

## Mutual Targeting of Mediator and the TFIID Kinase Kin28\*

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**In *Saccharomyces cerevisiae*, Kin28 is a member of the cyclin-dependent kinase family. Kin28 is a subunit of the basal transcription factor holo-TFIID and its trimeric sub-complex TFIID. Kin28 is the primary kinase that phosphorylates the RNA polymerase II (RNA pol II) C-terminal domain (CTD) within a transcription initiation complex. Mediator, a global transcriptional co-activator, dramatically enhances the phosphorylation of the CTD of RNA pol II by holo-TFIID *in vitro*. Using purified proteins we have determined that the subunits of TFIID are sufficient for Mediator to enhance Kin28 CTD kinase activity and that Mediator enhances phosphorylation of a glutathione S-transferase-CTD fusion protein, despite the absence of multiple Mediator and/or TFIID interactions with polymerase. Mediator does not stimulate the activity of several other CTD kinases, suggesting that the specific enhancement of TFIID kinase activity results in Kin28 being the primary CTD kinase at initiation. In addition, we have found that Kin28 phosphorylates Mediator subunit Med4 in an assay, including purified holo-TFIID, and either Mediator or recombinant Med4 alone. Furthermore, Kin28 appears to be, at least in part, responsible for the phosphorylation of Med4 *in vivo*. We have identified Thr-237 as the site of phosphorylation of Med4 by Kin28 *in vitro*. The mutation of Thr-237 to Ala has no effect on the growth of a yeast strain under normal conditions but confirms that Thr-237 is also the site of Med4 phosphorylation *in vivo*.**

TFIID is a RNA pol II<sup>1</sup> general transcription factor that, when purified to homogeneity, is composed of nine subunits conserved from yeast to humans. An intact nine-subunit complex, holo-TFIID, is fully capable of satisfying the TFIID requirements for transcription in purified systems derived from yeast and mammalian cells (1, 2). In the yeast *Saccharomyces cerevisiae* the genes encoding all nine subunits are essential for

viability. Structurally, TFIID can be divided into sub-complexes that may have distinct functional roles in the cell. "Core" TFIID consists of Rad3, Tfb1, Tfb2, Ssl1, and Tfb4, as well as a sixth subunit, Ssl2, that is loosely bound to the core and is often sub-stoichiometric in purified TFIID. Tfb3, Ccl1, and Kin28 make up a trimeric holo-TFIID sub-complex called TFIID (3). In *S. cerevisiae*, Kin28 and Ccl1 are a cyclin-dependent kinase (cdk)-cyclin-like pair that possesses the ability to phosphorylate the CTD of RNA pol II, whereas in higher eukaryotes the cdk-cyclin (Cdk7/cyclin H) components of TFIID also serve as a Cdk-activating kinase. It has recently been shown that TFIID exists separately from holo-TFIID in yeast extracts, but it is still unclear what function TFIID, as an independent entity, has *in vivo* (4). Despite extensive studies (for review see Ref. 5), clearly defining the role of the kinase activity of Kin28 in transcription has remained elusive.

The kinase activity of Kin28 is essential for viability (6), and a whole genome analysis, using the temperature-sensitive mutant strain *kin28-ts3*, has shown a decrease in the mRNA levels of nearly all RNA pol II-transcribed genes at the non-permissive temperature (7). It is unclear, however, whether this decrease results from defects at transcription initiation or one of the many other processes regulated and targeted by the dynamic phosphorylation of the CTD of RNA pol II. Kin28 has been shown to be the primary CTD kinase at initiation *in vivo* (6, 8–10), but the CTD and its phosphorylation state also play an important role in transcription elongation, mRNA processing, and mRNA export (11), all of which could affect steady-state mRNA levels. Small molecules that specifically inhibit the kinase activity of Kin28 cause a decrease in elongating polymerases *in vivo* and a defect in transcription in yeast nuclear extracts (6). However, the role of Kin28 in transcription is not straightforward, because basal transcription reactions reconstituted from purified factors require neither the Kin28 kinase activity nor the CTD itself (12, 13).

