriched in pre-mRNA processing components (reviewed by Spector, 1993). The SC35 antibody, which recognizes a non-snRNP splicing assembly factor of the ser-arg (SR) family and commonly serves to define nuclear speckles, was used in immunolocalization experiments to determine whether the regions containing the highest concentration of Cdk9 and cyclin T1 coincided with SC35-containing speckle regions. As seen in Fig. 4A, Cdk9 was present in the general vicinity of SC35, although direct overlap was limited. Cyclin T1, however, showed significant overlap and in some regions the areas of staining were superimposed extensively.

To determine whether the speckle pattern was specific to TAK (i.e. the Cdk9-cyclin T1 complex) or whether this distribution was shared by other P-TEFb complexes, we analyzed the localization of cyclin T2b and found that it gave a similar staining pattern to cyclin T1 and colocalized with SC35 (Fig. 4A). We have found that cyclin T2a is also present in speckle regions with a distribution similar to the other cyclin T proteins (data not shown). Therefore, all the different known cyclin T-containing P-TEFb complexes are found at speckle regions.

Spliceosomes consist of proteins from two general classes, the SR family and the Sm snRNP family. To determine whether cyclin T1 associates with components of the spliceosome in addition to SC35, we tested antibodies directed at a matrix-associated SR family protein (SR m160) or the Sm group of proteins (Y12), and an antibody that recognizes a phospho-epitope of SR proteins but does not give a speckled pattern of staining under the fixation method used in this study (mAb104). We found that cyclin T1 largely colocalized with antibodies SR160 and Y12, which gave a speckled labeling pattern, but did not extensively colocalize with 'dots' labeled with the mAb104 antibody (Fig. 4B). Collectively, these results indicate that Cdk9 and especially cyclins T1 and T2 are present at nuclear speckle domains.

Effects of transcriptional inhibitors on Cdk9 and cyclin T1 localization

When cells are treated with transcriptional inhibitors, splicing activity is reduced and speckles labeled with SC35 become fewer in number, enlarged and rounded (O'Keefe et al., 1994). We were therefore interested to learn whether the association of Cdk9 and cyclin T1 with speckles was dependent on the transcriptional activity of the cell. HeLa cells were treated with actinomycin D (act. D), which blocks Pol II-dependent transcription by binding to the guanine residues in DNA (Drapkin et al., 1994). Cells were simultaneously stained for Cdk9, cyclin T1 and SC35. As shown in Fig. 5, Cdk9 and especially cyclin T1 coalesced into larger speckles coinciding with SC35 labeling. Another inhibitor of transcription, DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole), specifically prevents RNA Pol II-directed transcription by inhibiting the activity of kinases, with Cdk9 activity being exquisitely sensitive (Zandomeni et al., 1986; Herrmann and Rice, 1995; Marshall et al., 1996; Mancebo et al., 1997). In cells treated with DRB, cyclin T1 tightly colocalized with enlarged speckles and, although Cdk9 was present at speckles, it did not appear to be highly concentrated in these regions (Fig. 5). These results indicate that the association of at least cyclin T1 with speckles is not dependent on active transcription.

A region in the central portion of cyclin T1 is important for association of Cdk9 and cyclin T1 with speckle regions

The finding that, in DRB-treated cells, cyclin T1 was associated with speckle domains, whereas Cdk9 appeared not to become enriched in these regions, suggested that the cyclin T1 component of TAK/P-TEFb is responsible for the association of the kinase complex with nuclear speckles. To test this idea, we transiently transfected HeLa cells with a

