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# Phosphorylated Positive Transcription Elongation Factor b (P-TEFb) Is Tagged for Inhibition through Association with 7SK snRNA*

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**The positive transcription elongation factor b (P-TEFb), comprising CDK9 and cyclin T, stimulates transcription of cellular and viral genes by phosphorylating RNA polymerase II. A major portion of nuclear P-TEFb is sequestered and inactivated by the coordinated actions of the 7SK snRNA and the HEXIM1 protein, whose induced dissociation from P-TEFb is crucial for stress-induced transcription and pathogenesis of cardiac hypertrophy. The 7SK·P-TEFb interaction, which can occur independently of HEXIM1 and does not by itself inhibit P-TEFb, recruits HEXIM1 for P-TEFb inactivation. To study the control of this interaction, we established an *in vitro* system that reconstituted the specific interaction of P-TEFb with 7SK but not other snRNAs. Using this system, together with an *in vivo* binding assay, we show that the phosphorylation of CDK9, on possibly the conserved Thr-186 in the T-loop, was crucial for the 7SK·P-TEFb interaction. This phosphorylation was not caused by CDK9 autophosphorylation or the general CDK-activating kinase CAK, but rather by a novel HeLa nuclear kinase. Furthermore, the stress-induced disruption of the 7SK·P-TEFb interaction was not caused by any prohibitive changes in 7SK but by the dephosphorylation of P-TEFb, leading to the loss of the key phosphorylation important for 7SK binding. Thus, the phosphorylated P-TEFb is tagged for inhibition through association with 7SK. We discuss the implications of this mechanism in controlling P-TEFb activity during normal and stress-induced transcription.**

7SK is an abundant ($2 \times 10^5$ copies/cell) and evolutionarily conserved small nuclear RNA (snRNA)[1] of ~330 nucleotides (1–4). It is transcribed by RNA polymerase (Pol) III from one or more genes belonging to a family of interspersed repeats in the mammalian genome (2, 5). The high conservation and abundance of 7SK suggest an important physiological function of this RNA. In searching for nuclear factors that can bind to and regulate the activity of human positive transcription elongation factor b (P-TEFb), we and others (6, 7) have identified 7SK as a specific P-TEFb-associated factor. Consisting of CDK9 and cyclin T1 (CycT1) (8, 9), P-TEFb strongly enhances the processivity of RNA Pol II by phosphorylating the C-terminal domain (CTD) of Pol II and antagonizing the actions of negative elongation factors (10–13). Studies employing RNA interference in *Caenorhabditis elegans* and a pharmacological inhibitor of CDK9 in mammalian cells implicate P-TEFb as essential for expression of most protein-coding genes (10, 14).

P-TEFb also functions as a specific host cellular cofactor for the HIV-1 Tat protein. Stimulation of Pol II elongation is essential for HIV transcription, during which P-TEFb is recruited to the nascent mRNA by Tat (15, 16). Tat binds to CycT1 and recruits P-TEFb through formation of a stable ternary complex containing P-TEFb, Tat, and the HIV TAR RNA structure located at the 5′-end of the nascent viral transcript. Once recruited, P-TEFb phosphorylates the CTD and stimulates the production of the full-length HIV-1 transcripts.

It is important to point out that not all forms of P-TEFb in the cell can display transcriptional activity. In fact, at least half of P-TEFb in HeLa cells are sequestered in the 7SK snRNP, where their kinase and transcriptional activities are suppressed (6, 7). Besides P-TEFb and 7SK, the snRNP has recently been shown to also contain HEXIM1 (17, renamed as MAQ1 in 18). HEXIM1 has previously been identified as a nuclear protein whose expression is rapidly induced in cells treated with hexamethylene bisacetamide (HMBA) (19), a potent inducer of cell differentiation (20). While the binding of 7SK to P-TEFb can occur in the absence of HEXIM1, the binding of HEXIM1 to P-TEFb is strictly 7SK-dependent (17). Furthermore, the 7SK binding alone is not sufficient to inhibit P-TEFb. P-TEFb is inhibited by HEXIM1 in a process that specifically requires 7SK for bridging the HEXIM1·P-TEFb interaction. This allows HEXIM1 to inhibit transcription both *in vivo* and *in vitro* (17).

The amount of the HEXIM1/7SK-bound P-TEFb does not remain unchanged. In fact, treatment of cells with several stress-inducing agents rapidly releases 7SK and HEXIM1 from P-TEFb (6, 7, 17, 18). These agents have been shown to stimulate CTD phosphorylation and HIV transcription (21, 22). Thus, the induced release of 7SK/HEXIM1 and activation of P-TEFb may contribute directly to the stress-induced HIV and cellular gene expression.

The activation of P-TEFb through release of 7SK also contributes to the pathogenesis of cardiac hypertrophy, a disease characterized by the enlargement of cardiac myocytes due to a global increase in RNA and protein contents. P-TEFb activity is limiting for normal cardiac growth. Hypertrophic signals induce the dissociation of 7SK (presumably also HEXIM1) from

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[1] The abbreviations used are: snRNA, small nuclear RNA; Pol, polymerase; GST, glutathione *S*-transferase; NE, nuclear extract; PP1, type I protein phosphatase; PAPP, potato acid phosphatase; DRB, 5,6-dichloro-1-$\beta$-D-ribofuranosylbenzimidazol; P-TEFb, positive transcription elongation factor b; CTD, C-terminal domain; CycT1, cyclin T1; HIV, human immunodeficiency virus; HMBA, hexamethylene bisacetamide.



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P-TEFb, activating P-TEFb to stimulate Pol II transcription (23, 24).

Given the importance of the 7SK·P-TEFb interaction in recruiting HEXIM1 for P-TEFb inactivation, we would like to understand how this interaction is regulated. This will aid the future elucidation of the signaling pathways that control the formation and disruption of the 7SK snRNP in response to physiological stimuli. Here, we report the establishment of an *in vitro* system that reconstituted the specific 7SK·P-TEFb interaction. Our data reveal that the phosphorylation of CDK9, on possibly the conserved Thr-186 in the T-loop, was crucial for this interaction. This phosphorylation was not caused by CDK9 autophosphorylation or the general CDK-activating kinase CAK, but rather by a yet-to-be-identified HeLa nuclear kinase. Finally, the stress-induced dissociation of 7SK from P-TEFb was not caused by any changes or modifications in 7SK but rather by the dephosphorylation of P-TEFb, resulting in the loss of the key phosphorylation important for 7SK binding.

## EXPERIMENTAL PROCEDURES

*In Vitro Reconstitution of 7SK·P-TEFb Interaction*—The reconstitution was performed in two main steps. During the immunoprecipitation step, 250 μg of nuclear extract (NE) prepared from either F1C2 (an engineered HeLa cell line stably expressing FLAG-tagged CDK9) (7) or HeLa cells (as a negative control) were incubated at 4 °C for 2 h with 10 μl of anti-FLAG-agarose beads (Sigma). After binding, the beads were washed extensively with buffer D (20 mM HEPES-KOH, pH 7.9, 15% glycerol, 0.2 mM EDTA, 0.2% Nonidet P-40, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) containing 0.3 M KCl (D0.3M), then buffer D0.8M, and finally D0.1M. The second wash with D0.8M stripped 7SK and HEXIM1 off P-TEFb. After virtually all the liquid was removed, the beads were preblocked at room temperature for 30 min with 15–20 μl of a blocking solution containing 5 mg/ml bovine serum albumin, 3 mg/ml poly(C) (Amersham Biosciences), and 2.5 mg/ml *Escherichia coli* tRNA.