Srb10/11 is a second cdk-cyclin-like pair in yeast that is a component of transcription initiation complexes formed in nuclear extracts (14). In an otherwise wild-type background, Srb10/11, which are encoded by non-essential genes, do not seem to be major contributors to the phosphorylation (6, 15) of the CTD *in vivo*. Genetic and genomic analyses show that Srb10/11 can have both negative (7) and positive (16) effects on the expression of certain subsets of genes *in vivo*. Experiments *in vitro* have shown that repression of transcription may originate from Srb10/11 phosphorylating the CTD prior to initiation and preventing the entry of RNA pol II into initiation complex, whereas Kin28 seems to phosphorylate the CTD only after the initiation complex has formed (17). Transcription in nuclear extracts was actually decreased by the specific inhibition of Srb10, but only in the absence of Kin28 kinase activity (6). What causes Kin28 and not Srb10/11 to be the primary CTD kinase at initiation is still an open question. Biochemical

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<sup>1</sup> The abbreviations used are: pol II, polymerase II; cdk, cyclin-dependent kinase; GST, glutathione S-transferase; CTD, C-terminal domain; DTT, dithiothreitol; 5-FOA, 5-fluoroorotic acid; RP, reversed-phase; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; TAP, tandem affinity purification; TEV, tobacco etch virus.



combinations described in the figures: holo-TFIIF (20 fmol), TFIIFK (20 fmol), Mediator (30 fmol), GST-CTD (300 fmol), RNA pol II (200 fmol), rSrb10/11 (400 fmol), and casein kinase 1 (25,000 units). Because small changes in the absolute magnitude of the Mediator enhancement between batches of reactions were possible, an individual control is shown for each experiment.

**Western Blot Analysis**—To examine the phosphorylation state of Med4 in the *KIN28* wild-type strain (GF260-2) and *kin28-ts3* temperature-sensitive strain (JGV105) we followed the protocol of Valay *et al.* (8) previously used to harvest and lyse these same strains for the analysis of the phosphorylation state of Rpb1p. In brief, yeast cultures were grown in YPD medium at 24 °C to an  $A_{600}$  of ~1.0, quickly shifted to 37 °C, and 5-ml samples were removed at 0, 20, and 60 min. For analysis of the *med4-T237A* mutant and the *MED4* wild-type control, the cells were grown in YPD medium to an  $A_{600}$  of ~1.2 at 30 °C. The cells were harvested and disrupted as described (8) and resuspended in 200  $\mu$ l of loading dye. Protein samples of ~2  $\mu$ l were loaded onto an 8% (w/v) polyacrylamide gel, transferred to polyvinylidene difluoride membrane (Amersham Biosciences), and probed with polyclonal  $\alpha$ -Med4 serum.

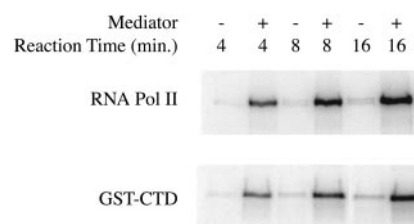
**Genetic Manipulations**—A BamHI-XhoI fragment containing the *MED4* gene and its native promoter was cut out of pGM23 (22) and cloned into these same sites in pRS315 (40) to construct the vector pMED4 (*LEU2 MED4*). Starting from pMED4 the Stratagene QuikChange kit was used to mutate Thr-237 to alanine to create pMED4 T237A (*LEU2 med4-T237A*), or a stop codon to create pMED4  $\Delta$ 237 (*LEU2 med4- $\Delta$ 237*).