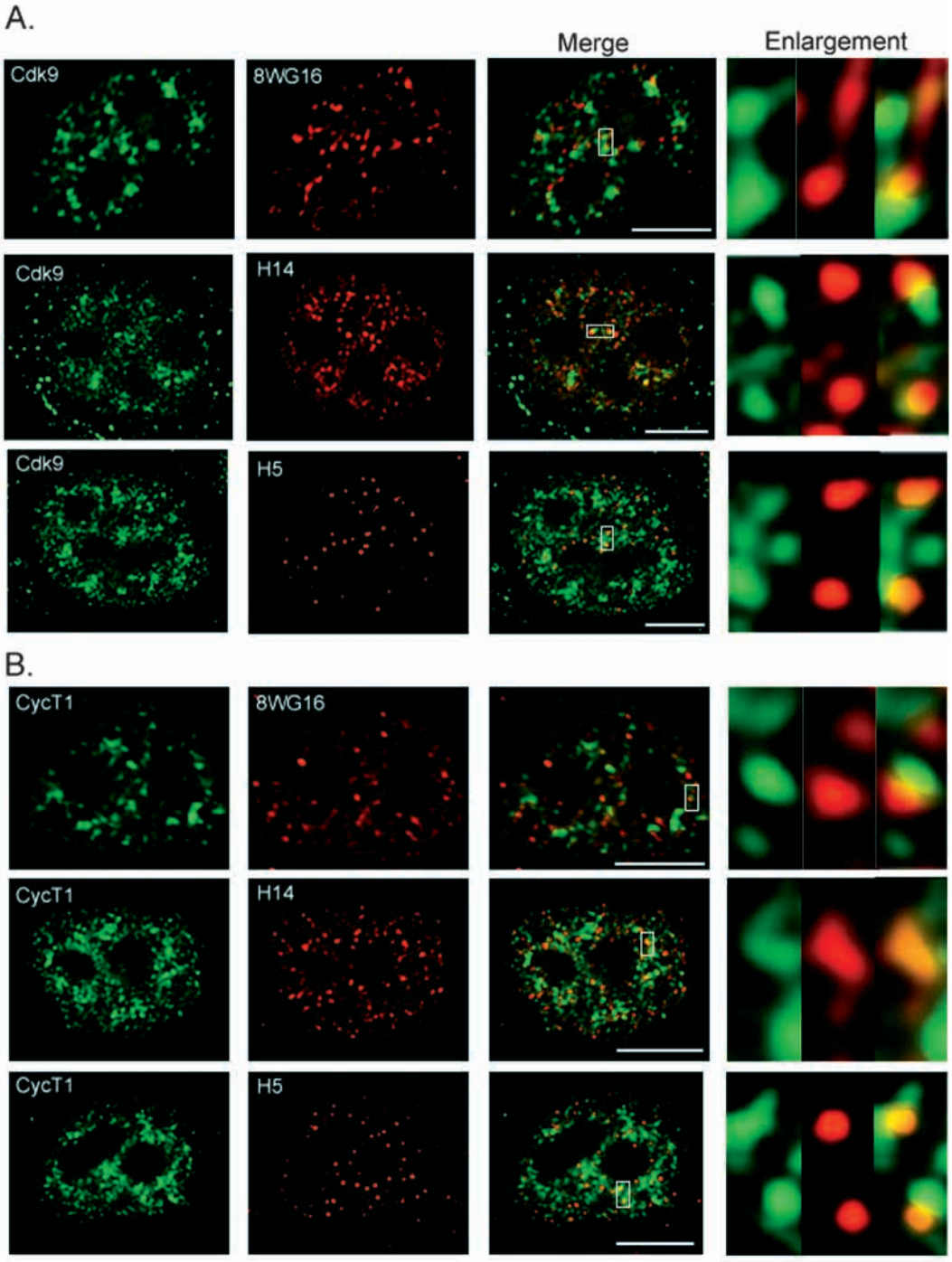

Fig. 3. Colocalization of Cdk9 and cyclin T1 with RNA Pol II. Dual labeling of HeLa cells with antibodies 8WG16, H14, or H5 that recognize different phosphorylated forms of RNA Pol II and Cdk9 (A) or cyclin T1 (B). Particularly apparent in the enlarged images, Cdk9 and cyclin T1 labeling partially overlaps with labeling by all the Pol II antibodies. Images were pseudocolored using Adobe Photoshop so that Cdk9 or cyclin T1 is shown in green and Pol II is shown in red. The box indicates the area that is enlarged in the panels in the right column. The horizontal box was rotated 90° clockwise to display the enlarged image. Bar, 5 μm.

series of epitope-tagged cyclin T1 deletion mutants ranging from a construct containing only the first 254 amino acid (HA-CycT1-254) residues to full length cyclin T1 (HA-CycT1-726) (Fig. 6A). All of the mutants tested retain the cyclin box region that is located at the N-terminus of cyclin T1 and is necessary for interaction with Cdk9 (Garber et al., 1998). With the exception of the smallest mutant, HA-CycT1-254, all the mutants also contain the Tat recognition motif (TRM), which is required for the interaction of cyclin T1 with Tat and TAR; therefore all the mutants are functional for Tat-mediated transactivation except for the HA-CycT1-254 mutant (Garber et al., 1998).

We examined the localization of the different HA-cyclin T1 constructs as well as their colocalization with endogenous Cdk9 and found two different patterns of localization using these mutants. The first group includes the mutants that contain at least the first 533 amino acid residues of cyclin T1. These mutants produced a speckled pattern of staining that was similar to that observed for endogenous cyclin T1 although the labeled foci were perhaps somewhat larger and more punctate than that seen by immunostaining of the endogenous protein. (This group is represented by HA-CycT1-708 and HA-CycT1-533 in Fig. 6B.) Overexpression of these cyclin T1 mutants did not significantly alter the pattern of Cdk9 localization in most