During the reconstitution step, the preblocked beads were incubated at 30 °C for 30 min with 100 μg of HeLa NE in buffer D0.1M plus 0.5 mM ATP and 5 mM $MgCl_2$ or 100 μg of HeLa NE depleted of the endogenous ATP by treatment with hexokinase and glucose (25). After the incubation, the beads were washed extensively with buffer D0.4M and then D0.1M. The reconstituted 7SK snRNP was finally eluted off the beads with 0.4 mg/ml of FLAG peptide (Sigma) prepared in D0.1M.

RNA used in the specified reconstitution reactions was transcribed *in vitro* from a 7SK DNA template by T7 RNA polymerase (Stratagene) or extracted from HeLa NE or the D0.8M-stripped 7SK fraction with phenol and chloroform. After precipitation with ethanol, the RNA pellet was dissolved in D0.1M.

*Phosphatase Treatment of Immobilized P-TEFb*—After washing away nonspecific factors with D0.3M, the immobilized 7SK(+) P-TEFb was washed with 90 mM sodium citrate and incubated with 0.3 units of PAPP (potato acid phosphatase, Sigma) in 50 μl. After incubation at 30 °C for 45 min, the complex was washed with D0.3M and then eluted by Flag peptide. For treatment of the immobilized 7SK/HEXIM1-free P-TEFb with PP1 (type 1 protein phosphatase, NEB), the D0.8M-stripped P-TEFb was washed with the PP1 solution (50 mM Tris-HCl pH 7.0, 0.1 mM EDTA, 5 mM dithiothreitol, 0.01% Nonidet P-40, and 2 mM $MnCl_2$) and then incubated at 30 °C with 12.5 units of PP1 in 20 μl for 45 min. After washing with D0.3M, the dephosphorylated P-TEFb was used in the reconstitution reactions.

*Affinity Purification of CDK9, CycT1, and Their Associated Factors for Northern and Western Analyses*—FLAG-tagged wild-type and mutant CDK9 and CycT1 and their associated factors were isolated by anti-FLAG immunoprecipitation from NE of transfected HeLa cells. After incubation at 4 °C for 2 h, the immunoprecipitates were washed with D0.3M. The FLAG peptide-eluted materials were analyzed by Western blotting with anti-FLAG (M2, Sigma), anti-CDK9, and anti-CycT1 antibodies (Santa Cruz Biotechnology), and Northern blotting using the full-length 7SK antisense RNA as a probe.

*In Vitro Kinase Reactions*—P-TEFb containing wild-type or mutant CDK9-FLAG were immunoprecipitated from NE of transfected HeLa cells, washed with D0.8M to remove the associated 7SK and HEXIM1, and eluted with FLAG peptide prepared in D0.1M. The kinase reactions contained P-TEFb, 1 μg of immobilized GST-CTD, 5 mM $MgCl_2$, 50 μM cold ATP, and 1 μl of [$\gamma$-$P^{32}$]ATP (3000 Ci/mmol) and were incubated at 30 °C for 25 min in 20-μl volume. The phosphorylated GST-CTD was analyzed by SDS-PAGE followed by autoradiography.

*Treatment of Cells with Drugs*—The HeLa-based F1C2 cells ($3 \times 10^6$) expressing CDK9-FLAG (7) were seeded in 15-cm dishes 1 day before the treatment. 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazol (DRB) (100 μM), H7 (100 μM), staurosporine (0.5 μM), and actinomycin D (1.2 μg/ml) were added to the culture medium for 1 h. NE was prepared from the treated cells and subjected to further analysis.

## RESULTS

*Phosphorylation-dependent Interaction of 7SK with P-TEFb*—To investigate the mechanism controlling the 7SK·P-TEFb interaction, we asked whether protein phosphorylation might play a role in this process. Previously, it has been shown that treatment of HeLa cells with the kinase inhibitor DRB causes a rapid dissociation of 7SK from P-TEFb (6). To determine whether other kinase inhibitors such as staurosporine and H7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine) would produce a similar effect, a HeLa-based cell line (F1C2) stably expressing CDK9-FLAG (7) was treated with DRB, staurosporine, H7, or the control solvent $Me_2SO$ (DMSO). CDK9-FLAG and its associated CycT1 and 7SK were immunoprecipitated from the NE of the treated cells and analyzed by Western and Northern blotting. All three kinase inhibitors significantly weakened the binding of 7SK to P-TEFb without affecting the CycT1-CDK9 interaction or the steady state levels of CDK9, CycT1, and 7SK in NE (Fig. 1*A*), suggesting that a protein phosphorylation event may be crucial in promoting the binding of 7SK to P-TEFb.

To determine whether a direct phosphorylation of a component of the 7SK·P-TEFb complex is required for this binding, we incubated the immobilized 7SK-bound P-TEFb with PAPP. While the CycT1-CDK9 interaction remained unaffected, 7SK was dissociated from P-TEFb following the incubation with PAPP and the subsequent washes (Fig. 1*B*, *left panel*). This effect was blocked by phosphatase inhibitors (data not shown). The PAPP preparation was free of any RNases and did not degrade 7SK when incubated with the peptide-eluted 7SK·P-TEFb complex in solution (Fig. 1*B*, *right panel*). Together, these *in vivo* and *in vitro* data implicate the phosphorylation of a component of the 7SK-bound P-TEFb as important for the binding of 7SK to P-TEFb.

*Requirement of Both CDK9 and CycT1 Subunits but Not CycT1 Phosphorylation for 7SK·P-TEFb Binding*—CDK9 and CycT1 are both phosphoproteins, and CycT1 is phosphorylated by CDK9 in the C-terminal half (26, 27) (Fig. 2*A*). To determine whether the phosphorylation of CycT1 is important for the 7SK·P-TEFb binding, a C-terminally truncated CycT1-(1–333)-FLAG lacking all the phosphorylation sites (26, 27) was analyzed for its binding to CDK9 and 7SK in anti-FLAG immunoprecipitates derived from NE of transfected HeLa cells. To test the contribution of CDK9 to the 7SK·P-TEFb binding, two additional CycT1 mutants 1–254 and 2M were also analyzed (Fig. 2*A*).

Wild-type CycT1 and its two C-terminal deletion mutants 1–333 and 1–254 all contained an intact cyclin-box, and as expected, were able to bind to CDK9 (Fig. 2*B*). Although 1–333 was expressed to a higher level than wild-type CycT1 and 1–254 based on per microgram of DNA transfected, a similar level of CDK9 was found to co-precipitate with all three CycT1 molecules, indicating the formation of a similar amount of the CycT1/CDK9 heterodimers (Fig. 2*B*). Under these conditions, 7SK bound strongly to the P-TEFb containing either wild-type CycT1 or 1–333 but not 1–254. Thus, a region between amino acids 255 and 333, which is C-terminal to the Tat/TAR recognition motif (TRM) (28), contributed to 7SK·P-TEFb binding. However, the C-terminal phosphorylation of CycT1 after residue 334 was clearly dispensable for the binding. Finally, unlike