A diploid yeast strain (accession number Y22430, EUROSCARF, Frankfurt), that was heterozygous for *MED4* deletion (BY4743; *MAT a $\alpha$* ; *his3 $\Delta$ 1/his3 $\Delta$ 1*; *leu2 $\Delta$ 0/leu2 $\Delta$ 0*; *lys2 $\Delta$ 0/LYS2*; *MET15/met15 $\Delta$ 0*; *ura3 $\Delta$ 0/ura3 $\Delta$ 0*; *med4::kanMX4/MED4*) was transformed with pGM23 (*MED4 URA*) and sporulated, and tetrads were dissected on YPD agar and scored for survival on 5-fluoroorotic acid (5-FOA) and G418. Using a spore that was G418-resistant and subject to killing by 5-FOA (*MAT a $\alpha$* ; *his3 $\Delta$ 1*; *leu2 $\Delta$ 0*; *LYS2*; *met15 $\Delta$ 0*; *ura3 $\Delta$ 0*; *med4::kanMX4*; pGM23 (*MED4 URA*)), the pMED4 (*LEU2 MED4*), pMED4 T237A (*LEU2 med4-T237A*), or pMED4  $\Delta$ 237 (*LEU2 med4- $\Delta$ 237*) plasmids were shuffled as in a previous study (41), and survival was scored on 5-FOA.

**Mass Spectrometry**—Gel-resolved proteins were digested with trypsin, batch-purified on a reversed-phase (RP) micro-tip, and an aliquot analyzed by matrix-assisted laser desorption/ionization (MALDI) reflectron time-of-flight (TOF) mass spectrometry (MS) (UltraFlex TOF/TOF; BRUKER Daltonics; Bremen, Germany) for peptide mass fingerprinting, as described (42, 43). This served to confirm the identity of the proteins and to locate possible differences between the tryptic peptide maps of the phosphorylated and unphosphorylated forms. The remainder of the RP-eluted digest mixtures were then subjected to immobilized gallium(III) affinity chromatography for selective capture of phosphopeptides, followed by elution with phosphate buffer, desalting over an RP tip, and a second round of MALDI-reflectron TOF MS (44). Peak *m/z* values were matched to the protein sequence, allowing for the likely presence of one or more phosphate groups. Mass spectrometric sequencing of the putative phosphopeptides was then carried by MALDI-TOF/TOF (MS/MS) analysis using the UltraFlex instrument in "LIFT" mode. Fragment ion spectra were inspected for *a*<sup>+</sup>, *b*<sup>+</sup>, and *y*<sup>+</sup> ions to compare with the computer-generated fragment ion series of the predicted tryptic peptides to locate the exact position of either phosphoserine or phosphothreonine.

## RESULTS

**A GST-CTD Fusion Protein Is Sufficient as a Substrate for Mediator-enhanced TFIIF Kinase Activity**—Both the CTD (21, 22) itself and the core structure of RNA pol II (45) have been implicated as being important for the interaction of Mediator with the polymerase. Additionally, it has been found that TFIIF physically interacts directly with RNA pol II (46). To determine whether the CTD was sufficient for Mediator-enhanced TFIIF kinase activity or whether additional interactions with the body of RNA pol II were required, we expressed and purified a GST-CTD fusion protein and assayed its phosphorylation. This construct includes not only the CTD repeats, but also an apparently unstructured (47) acidic domain (38) directly preceding the CTD repeats. The longer construct was used to assure that the GST module did not interfere with any possible interactions. In addition to purification of the GST-



**FIG. 1. The core subunits of RNA pol II are not required for Mediator-enhanced phosphorylation of the CTD.** Kinase assays were performed with purified holo-TFIIF and either purified core RNA Pol II or GST-CTD for 4, 8, and 16 min in the presence or absence of purified Mediator. Reaction products were analyzed by 10% SDS-PAGE, and an autoradiogram was taken from the dried gel using a PhosphorImager.