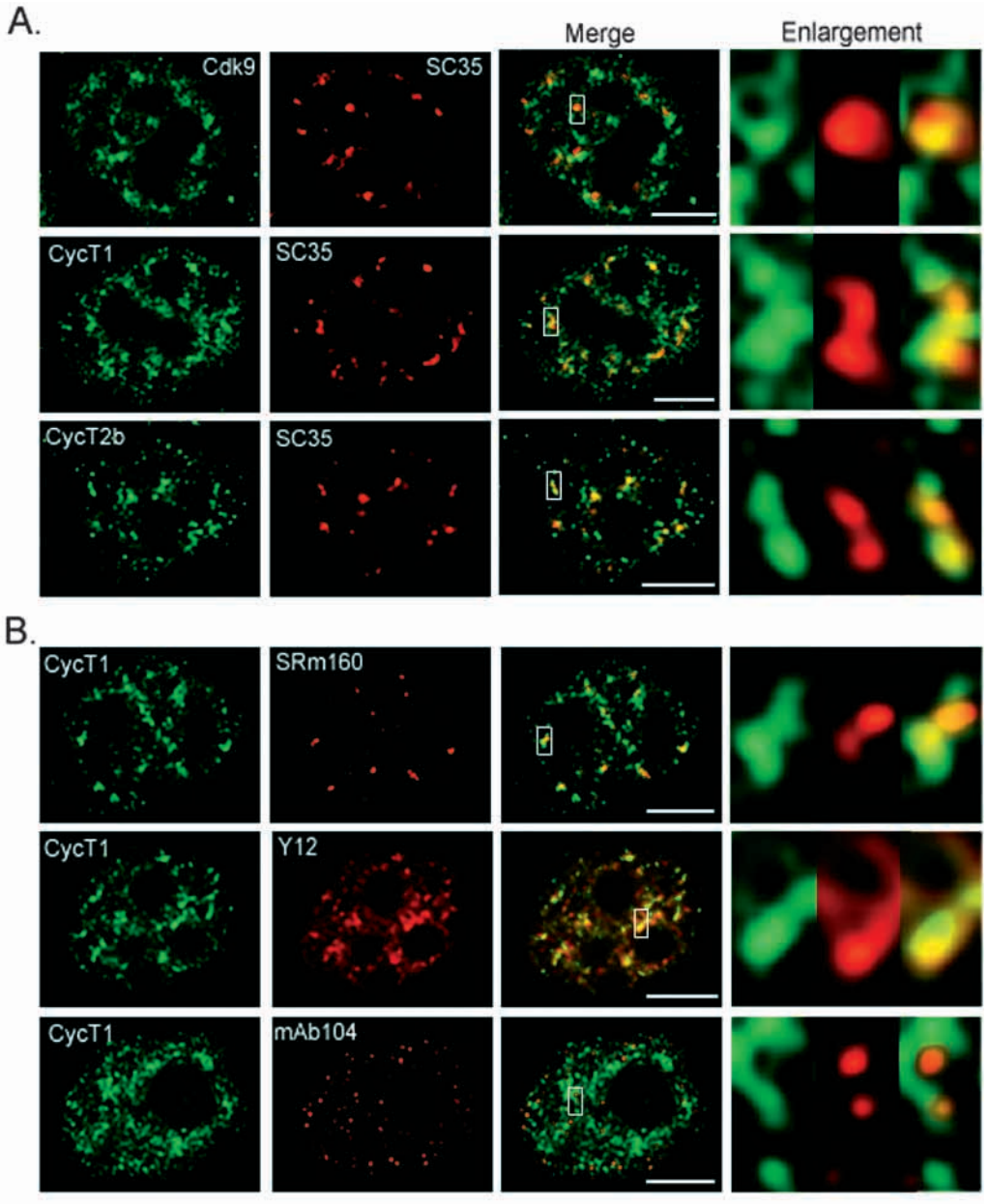

Fig. 4. Colocalization of Cdk9, cyclin T1 and cyclin T2b with splicing factors. (A) Dual labeling of HeLa cells with antibodies directed against Cdk9, cyclin T1 or cyclin T2b, and the SC35 antibody that recognizes an SR spliceosome component. (B) Dual staining for cyclin T1 and antibodies directed against spliceosome components SRm160, Y12 or mAb104. Apparent particularly in the enlarged images, Cdk9 labeling partially overlaps with SC35, but cyclin T1 and T2b show relatively more extensive overlap with SC35. Cyclin T1 also shows extensive colocalization with SRm160 and Y12. Images were pseudocolored using Adobe Photoshop so that Cdk9 or cyclin T1 is shown in green and Pol II is shown in red. The box indicates the area that is enlarged in the panels in the right column. Bar, 5 μm.

cells, although in some cells we observed a brighter pattern of 'speckling' than that seen in surrounding non-transfected cells.

A different labeling pattern was observed for the remainder of the mutants tested, which contained up to the first 433 amino acid residues of cyclin T1. These mutants gave a diffuse pattern of labeling without significant accumulation of high signal foci (shown in Fig. 6B for HA-CycT1-433 and HA-CycT1-272). Overexpression of this class of HA-CycT1 mutants resulted in a more diffuse pattern of Cdk9 localization. These results imply that the cyclin box and Tat recognition motif are not sufficient for localization to speckles and that a region between amino acid residues 433 and 533 is important for localization of cyclin T1 to the speckle compartment.

Cyclin T1 recruits Cdk9 and the HIV Tat protein to speckle domains

The accumulation of HA-CycT1 at SC35-containing domains raised the possibility that cyclin T1 directs its catalytic partner Cdk9 to speckles. To test this idea, we transfected cells with a construct that expresses functional Cdk9 fused to green fluorescent protein (GFP). In these cells, GFP-Cdk9 localized in a diffuse pattern throughout the nucleus, including the nucleolar regions, with some foci of higher accumulation suggestive of speckles (Fig. 7). The much more diffuse pattern of GFP-Cdk9 expression than that seen by immunolabeling of the endogenous Cdk9 protein is consistent with the idea that the binding sites at the speckle regions might be saturated by the high levels of GFP-Cdk9 produced in transient expression assays. To increase the potential binding sites at speckles, we cotransfected GFP-Cdk9 with HA-CycT1-708, which is directed to speckles. In cells coexpressing GFP-Cdk9 and HA-CycT1-708, we found a high degree of colocalization of GFP-Cdk9 with SC35, and reduced accumulation at the nucleolar regions. The enrichment of GFP-Cdk9 at speckles was not

observed when GFP-Cdk9 was cotransfected with the HA-Cyc-T1-272 mutant, which is not targeted to speckles. This result indicates that cyclin T1 can recruit Cdk9 to the speckle compartment.