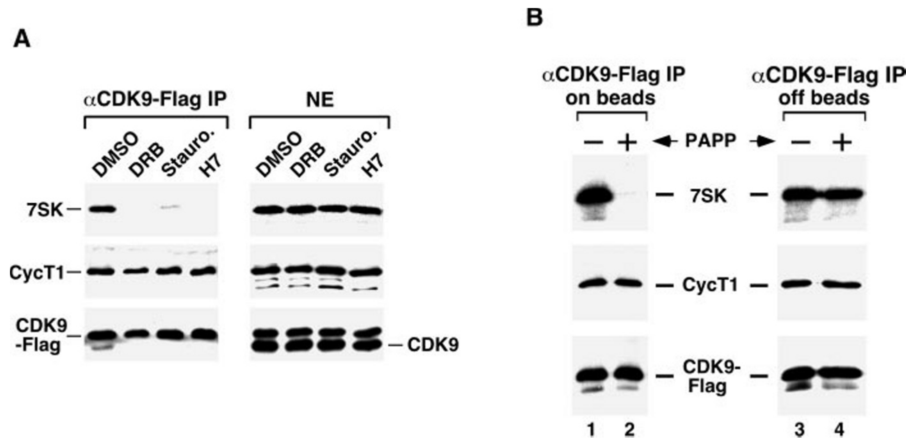


FIG. 1. **Phosphorylation-dependent interaction between 7SK and P-TEFb *in vivo*.** *A*, treatment of cells with kinase inhibitors disrupts 7SK·P-TEFb binding without affecting nuclear 7SK level and CycT1/CDK9 dimer formation. F1C2 cells containing CDK9-FLAG were treated with the indicated kinase inhibitors or the solvent $Me_2SO$. CDK9-FLAG and its associated 7SK and CycT1 (*αCDK9-Flag IP*) were immunoprecipitated from NE and analyzed by Northern and Western blotting (*left panel*). The levels of 7SK, CycT1, CDK9, and CDK9-FLAG in F1C2 NE were also examined (*right panel*). *B*, PAPP dissociates 7SK from P-TEFb *in vitro*. The 7SK-bound P-TEFb, either immobilized on anti-FLAG beads via CDK9-FLAG (*lanes 1* and *2*) or eluted off the beads with FLAG peptide (*lanes 3* and *4*), was incubated with or without PAPP. For *lanes 1* and *2*, after the incubation, the complex was washed with D0.3M and eluted. The compositions of the PAPP-treated complexes were analyzed by Northern and Western blotting.

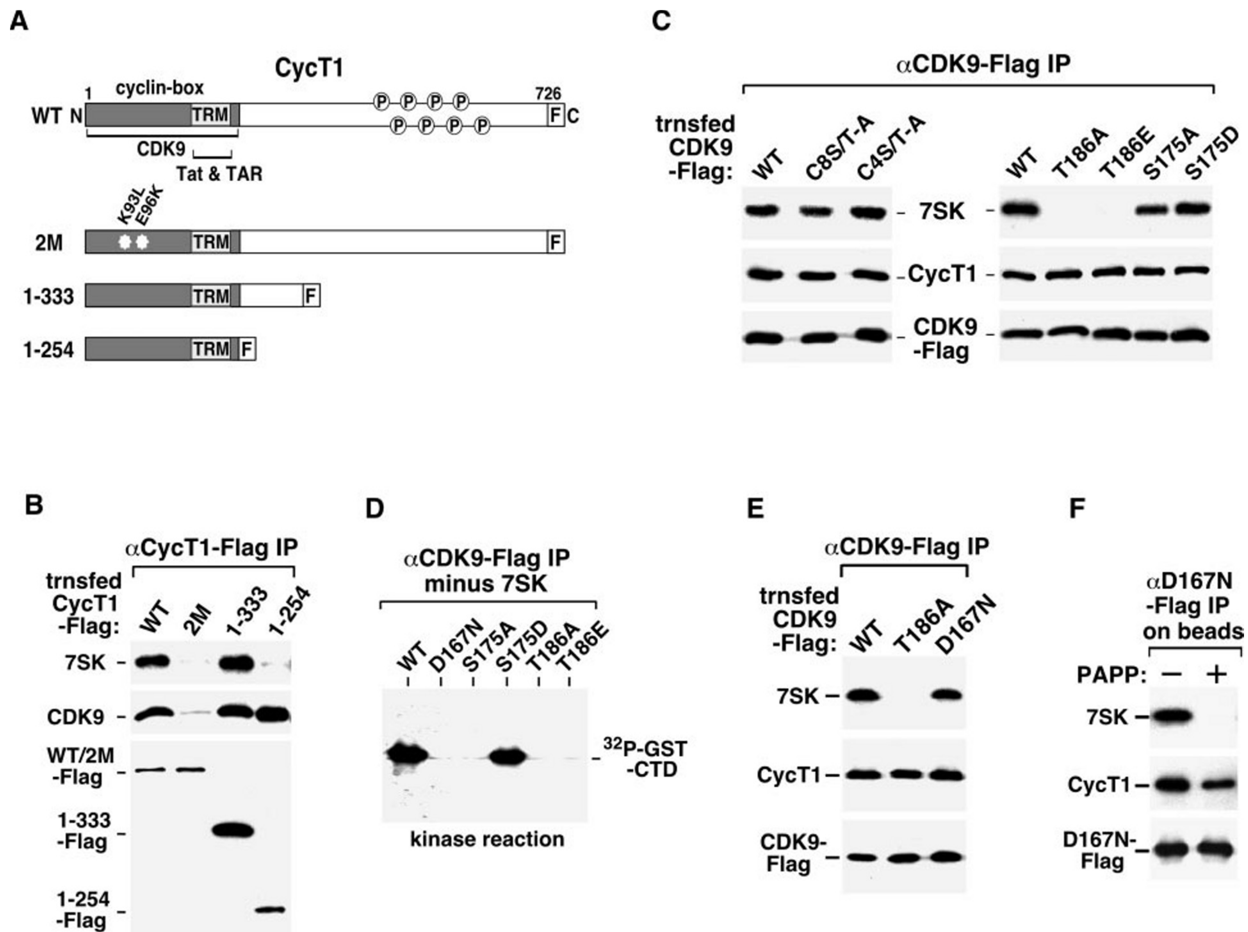


FIG. 2. **Phosphorylation of CDK9 on possibly Thr-186 is important for 7SK·P-TEFb interaction *in vivo*.** *A*, domain structures and phosphorylation patterns of wild-type CycT1, mutant 2M containing point mutations in the cyclin-box, and truncated CycT1-(1–333) and -(1–254) lacking all potential phosphorylation sites. *TRM*, Tat/TAR-recognition motif. *F*, FLAG tag. *B*, requirement of both CycT1 and CDK9 subunits but not CycT1 phosphorylation for 7SK·P-TEFb binding. FLAG-tagged wild-type and mutant CycT1 and their associated CDK9 and 7SK were immunoprecipitated from the transfected HeLa NE and analyzed by Western and Northern blotting. *C*, Thr-186 in CDK9 T-loop is crucial for 7SK·P-TEFb binding. FLAG-tagged wild-type and mutant CDK9 and their associated CycT1 and 7SK were isolated from transfected HeLa cells and analyzed as in *B*. *D*, CTD kinase activities of wild-type and mutant P-TEFb. 7SK/HEXIM1-free P-TEFb containing wild-type or mutant CDK9 were normalized for their CycT1/CDK9 levels and examined in kinase reactions for phosphorylation of GST-CTD. The $^{32}$P-labeled GST-CTD was analyzed by SDS-PAGE followed by autoradiography. *E*, CDK9 kinase activity is not required for 7SK·P-TEFb interaction. The binding to 7SK by the transfected wild-type and mutant CDK9 was analyzed as in *B*. *F*, phosphorylation-dependent 7SK-binding by kinase-defective P-TEFb. The immobilized 7SK-bound P-TEFb containing D167N-FLAG was treated with or without PAPP, washed, eluted, and analyzed by Western and Northern blotting.

the HIV TAR RNA, which can interact with only the monomeric CycT1 in conjunction with Tat (9, 28), 7SK appeared to require also CDK9 for binding to the CycT1/CDK9 heterodimer. For example, the mutant CycT1(2M), with Lys-93 changed to Leu and Glu-96 to Lys within the cyclin-box, bound to neither CDK9 (29) nor 7SK (Fig. 2*B*).