Cyclin T1 also directly interacts with the HIV Tat protein (Wei et al., 1998). Tat has been shown to localize to the nucleus, concentrating in the nucleolus of transfected and infected cells (Cullen et al., 1988; Luznik et al., 1995; Stauber and Pavlakis, 1998), although in one study Tat was reported to distribute in a speckled pattern (Chun et al., 1998). We wondered whether cyclin T1 could recruit Tat to speckle regions. To examine this possibility, we transfected a functional GFP-Tat expression plasmid into cells and found that it localized predominantly to the nucleolus with some expression in the nucleoplasm, consistent with previous reports. However, when GFP-Tat was cotransfected with HA-CycT1-708, a distinctly speckled pattern was observed in addition the nucleolar labeling. The GFP-Tat 'speckles' colocalized well with SC35. The speckled pattern was not seen when GFP-Tat was coexpressed with HA-CycT1-272 or when a mutant version of Tat, GFP-Pro18, which is deficient for cyclin T1 binding, was used. These results indicate that cyclin T1 can recruit the HIV Tat protein to nuclear speckles and that this targeting is dependent on the region of cyclin T1 that directs it to speckles and a specific interaction between Tat and cyclin T1.

In summary, we have shown that the TAK/P-TEFb subunits Cdk9 and cyclin T1 are present at SC35-containing speckle domains and that a region in the central portion of cyclin T1 is important for directing the complex to nuclear speckles.

DISCUSSION

Although control of transcription at the level of initiation has long been known to be an important regulatory step, the significance of regulation at the level of elongation has been appreciated only recently and might be important in connecting the processes of transcription and mRNA processing. The subcellular distribution of basal transcription factors and activator proteins, as well as pre-mRNA splicing factors has been examined (Spector, 1993; Grande et al., 1997; Stenoien et al., 2000), but little is known about the subcellular localization of factors involved in transcriptional elongation or their association with components of the transcriptional and mRNA processing machinery at the single cell level.

Here we investigated the localization of Cdk9 and cyclin T,

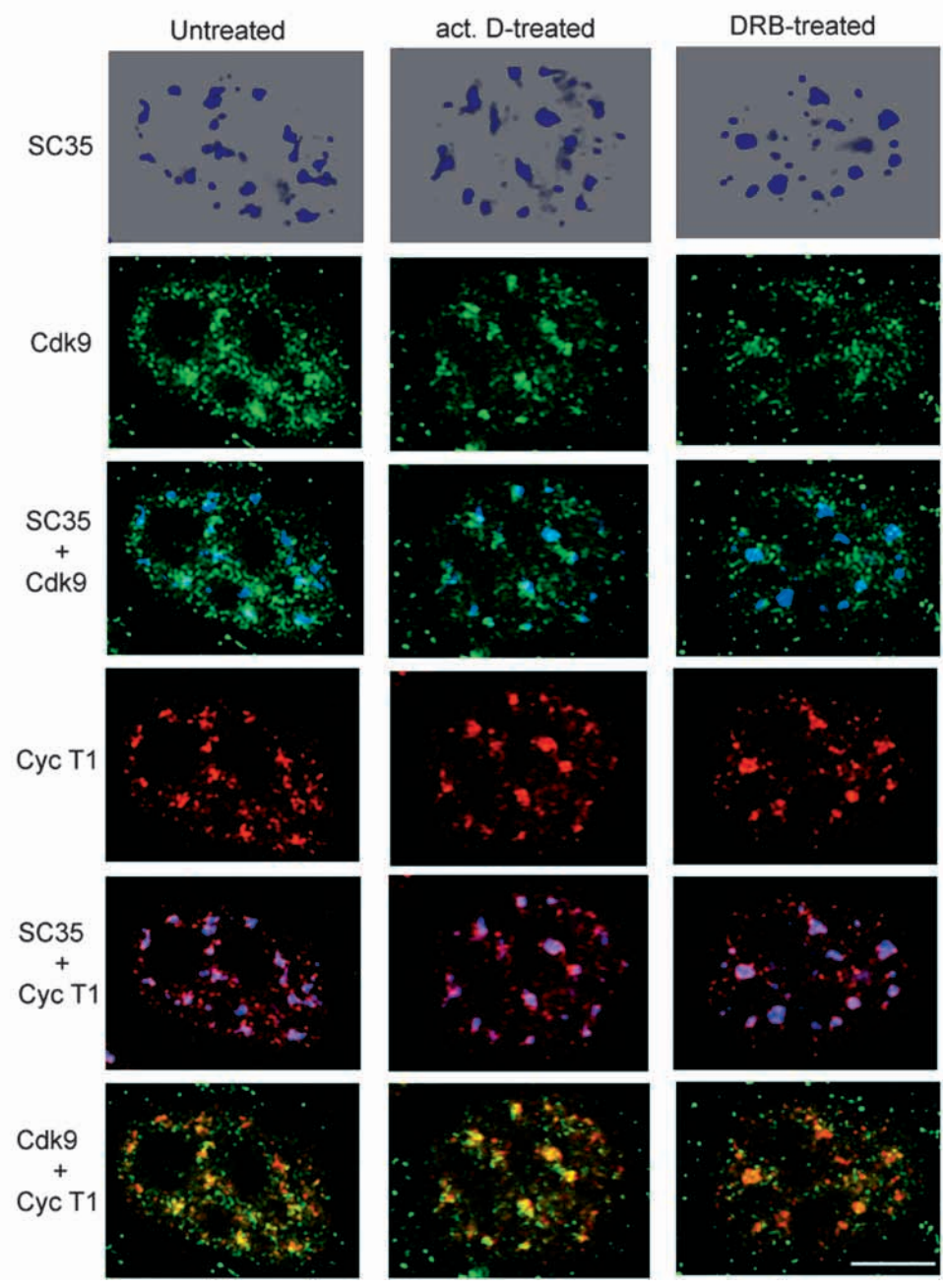

Fig. 5. Localization of Cdk9 and cyclin T1 is perturbed following treatment with transcription and kinase inhibitors. HeLa cells were treated with 20 μg/ml actinomycin D (act. D) for 2 hours or with 100 μM DRB for 1 hour. Both drugs inhibit RNA Pol II-directed transcription but DRB also inhibits the activity of TAK/P-TEFb. Cells were triple stained for SC35 (blue), Cdk9 (green) and cyclin T1 (red). Merged images of SC35 with Cdk9 (SC35 + Cdk9) or cyclin T1 (SC35 + cyclin T1) and Cdk9 with cyclin T1 (Cdk9 + Cyc T1) are shown. Images were pseudocolored using Adobe Photoshop. As shown, cyclin T1 coalesces with SC35 into enlarged speckles with both act. D and DRB treatment, whereas Cdk9 colocalizes less extensively with SC35 relative to cyclin T1 in DRB-treated cells. Bar, 5 μm.

the two known subunits of elongation factor TAK/P-TEFb and found that Cdk9 and cyclins T1 and T2 were found in numerous foci throughout the non-nucleolar nucleoplasm in interphase cells but were present in highest abundance in speckle-like regions of the nucleus. The expression of Cdk9 and cyclin T1 and T2 was exclusively nuclear, indicating that the functional TAK/P-TEFb complex resides in the nucleus. Based on biochemical fractionation experiments, a recent study proposed that TAK/P-TEFb assembles in the cytoplasm (Ramanathan et al., 1999). Our data are not consistent with this model.

A small but significant portion of Cdk9 and cyclin T1 remained following extraction of chromatin and soluble material from cells, indicating that a subpopulation of Cdk9 and cyclin T1 is associated with the insoluble component termed the nuclear matrix. Proponents of this highly debated structure argue that a protein-based structural framework exists, somewhat analogous to the cytoskeleton, and serves to organize and partition nuclear substructures and macromolecules, such as chromosomes, RNA and speckles (reviewed by Nickerson et al., 1995). Factors involved in transcription and mRNA processing, including the hyperphosphorylated form of RNA Pol II and splicing factors SC35 and SRm160, have been found associated with this structure (Bregman et al., 1995; Wan et al., 1994). Recent results using fluorescence recovery after photobleaching (FRAP) techniques suggest that, whereas some proteins have been found to undergo very rapid exchange among different cellular subcompartments (Phair and Misteli, 2000), other proteins exhibit low mobilities, presumably due