*Requirement of Conserved Thr-186 in CDK9 T-loop for in Vivo 7SK·P-TEFb Binding*—Since CDK molecules have built-in structural flexibility that is most often controlled by phosphorylation (30, 31), we next investigated the requirement of CDK9 phosphorylation for the 7SK·P-TEFb binding. A group of Ser/Thr residues, located near the CDK9 C terminus in a region not conserved with other CDKs, are phosphorylated when CDK9 undergoes autophosphorylation (27). This induces a conformational change in P-TEFb critical for the formation of the P-TEFb·Tat·TAR complex (26, 27). As a member of the CDK family, the activity and conformation of CDK9 are also affected by phosphorylation on other amino acids, which are conserved among several or all CDK family members (31). For example, phosphorylation of a conserved threonine (Thr-186 in CDK9) located at the tip of a flexible loop (the T-loop) in all CDKs is known to induce a major movement of the loop and allow the access to the catalytic center by the substrate (32). Furthermore, a conserved Ser residue in the T-loops of CDK7 (Ser-164) and CDK9 (Ser-175) can also be phosphorylated (33, 34).

Next, FLAG-tagged CDK9 mutants containing point mutations at these phosphorylation sites were analyzed for their interactions with 7SK and CycT1 in transfected HeLa cells. Unlike the binding of P-TEFb to Tat/TAR, which requires the phosphorylation of several Ser/Thr residues near the CDK9 C terminus (27), substitution of 4 (C4S/T-A: Ser-347, Ser-353, Thr-354, and Ser-357) or 8 (C8S/T-A: Ser-329, Thr-330, Thr-333, Ser-334, Ser-347, Thr-350, Ser-353, and Thr-354) Ser/Thr in and around this region with Ala had no or only minor effect on CDK9 binding to 7SK and CycT1 (Fig. 2*C*). Similarly, the Ser-175 to Ala (S175A) mutation in the T-loop only slightly decreased the binding to 7SK, which was fully restored when Ser-175 was changed to Asp (S175D). In contrast, mutation of Thr-186 to either Ala (T186A) or Glu (T186E) completely abolished the binding to 7SK, although neither mutation significantly affected the CycT1-CDK9 interaction. Thus, among all the known phosphorylation sites in CDK9, Thr-186 was the only one essential for the 7SK·P-TEFb binding. Since this conserved threonine is invariably phosphorylated in all activated CDKs, the phosphorylation-dependent 7SK·P-TEFb binding could potentially be attributed to the phosphorylation of Thr-186.

*The Phosphorylation Critical for 7SK·P-TEFb Binding Is Not Caused by CDK9 Autophosphorylation*—We next performed kinase reactions to examine whether the T-loop mutations could affect P-TEFb ability to phosphorylate GST-CTD. Prior to the reactions, the immunoprecipitated CycT1/CDK9-FLAG were washed with a high salt (0.8 M KCl) buffer, which removed the associated 7SK and HEXIM1 without disrupting the CycT1-CDK9 binding (see below). In kinase reactions, the T186A, T186E, and S175A mutations all inactivated P-TEFb, whereas S175D fully restored P-TEFb kinase activity (Fig. 2*D*), revealing a requirement for a negatively charged amino acid at position 175. More importantly, the ability of S175A to inactivate the P-TEFb kinase without significantly affecting P-TEFb binding to 7SK suggests that the CDK9 kinase activity is not required for the binding.

This notion was further supported by the observation that another P-TEFb complex containing the D167N mutant CDK9 was completely inactive as a kinase (Fig. 2*D*), but fully capable of binding to 7SK (Fig. 2*E*). Moreover, as with the wild-type 7SK-bound P-TEFb, treatment of the D167N-containing complex with phosphatase also released 7SK (Fig. 2*F*), indicating that phosphorylation was required for this kinase-defective P-TEFb to bind to 7SK. Because D167N cannot phosphorylate itself or any associated proteins, it is possible that the phosphorylation, possibly on Thr-186, was caused by another unrelated kinase. This issue will be revisited later.

*In Vitro Reconstitution of the 7SK·P-TEFb Interaction*—To further study the control of the 7SK·P-TEFb interaction, we decided to reconstitute this interaction *in vitro*. Since CDK9 phosphorylation has been implicated as critical for the interaction, we tried to assemble the 7SK·P-TEFb complex from P-TEFb that had acquired the key phosphorylation *in vivo*. We took advantage of an observation (35) that 7SK could be washed off the immobilized P-TEFb with buffers containing greater than 0.6 M KCl (*e.g.* 0.8 M KCl in buffer D0.8M), whereas the CycT1/CDK9-FLAG dimer was mostly unaffected and remained tightly bound to the beads (Fig. 3*A*). HEXIM1, which bound to P-TEFb through 7SK, was also stripped off P-TEFb by the same D0.8M buffer (17).

The 7SK/HEXIM1-free P-TEFb thus obtained was then incubated with HeLa NE, protein-free total RNA extracted from NE, purified native 7SK RNA or heat-denatured (Δ) 7SK to reconstitute the 7SK·P-TEFb interaction (Fig. 3*B*). The purified 7SK was from a fraction (termed 7SK fraction) that was stripped off the immobilized P-TEFb by buffer D0.8M (Fig. 3*B*). After the incubation and washing with buffer D0.4M, the reconstituted 7SK·P-TEFb complex was eluted with FLAG peptide and analyzed by Western and Northern blotting. The control reactions in *lanes 6–10*, Fig. 3*C* used HeLa NE in anti-FLAG immunoprecipitation, which retained no P-TEFb on the beads. Compared with these reactions, the 7SK-free CycT1/CDK9-FLAG derived from F1C2 NE interacted with 7SK from all four sources (Fig. 3*C*, *lanes 2–5*), which contained a similar level of 7SK (*lanes 11–14*). Even the heat-denatured 7SK interacted with P-TEFb, probably because the RNA quickly adopted an active conformation once in the reaction. Thus, no additional protein from HeLa NE was required for the reconstituted 7SK·P-TEFb interaction.

*Dependence on Thr-186 for Reconstituted 7SK·P-TEFb Interaction*—To test the specificity of the reconstituted 7SK·P-TEFb interaction, we examined whether Thr-186 in the CDK9 T-loop was required. P-TEFb containing wild-type or mutant (T186A or D167N) CDK9-FLAG was immunoprecipitated from the transfected HeLa cells and then washed with D0.8M to remove 7SK/HEXIM1. While both wild-type and the D167N P-TEFb bound to 7SK present in total RNA and HeLa NE, the T186A P-TEFb failed (Fig. 4*A*). Thus, like the situation *in vivo* (Fig. 2*E*), Thr-186 was also required for the 7SK·P-TEFb binding *in vitro*. Moreover, a Thr-186-dependent binding could be detected between P-TEFb and the *in vitro* transcribed 7SK by T7 RNA polymerase (Fig. 4*A*). This binding was as efficient as the one involving the native HeLa 7SK (data not shown), ruling out a significant role of any potential 7SK modification in the binding.