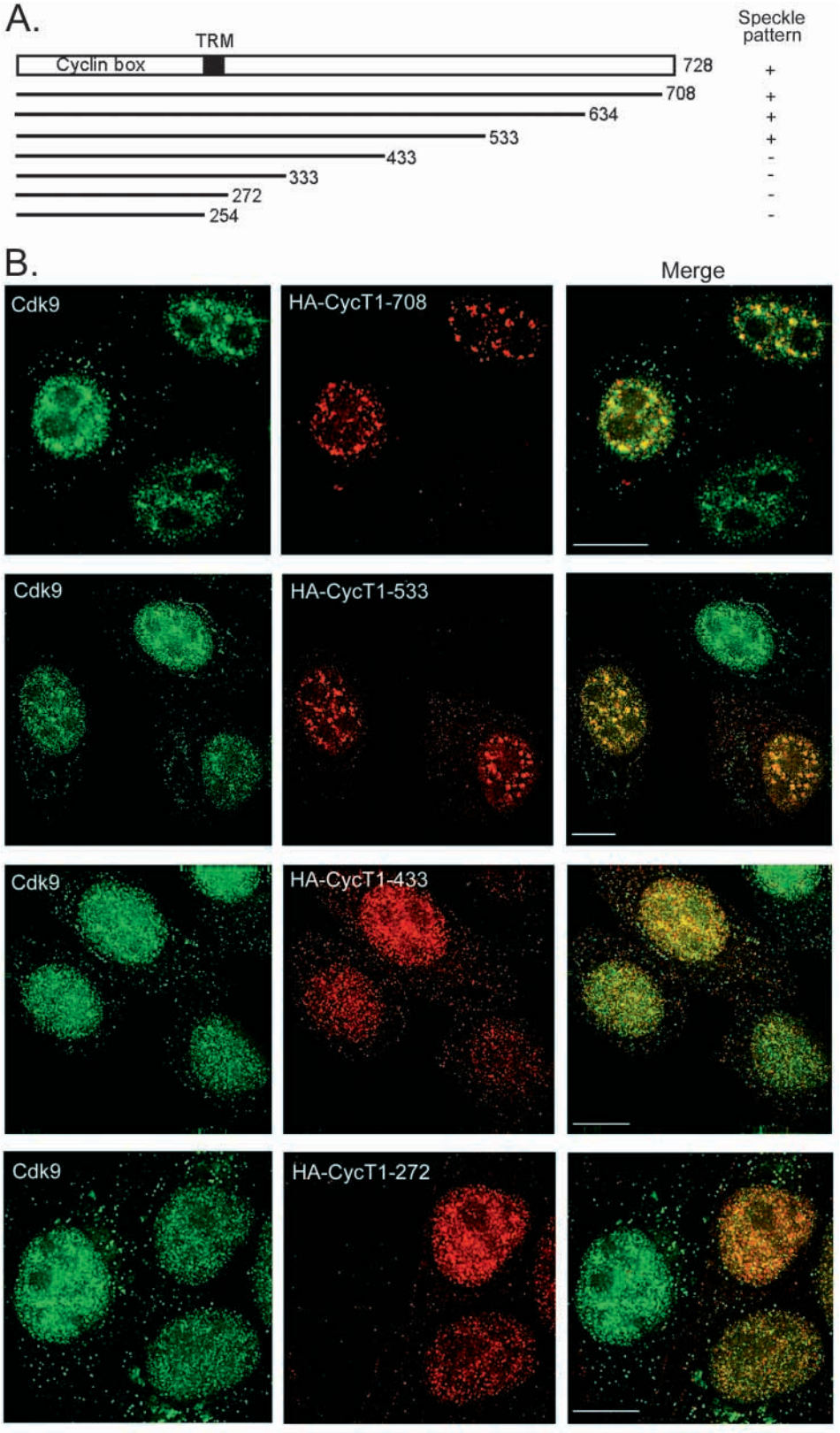

Fig. 6. Localization of transiently transfected cyclin T1 mutant proteins. (A) Diagrammatic representation of HA-tagged cyclin T1 mutants used in this study. Constructs 728, 708, 272 and 254 were obtained from K. Jones (Garber et al., 1998) and the 634, 533, 433 and 333 constructs were obtained from Q. Zhou (Fong and Zhou, 2000). (B) HeLa cells were transfected with 200 ng of the HA-tagged cyclin T1 constructs HA-CycT1-708, HA-CycT1-533, HA-CycT1-433, or HA-CycT1-272. Cells were fixed at 20 - 24 hr following transfection and dual labeled with antibodies directed against Cdk9 (green) or the HA epitope (red). A speckled pattern is observed for HA-CycT1-708 and HA-CycT1-533, but HA-CycT1-433 and HA-CycT1-272 are more diffusely dispersed throughout the nucleoplasm and result in a more diffuse pattern of Cdk9 staining. Bar, 10 μm.

to interactions with more ‘fixed’ or relatively immobile nuclear structures (Kruhlak et al., 2000; Stenoien et al., 2001; Shopland and Lawrence, 2000). Determination of the relative mobilities of Cdk9 and cyclin T1 using FRAP techniques should be informative.

The speckled pattern of Cdk9 distribution is in contrast to that of two other members of the Cdk family that are known to be involved in transcriptional regulation, Cdk7 and Cdk8. The latter proteins exhibit a much more evenly dispersed pattern of non-nucleolar nucleoplasmic distribution, with Cdk7 found also in Cajal bodies (Jordan et al., 1997; Herrmann, unpublished). Although all three kinases are capable of phosphorylating the CTD, their function at specific points during transcription may be determined by their location within subnuclear compartments. Interestingly, the Cdk9-related protein PITSLRE localizes to both the nucleoplasm and

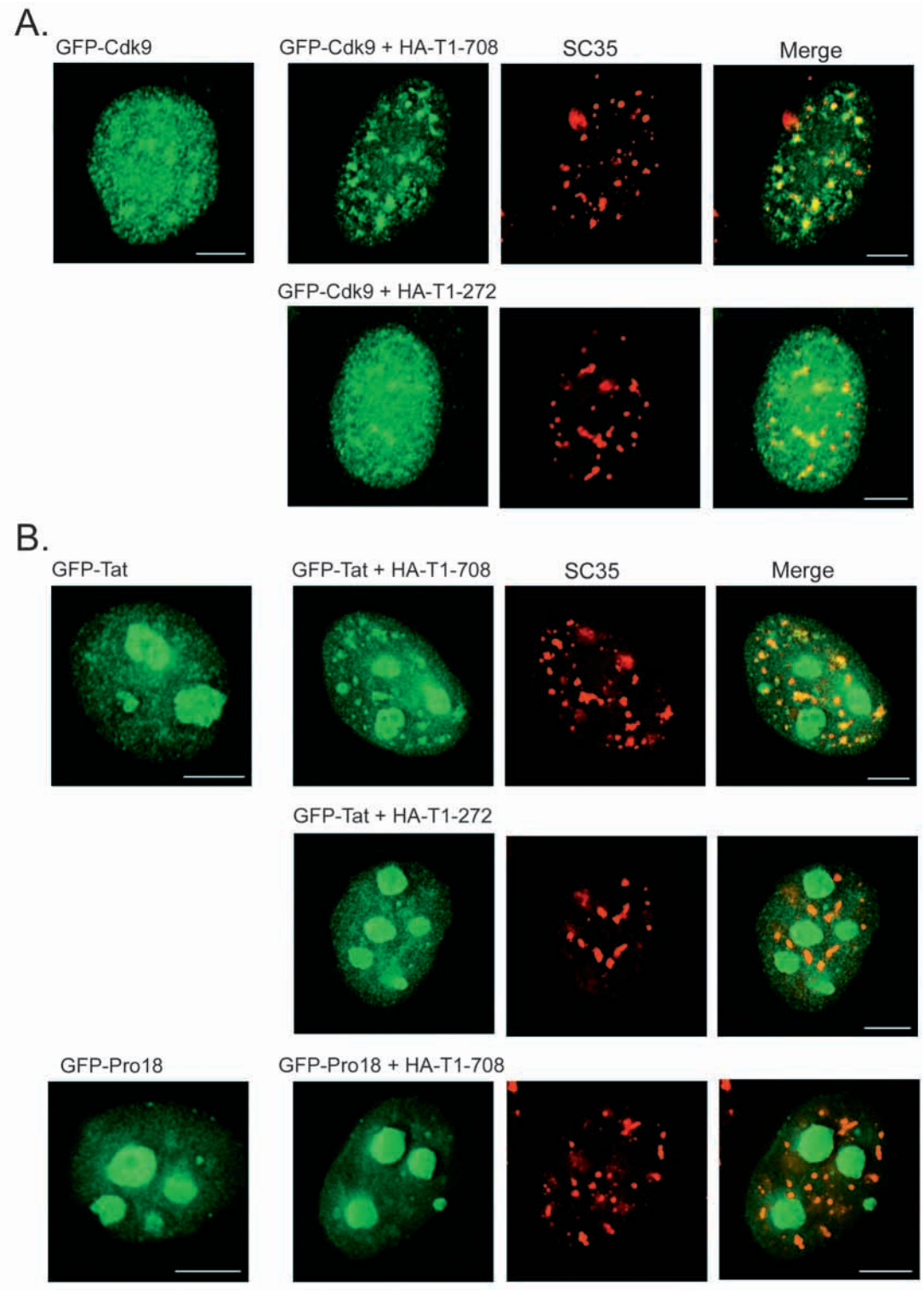

Fig. 7. Recruitment of Cdk9 and HIV Tat to nuclear speckles by cyclin T1. (A) HeLa cells were transfected with 100 ng of GFP-Cdk9 alone (top row, left panel), fixed at approximately 22 hours post-transfection, and visualized using the FITC channel. In the right-hand three panels, GFP-Cdk9 was cotransfected with HA-CycT1-708 (top row) or HA-CycT1-272 (second row). GFP-Cdk9 is shown in green and immunolabeling of SC35 is shown in red. In the presence of HA-CycT1-708, but not HA-CycT1-272, GFP-Cdk9 takes on a hyperspeckled appearance. (B) HeLa cells were transfected with 100 ng of GFP-Tat or the transactivation-defective mutant GFP-Pro18 (left panels). In the right-hand three panels, GFP-Tat or GFP-Pro18 were cotransfected with HA-CycT1-708 or HA-CycT1-272. GFP-Tat and GFP-Pro18 are shown in green and immunolabeling of SC35 is shown in red. GFP-Tat is present predominantly in the nucleolus but, in cells expressing HA-CycT1-708, GFP-Tat also appears in speckles that colocalize with SC35. We have confirmed that GFP-Tat colocalizes with HA-CycT1-708 in transfected cells (not shown). Bar, 5 μm.

nuclear speckle regions and is found in soluble and insoluble pools (Loyer et al., 1998), but its cyclin partner has not yet been identified and it is unclear whether PITSLRE functions during transcription.

Colocalization of Cdk9 and cyclin T1 with the mRNA transcription/processing machinery

A physiologically important substrate of TAK/P-TEFb is thought to be the CTD of RNA Pol II. However, it is not clear whether TAK/P-TEFb acts on the completely hypophosphorylated form or on more intermediately phosphorylated forms that may have been generated as a result of prior modification by other CTD kinases such as the Cdk7 complex. Thus, we examined the interaction of Cdk9 and cyclin T1 with all of the phosphorylated forms. We found colocalization of Cdk9 and cyclin T1 with antibodies that recognize hypo-, intermediate, and hyperphosphorylated Pol II, although the extent of colocalization was limited to a few foci per focal plane. This indicates that the majority of Cdk9 and cyclin T1 is not associated with Pol II and, conversely, that the bulk of Pol II-containing foci do not contain Cdk9 and cyclin T1. Other transcription factors examined also display little spatial overlap with RNA Pol II (Grande et al., 1997; Stein and Berezney, 2000; Stenoien et al., 2000) The low level of co-association of TAK/P-TEFb with RNA Pol II argues against the concept that TAK/P-TEFb is stably associated with the RNA Pol II holoenzyme or elongation complexes. Our results are consistent with the finding of Lis and colleagues that cyclin T1 colocalized with the 8WG16 and H14 antibodies at sites on the polytene chromosome of *Drosophila* but that cyclin T1 was not present at the majority of sites containing Pol II (Lis et al., 2000). By extension of the recent findings that some nuclear proteins are highly mobile (Phair and Misteli, 2000; Kruhlak et al., 2000; Stenoien et al., 2001), the interaction between TAK/P-TEFb and RNA Pol II could be very dynamic and short-lived.