*Specific Reconstituted Interaction of P-TEFb with 7SK but Not Other snRNAs*—To further test the specificity of the reconstituted 7SK·P-TEFb interaction, we asked whether other snRNA species could also bind to P-TEFb in the reconstitution reactions. Data in Fig. 4*B* indicate that 7SK from both HeLa NE and the total RNA fraction bound to P-TEFb efficiently. In contrast, the spliceosomal U1 and U2 snRNA from these two sources failed to bind to P-TEFb in the same reactions. This specificity was especially striking given that U1 and U2 were highly abundant in the input materials (*lanes 5* and *6*) but only a trace amount of U1 from HeLa NE-bound non-specifically to

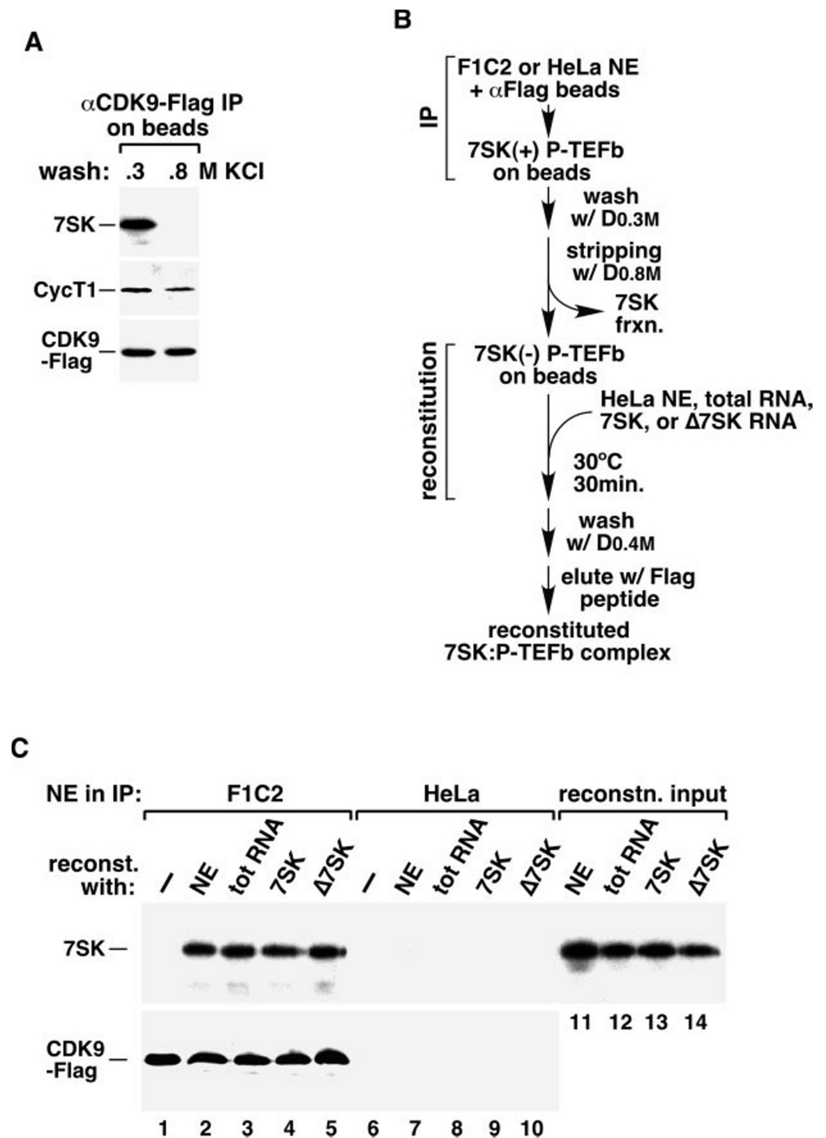


FIG. 3. **Reconstitution of 7SK·P-TEFb interaction *in vitro*.** *A*, high salt disrupts 7SK·P-TEFb interaction. The immobilized 7SK-bound P-TEFb was washed with Buffer D containing either 0.3 or 0.8 M KCl, eluted, and analyzed by Western and Northern blotting. *B*, a procedure for reconstituting 7SK·P-TEFb interaction *in vitro*. Anti-FLAG immunoprecipitates from F1C2 NE (containing CDK9-FLAG) or HeLa NE (a negative control) was washed with D0.8M to yield the immobilized 7SK(−) P-TEFb. This was then incubated with HeLa NE, protein-free total RNA, purified native or heat-denatured (Δ) 7SK to reconstitute the 7SK·P-TEFb complex. After extensive washing, the reconstituted complex was eluted with FLAG peptide. *C*, reconstitution of 7SK·P-TEFb binding does not require other nuclear proteins. Northern and Western analyses of 7SK and CDK9-FLAG in the complexes reconstituted under the various conditions described in *B*. 7SK in the input materials (5%) was examined by Northern blotting in *lanes 11–14*.

the beads independently of P-TEFb (*lanes 1* and *2*).

*7SK Binds to Phosphorylated P-TEFb in Vitro*—To determine whether ATP could affect the reconstituted 7SK·P-TEFb interaction, we incubated the immobilized 7SK(−) P-TEFb with HeLa NE either depleted of the endogenous ATP or containing exogenously added ATP (Fig. 5*A*). The extract used in the ATP(−) condition had been dialyzed to remove the endogenous ATP, and then treated with hexokinase and glucose to further reduce the ATP level to below 10 $\mu$M (25). Compared with this condition, addition of ATP consistently increased the 7SK·P-TEFb binding by about 2-fold (Fig. 5*B*, *lanes 1* and *2*). In HeLa NE, ~50% of P-TEFb are 7SK-free (6, 7), probably because they lack the key phosphorylation to bind to 7SK. Therefore, the ATP-dependent 2-fold increase in 7SK binding was significant and could be due to the phosphorylation of these 7SK(−) P-TEFb, which then activated their 7SK binding ability. In support of this idea, we found that upon removal of the 7SK-bound P-TEFb from NE (through anti-HEXIM1 depletion as it is part of the 7SK·HEXIM1·P-TEFb snRNP) (17), the remaining 7SK(−) P-TEFb failed to bind to 7SK unless it was phosphorylated in the presence of ATP (Fig. 5*C*).

Further support of a phosphorylation-dependent 7SK·P-TEFb binding *in vitro* came from the observation that dephosphorylation of the 7SK(−) P-TEFb by recombinant PP1 prior to the reconstitution reaction (see procedure in Fig. 5*A*) markedly reduced the amount of 7SK bound to P-TEFb (Fig. 5*B*, compare *lanes 1* and *3*). Addition of ATP into the reaction dramatically increased the 7SK binding (*lane 4*). It is likely that the PP1-treated P-TEFb was re-phosphorylated under this condition, which restored its 7SK binding ability (compare *lanes 2* and *4*).

*P-TEFb Is Phosphorylated by a Non-CAK HeLa Nuclear Kinase*—What could be the kinase that phosphorylated P-TEFb? The phosphorylation-dependent binding of 7SK to the kinase-inactive D167N- or S175A-P-TEFb (Fig. 2, *C* and *E*) suggests that the relevant kinase was present in the HeLa nucleus and different from CDK9. Further support for the existence of a key kinase in HeLa NE came from the observation that if protein-free total RNA was used in place of HeLa NE in the reconstitution reaction, no ATP-dependent increase in 7SK binding was observed regardless of whether the PP1-treated or untreated P-TEFb was used (Fig. 5*B*, *lanes 5–8*). Because the intermolecular CDK9 autophosphorylation could occur under these conditions (data not shown), its inability to promote the ATP-dependent 7SK·P-TEFb binding rules out its involvement in this process.

Several CDKs (CDC2, CDK2, and CDK4) that control cell cycle transitions are phosphorylated on the T-loop threonines by the CDK-activating kinase CAK (reviewed in Ref. 36), which consists of CDK7, cyclin H, and MAT1. Given that the phosphorylation of CDK9 on possibly Thr-186 promoted the 7SK·P-

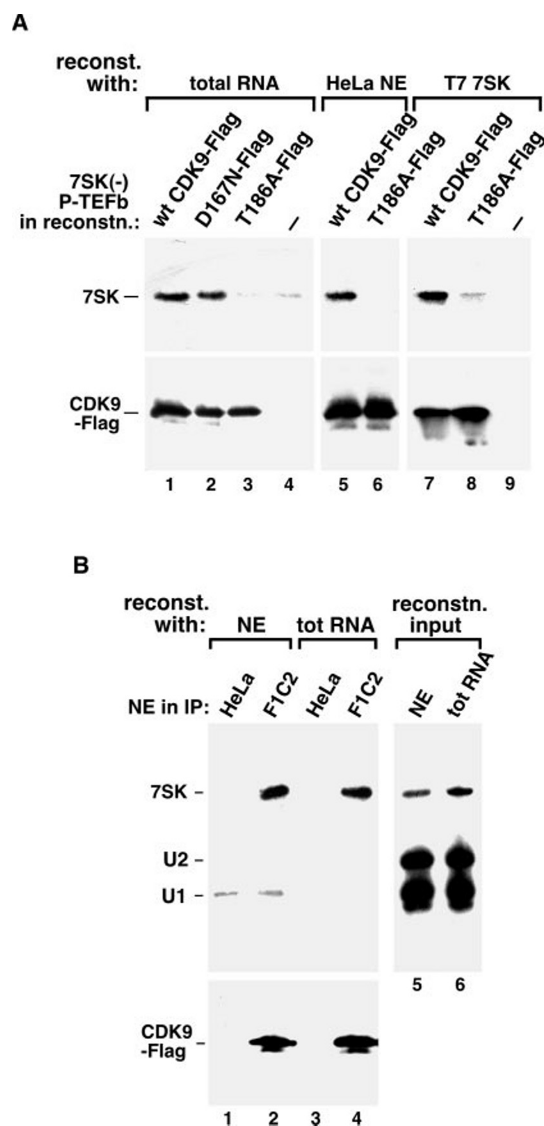


FIG. 4. **Dependence on Thr-186 in CDK9 T-loop for reconstituted interaction of P-TEFb with 7SK but not other snRNA species.** *A*, Thr-186-dependent binding of P-TEFb to both native and *in vitro* synthesized 7SK *in vitro*. P-TEFb containing FLAG-tagged wild-type or mutant CDK9 was immunoprecipitated from transfected HeLa NE and then stripped with high salt. The immobilized 7SK(−) P-TEFb was incubated with protein-free total RNA (*lanes 1–4*), HeLa NE (*lanes 5* and *6*), or *in vitro* transcribed 7SK (T7 7SK, *lanes 7–9*). The levels of 7SK and CDK9 in the reconstituted 7SK·P-TEFb were analyzed by Northern and Western blotting. *B*, specific binding of P-TEFb to 7SK but not U1 or U2 snRNAs *in vitro*. The reactions contained HeLa NE or total RNA and anti-FLAG beads with (F1C2) or without (HeLa) the associated P-TEFb as indicated. The reconstituted complexes were analyzed for the presence of 7SK, U1, U2, and CDK9-FLAG. RNAs in 5% of the input materials were shown in *lanes 5* and *6*.

TEFb binding, we asked whether CAK could replace the HeLa nuclear kinase to promote the ATP-dependent binding *in vitro*. The affinity-purified CAK (via anti-CDK7-HA IP) was active as shown by its phosphorylation of GST-CTD (Fig. 5*B*, *lane 12*). However, unlike the kinase in HeLa NE, it failed to induce the binding of 7SK to the PP1-treated P-TEFb in the presence of ATP (compare *lanes 10* and *4*), Thus, the CDK9 phosphorylation critical for the 7SK·P-TEFb binding was likely mediated by a non-CAK nuclear kinase.

*Stress-inducing Agents Disrupt 7SK·P-TEFb Binding by Causing P-TEFb Dephosphorylation*—Treatment of HeLa cells with certain stress-inducing agents such as actinomycin D and DRB causes the release of 7SK and HEXIM1 from P-TEFb, increasing the cellular level of active P-TEFb for stress-induced gene expression (6, 7, 17, 18). When tested in reconstitution reactions, 7SK extracted from both untreated and actinomycin D- or DRB-treated cells bound to P-TEFb with a similar efficiency (Fig. 6*A*), suggesting that the treatment did not cause any changes in 7SK to prevent its binding to P-TEFb. In contrast, P-TEFb isolated from only the untreated but not the treated cells bound to 7SK (Fig. 6*B*, *lanes 2–4*). Importantly, incubation of the latter form of P-TEFb with ATP and HeLa NE containing the critical kinase for P-TEFb restored the 7SK·P-TEFb binding (*lanes 6–8*), suggesting that the stress-induced dissociation of 7SK from P-TEFb may be caused by the loss of the key phosphorylation on P-TEFb important for 7SK binding.

## DISCUSSION

The sequestration of a major portion of cellular P-TEFb into the 7SK snRNP and the induced release of P-TEFb from this sequestration constitute a fast responsive regulatory circuit to control the nuclear level of active P-TEFb for normal and stress-induced transcription. Within the snRNP, 7SK plays a structural role in mediating the interaction of HEXIM1 with P-TEFb (17). Although 7SK can bind to P-TEFb independently of HEXIM1, this binding alone does not inhibit P-TEFb. Inactivation of P-TEFb requires HEXIM1, which interacts with only the 7SK-bound P-TEFb (17). Given the importance of the 7SK·P-TEFb binding in recruiting HEXIM1, we investigated how the binding is regulated. Using an *in vitro* system that reconstituted the specific binding of P-TEFb to 7SK but not other snRNA species, together with an *in vivo* binding assay, we demonstrate that the phosphorylation of P-TEFb by a non-CAK HeLa nuclear kinase promotes the 7SK·P-TEFb binding.

The previously identified C-terminal phosphorylation of CDK9, which is essential for forming the P-TEFb·Tat·TAR ternary complex (26, 27), was not required for the 7SK·P-TEFb binding (Fig. 2*C*). Instead, Thr-186 of the CDK9 T-loop was important. This conserved threonine, whose phosphorylation controls the conformation and activity of all CDKs, is one of the most important regulatory residues in a CDK molecule (30, 31). Several observations have led us to propose that the phosphorylated Thr-186 contributes directly to the phosphorylation-dependent 7SK·P-TEFb binding. For example, the C-terminally truncated CycT1 lacking all the phosphorylation sites (Fig. 2*B*), the *in vitro*-synthesized 7SK (Fig. 4*A*), and the immobilized P-TEFb stripped of the associated HEXIM1/7SK and then dephosphorylated by PP1 (Fig. 5*B*) could all be assembled into the 7SK·P-TEFb complex in a phosphorylation-dependent manner. These results are consistent with the notion that the phosphorylation of CDK9, but not CycT1, HEXIM1, or 7SK, is critical for the 7SK·P-TEFb binding. Furthermore, among all the known phosphorylated residues in CDK9, Thr-186 was the only one important for the binding both *in vivo* and *in vitro* (Figs. 2, *C* and *E* and 4*A*).

The possibility that 7SK specifically targets P-TEFb with the phosphorylated T-loop and thus an accessible catalytic site has important implications for the control of P-TEFb activity. As the T-loop phosphorylation is generally required for CDK activation (30, 31), it is expected that P-TEFb will become active once Thr-186 is phosphorylated. This event, although critical for CDK9 activation, could also attract 7SK/HEXIM1 and lead to the inactivation of P-TEFb. To resolve this apparent paradox, we speculate that a competing mechanism may work next to the elongating polymerase to prevent 7SK/HEXIM1 from binding to the phosphorylated P-TEFb and maintain P-TEFb in an active state for transcription. On the HIV-1 template, the actions of Tat and TAR may provide just such a mechanism. The P-TEFb·Tat·TAR ternary complex is known to contain an active P-TEFb that phosphorylates Pol II and stimulates elon-