In contrast with the degree of colocalization of Cdk9 and cyclin T1 with RNA Pol II, the extent of overlap between the TAK/P-TEFb subunits with splicing factors residing in speckle regions of the nucleus was much more considerable, especially for cyclin T1. Cyclin T1, and to a lesser degree Cdk9, colocalized with spliceosome components of both the SR and Sm groups of splicing factors. The presence of splicing factors in speckle regions appears to be important for their association with TAK/P-TEFb because mAb104, which decorates members of the SR family of splicing factors in a ‘dot’ pattern, did not show extensive overlap with Cdk9 or cyclin T1.

Results from several experiments suggest that it is the cyclin T component of TAK/P-TEFb that directs the kinase complex to nuclear speckles. First, the degree of colocalization of cyclin T1 with splicing factors present at speckles is greater than that for Cdk9. Second, in the presence of DRB, little Cdk9 is found coincident with SC35, whereas cyclin T1 is present in enlarged speckles at high concentrations. Third, when cyclin T1 is transiently expressed it produces a highly speckled pattern, whereas transiently-expressed Cdk9 appears to be more diffusely distributed. In addition, the pattern of endogenous Cdk9 labeling becomes more diffuse upon overexpression of the C-terminal truncated cyclin T1 mutants that fail to accumulate in speckle regions. Finally, Cdk9 and the HIV Tat protein become enriched at speckles when cyclin T1 is overexpressed. Biochemical experiments will be required to determine the factor present in speckle domains that directly interacts with TAK/P-TEFb. Co-immunoprecipitation experiments have thus far failed to identify an interaction between Cdk9 or cyclin T1 with a specific spliceosome components (Herrmann, unpublished).

Subnuclear localization of Tat

In the majority of studies where Tat localization has been examined, Tat was reported to be present primarily in the nucleolus of transfected and infected cells (Cullen et al., 1988, Luznik et al., 1995, Stauber and Pavlakis 1998). In contrast to the nucleolar localization seen when Tat was overexpressed, Chun et. al. observed that Tat produced a speckled pattern of staining that was partially coincident with SC35 staining when expressed at lower levels (Chun et al., 1998). It is likely that the level of Tat, when overexpressed in transfected cells, greatly exceeds the level of endogenous cyclin T1 so that binding of Tat to cyclin T1 becomes saturated. In these cells, only a small proportion of Tat may be recruited to speckle domains resulting in an accumulation of the highly basic Tat protein at nucleoli and the intense nucleolar staining that is often observed. Consistent with this idea, overexpression of cyclin T1 directs higher levels of GFP-Tat to speckle domains (Fig. 7). The precise subnuclear localization of Tat when expressed at levels found during HIV infection in cell types that are natural targets of HIV (i.e. T cells and macrophages) remains to be established.

We and others have reported that cyclin T1 levels are upregulated during T-cell activation and monocyte differentiation (Herrmann et al., 1998, Garriga et al., 1998), conditions that are necessary for high levels of HIV replication. Conceivably, an increase in endogenous levels of cyclin T1 in these cell types could result in recruitment of Tat to speckles and provide a mechanism for regulating the intranuclear localization, and perhaps, function of Tat.

Possible functional roles of TAK/P-TEFb in speckle domains

The functional significance of nuclear speckles is still somewhat unclear but they have been proposed to function as storage and/or assembly areas for splicing components (Spector, 1996). By analogy with splicing factors, Cdk9 and cyclin T1 could be stored in these areas or available for assembly into larger macromolecular structures. It is also possible, however, that speckles are a site of TAK/P-TEFb function. A potential function for TAK/P-TEFb at speckles might be for the transcription of specific genes that require the elongation processivity function of this kinase complex. Thus, certain genes might be present at speckle domains to allow TAK/P-TEFb-enhanced elongation to occur. Transcription of some genes has been detected at the periphery of speckles or within the speckles themselves (Huang and Spector, 1991; Smith et al., 1999; Wei et al., 1999). Alternatively, by directly phosphorylating spliceosome components, TAK/P-TEFb could play a role in regulating the equilibrium of splicing factors between the speckle regions and the nucleoplasm or be involved in the process of spliceosome assembly. It is known that the accumulation and release of splicing factors is controlled by cycles of phosphorylation and dephosphorylation, and several kinases have been identified that cause release of

splicing factors from speckles when overexpressed (Colwill et al., 1996; Gui et al., 1994; Wang et al., 1998). Finally, TAK/P-TEFb could regulate the distribution of splicing factors through its known ability to phosphorylate the CTD of RNA Pol II. The displacement of negative elongation factors from the CTD by TAK/P-TEFb in the vicinity of high concentrations of splicing factors present at speckles could promote the efficient interaction of splicing factors with the CTD. This hypothesis is attractive because it would provide a mechanism for effectively coupling transcription elongation with splicing. Further work will be required to test these ideas.

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