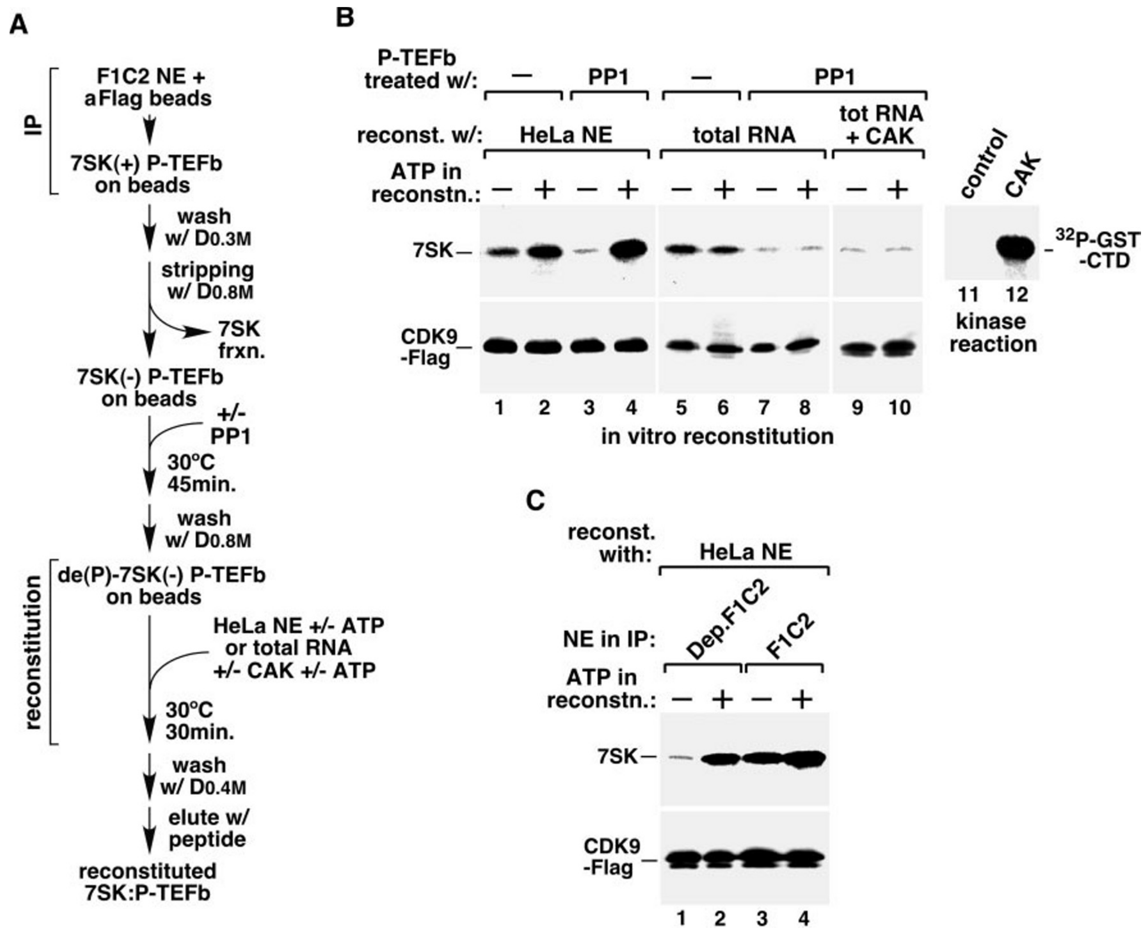


FIG. 5. **7SK targets P-TEFb phosphorylated by a non-CAK HeLa nuclear kinase.** *A*, procedure for generating the immobilized 7SK(−) P-TEFb, which was dephosphorylated with PP1, washed extensively to remove PP1, and then incubated in the reconstitution reactions with HeLa NE ± ATP or protein-free total RNA ± purified CAK ± ATP. *B*, re-phosphorylation of PP1-treated P-TEFb by a non-CAK HeLa nuclear kinase is required for P-TEFb binding to 7SK. The 7SK·P-TEFb complexes reconstituted under the various conditions indicated in *A* were analyzed by Western and Northern blotting. *Lanes 11* and *12* show labeled GST-CTD phosphorylated by affinity-purified CAK (through anti-CDK7-HA IP) or a control fraction prepared from untransfected cells. *C*, native 7SK(−) P-TEFb in NE fails to bind to 7SK unless phosphorylated. Normal or HEXIM1-depleted F1C2 NE in which the 7SK/HEXIM1-bound P-TEFb was removed was used in immunoprecipitation to generate the immobilized P-TEFb for subsequent reconstitution with 7SK in HeLa NE ± ATP.

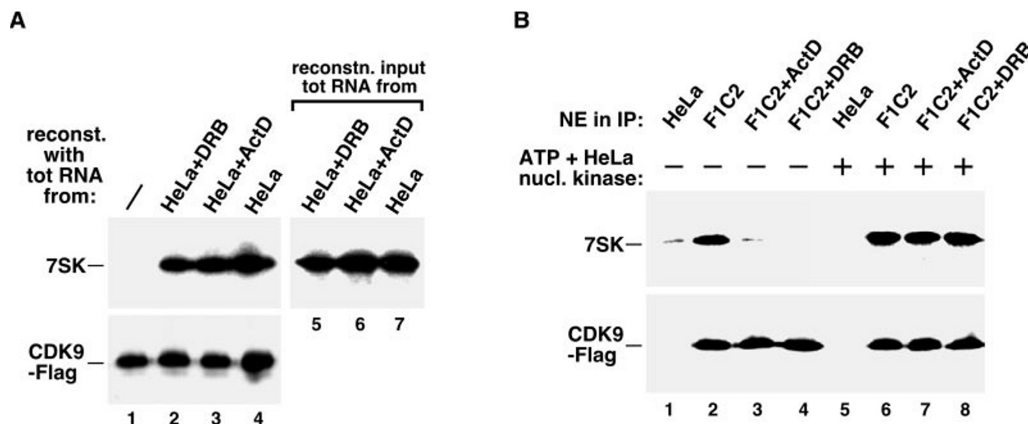


FIG. 6. **Stress-inducing drugs disrupt 7SK·P-TEFb binding by inducing P-TEFb dephosphorylation.** *A*, 7SK from drug-treated cells maintains P-TEFb-binding activity. Reconstitution reactions contained immobilized 7SK(−) P-TEFb and buffer alone (*lane 1*) or protein-free total nuclear RNA isolated from HeLa cells treated with or without the indicated drugs (*lanes 2–4*). The reconstituted 7SK·P-TEFb and 5% of the input RNA were analyzed by Northern and Western blotting. *B*, P-TEFb from drug-treated cells cannot reconstitute with 7SK unless phosphorylated by a HeLa nuclear kinase. Reactions contained no (*lanes 1* and *5*) or immobilized 7SK(−) P-TEFb isolated from untreated or drug-treated F1C2 cells (*lanes 2–4* and *6–8*) and total RNA without ATP (*lanes 1–4*) or HeLa NE plus ATP (*lanes 5–8*) as indicated. The reconstitution was monitored by Northern and Western analyses.

gation (15, 16). Recently, it has been shown that the formation of this complex precludes the binding of 7SK/HEXIM1 to P-TEFb (18).

By targeting the T-loop-phosphorylated P-TEFb that is poised to become active, 7SK and HEXIM1 have the potential to maximally control the nuclear level of active P-TEFb. In addition, this also renders the regulation of P-TEFb highly sensitive, as the dissociation of 7SK/HEXIM1 from only a small

fraction of P-TEFb in response to stress signals and environmental stimuli can lead to a significant induction of the overall P-TEFb activity in the cell. Consistent with these ideas, it has been noticed that although CDK9 and CycT1 are fairly abundant in the cell, the amount of the transcriptionally active P-TEFb appears to be limiting in a variety of cell types and under different conditions (8, 24, 37). Finally, by targeting CDK9 with the phosphorylated T-loop and consequently an "open" conformation, 7SK/HEXIM1 may physically block the access to the catalytic center, thereby inhibiting the CDK9 kinase. A direct test of this hypothesis requires the structural determination of the 7SK snRNP in the future.

Although our data so far strongly suggest a key role of the phosphorylated Thr-186 in 7SK snRNP formation, an ultimate confirmation of this will require the determination of the phosphorylation state of Thr-186 in both the 7SK/HEXIM1(−) and 7SK/HEXIM1(+) P-TEFb complexes. Unfortunately, this effort has been hampered by the difficulty of obtaining enough materials for analysis by mass spectrometry and the failure to generate phosphospecific antibodies that can recognize the phosphorylated CDK9 T-loop. Despite the lack of a final confirmation, one notion that has received strong support is that the phosphorylation crucial for the 7SK·P-TEFb binding was not caused by CDK9 itself or the general CDK-activating kinase CAK, but rather by a yet-to-be-identified HeLa nuclear kinase. Future identification of this kinase is important, as it may help uncover the signaling pathway that determines the level of cellular P-TEFb tagged for inhibition though association with 7SK. The newly established *in vitro* system that reconstitutes the specific 7SK·P-TEFb interaction will be very useful in this regard.

A process opposite to the sequestration of P-TEFb into the 7SK snRNP is the stress-induced dissociation of 7SK/HEXIM1 from P-TEFb (6, 7, 17, 18), in which an important role of the dephosphorylation of P-TEFb has been revealed (Fig. 6). This event could be caused by either the activation of a specific phosphatase or the suppression of the key nuclear kinase for P-TEFb. Consistent with a potential role of a phosphatase in this process, *in vitro* dephosphorylation of P-TEFb by PP1 disrupted the 7SK·P-TEFb binding (Fig. 5*B*). Interestingly, PP1 has been reported to stimulate HIV-1 transcription *in vivo* and *in vitro* (38–40). Future studies are necessary to determine whether PP1 may exert its stimulatory effect by releasing 7SK/HEXIM1 from P-TEFb *in vivo* and whether the cellular level or activity of PP1 may increase in response to various physiological stimuli that free P-TEFb from the 7SK snRNP.

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REFERENCES

1. Humphries, P., Russell, S. E. H., McWilliam, P., McQuaid, S., Pearson, C., and Humphries, M. M. (1987) *Biochem. J.* **24,** 281–284
2. Murphy, S., Altruda, F., Ullu, E., Tripodi, M., Silengo, L., and Melli, M. (1984) *J. Mol. Biol.* **177,** 575–590
3. Reddy, R., Henning, D., Subrahmanyam, C. S., and Busch, H. (1984) *J. Biol. Chem.* **259,** 12265–12270
4. Ullu, E., and Melli, M. (1982) *Nucleic Acids Res.* **10,** 2209–2223
5. Zieve, G., Benecke, B. J., and Penman, S. (1977) *Biochemistry* **16,** 4520–4525
6. Nguyen, V. T., Kiss, T., Michels, A. A., and Bensaude, O. (2001) *Nature* **414,** 322–325
7. Yang, Z., Zhu, Q., Luo, K., and Zhou, Q. (2001) *Nature* **414,** 317–322
8. Peng, J., Zhu, Y., Milton, J. T., and Price, D. H. (1998) *Genes Dev.* **12,** 755–762
9. Wei, P., Garber, M. E., Fang, S. M., Fischer, W. H., and Jones, K. A. (1998) *Cell* **92,** 451–462
10. Shim, E. Y., Walker, A. K., Shi, Y., and Blackwell, T. K. (2002) *Genes Dev.* **16,** 2135–2146
11. Renner, D. B., Yamaguchi, Y., Wada, T., Handa, H., and Price, D. H. (2001) *J. Biol. Chem.* **276,** 42601–42609
12. Wada, T., Takagi, T., Yamaguchi, Y., Watanabe, D., and Handa, H. (1998) *EMBO J.* **17,** 7395–7403
13. Yamaguchi, Y., Takagi, T., Wada, T., Yano, K., Furuya, A., Sugimoto, S., Hasegawa, J., and Handa, H. (1999) *Cell* **97,** 41–51
14. Chao, S.-H., and Price, D. H. (2001) *J. Biol. Chem.* **276,** 31793–31799
15. Jeang, K. T., Xiao, H., and Rich, E. A. (1999) *J. Biol. Chem.* **274,** 28837–28840
16. Price, D. H. (2000) *Mol. Cell Biol.* **20,** 2629–2634
17. Yik, J. H. N., Chen, R., Nishimura, R., Jennings, J. L., Link, A. J., and Zhou, Q. (2003) *Mol. Cell* **12,** 971–983
18. Michels, A. A., Nguyen, V. T., Fraldi, A., Labas, V., Edwards, M., Bonnet, F., Lania, L., and Bensaude, O. (2003) *Mol. Cell Biol.* **23,** 4859–4869
19. Ouchida, R., Kusuhara, M., Shimizu, N., Hisada, T., Makino, Y., Morimoto, C., Handa, H., Ohsuzu, F., and Tanaka, H. (2003) *Genes Cells* **8,** 95–107
20. Marks, P. A., Richon, V. M., and Rifkind, R. A. (1996) *Int. J. Hematol.* **63,** 1–17
21. Valerie, K., Delers, A., Bruck, C., Thiriart, C., Rosenberg, H., Debouck, C., and Rosenberg, M. (1988) *Nature* **333,** 78–81
22. Casse, C., Giannoni, F., Nguyen, V. T., Dubois, M.-F., and Bensaude, O. (1999) *J. Biol. Chem.* **274,** 16097–16106
23. Sano, M., and Schneider, M. D. (2003) *Cell Cycle* **2,** 99–104
24. Sano, M., Abdellatif, M., Oh, H., Xie, M., Bagella, L., Giordano, A., Michael, L. H., DeMayo, F. J., and Schneider, M. D. (2002) *Nat. Med.* **8,** 1310–1317
25. Zamore, P. D., Tuschl, T., Sharp, P. A., and Bartel, D. P. (2000) *Cell* **101,** 25–33
26. Fong, Y. W., and Zhou, Q. (2000) *Mol. Cell Biol.* **20,** 5897–5907
27. Garber, M. E., Mayall, T. P., Suess, E. M., Meisenhelder, J., Thompson, N. E., and Jones, K. A. (2000) *Mol. Cell Biol.* **20,** 6958–6969
28. 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) *Genes Dev.* **12,** 3512–3527
29. Bieniasz, P. D., Grdina, T. A., Bogerd, H. P., and Cullen, B. R. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 7791–7796
30. Pavletich, N. P. (1999) *J. Mol. Biol.* **287,** 821–828
31. Morgan, D. O. (1995) *Nature* **374,** 131–134
32. Russo, A. A., Jeffrey, P. D., and Pavletich, N. P. (1996) *Nat. Struct. Biol.* **3,** 696–700
33. Martinez, A. M., Afshar, M., Martin, F., Cavadore, J. C., Labbe, J. C., and Doree, M. (1997) *EMBO J.* **16,** 343–354
34. Larochelle, S., Chen, J., Knights, R., Pandur, J., Morcillo, P., Erdjument-Bromage, H., Tempst, P., Suter, B., and Fisher, R. P. (2001) *EMBO J.* **20,** 3749–3759
35. Shore, S. M., Byers, S. A., Maury, W., and Price, D. H. (2003) *Gene* (*Amst.*) **307,** 175–182
36. Harper, J. W., and Elledge, S. J. (1998) *Genes Dev.* **12,** 285–289
37. Liou, L. Y., Herrmann, C. H., and Rice, A. P. (2002) *J. Virol.* **76,** 10579–10587
38. Ammosova, T., Jerebtsova, M., Beullens, M., Voloshin, Y., Ray, P., Kumar, A., Bollen, M., and Nekhai, S. (2003) *J. Biol. Chem.* **278,** 32189–32194
39. Washington, K., Ammosova, T., Beullens, M., Jerebtsova, M., Kumar, A., Bollen, M., and Nekhai, S. (2002) *J. Biol. Chem.* **277,** 40442–40448
40. Bharucha, D. C., Zhou, M., Nekhai, S., Brady, J. N., Shukla, R. R., and Kumar, A. (2002) *Virology* **296,** 6–16