CTD fusion protein on glutathione-Sepharose, we also included a final purification on phenyl-Sepharose that cleanly removed shorter proteolytic fragments from the full-length fusion protein. Purified holo-TFIIF, with or without Mediator, was incubated in the reaction buffer, and the phosphorylation of the substrate was begun by adding RNA pol II or GST-CTD. Reactions were allowed to proceed for 4, 8, and 16 min to assure that no component became limiting and to detect any possible differences in the kinetics of CTD phosphorylation in the presence and absence of Mediator. At all three time points Mediator substantially increased the ability of TFIIF to phosphorylate the CTD whether it was attached to the core of RNA pol II or not (Fig. 1). In both cases there was an enhancement of ~20-fold in kinase activity. On the other hand, TFIIF phosphorylation of a synthetic peptide made up of just three heptapeptide CTD repeats was not enhanced by Mediator (data not shown). In these reactions the amount of substrate was normalized by the amount of phosphorylation in the absence of Mediator. Approximately 5-fold less RNA pol II than GST-CTD was required for equal phosphorylation by TFIIF in the absence of Mediator. This finding suggests that the polymerase is a slightly better substrate for the TFIIF kinase than the CTD alone. The magnitude of Mediator enhancement of TFIIF kinase activity, however, was not markedly changed by small increases in the amount of RNA pol II substrate.

**TFIIFK Contains the Molecular Information Required for Enhancement of CTD Kinase Activity by Mediator**—To determine if the TFIIFK sub-module of holo-TFIIF retained all of the components required for Mediator-enhanced Kin28 kinase activity, we isolated TFIIFK utilizing a modified version of the TAP purification described by Kornberg and colleagues (3). To assure that there were no sub-stoichiometric contaminating amounts of holo-TFIIF, we applied the TAP-purified TFIIFK to a Mono Q column. TFIIFK elutes from Mono Q at a much lower salt concentration (260 mM potassium acetate) than holo-TFIIF (750 mM potassium acetate) (48). A silver stain gel shows the final homogeneous TFIIFK (Fig. 2A). A Western blot (Fig. 2B) shows the absence (to within the level of detectability of the Western blot) of the Tfb1 subunit in the TFIIFK preparation as compared with the holo-TFIIF preparation used in Fig. 1. The Western blot was also used to normalize the amount of the kinase subunit (Kin28) when comparing holo-TFIIF and TFIIFK for Mediator-enhanced kinase assays. Using the GST-CTD as a substrate we compared the kinase activity of holo-TFIIF and TFIIFK in the presence or absence of Mediator. Mediator increased the kinase activity of TFIIFK with a similar fold enhancement to that of an equimolar amount of holo-TFIIF (Fig. 3). This finding indicates that the core subunits of TFIIF are unnecessary for Mediator enhancement of Kin28 kinase activity. Holo-TFIIF appears, in this assay, to have a slightly higher specific activity than TFIIFK. It is unclear, however, whether this is an intrinsic property of the complex or

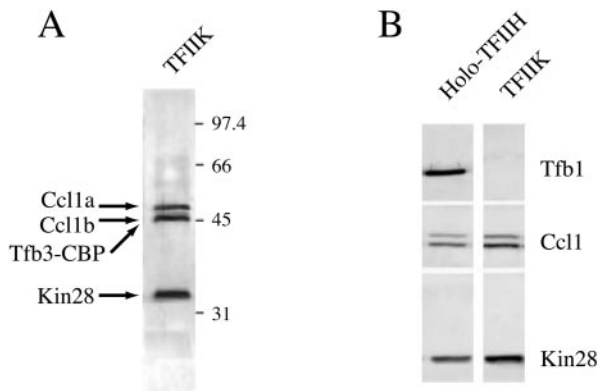


FIG. 2. Purified TFIIF is free of core TFIIF subunits. A silver-stained 10% SDS-PAGE analysis of purified TFIIF (A) and a Western blot analysis of purified holo-TFIIF and TFIIF (B) using antibodies against core TFIIF ( $\alpha$ -Tfb1p) and TFIIF ( $\alpha$ -Ccl1 and  $\alpha$ -Kin28) show the absence of core TFIIF subunits from the TFIIF preparation.

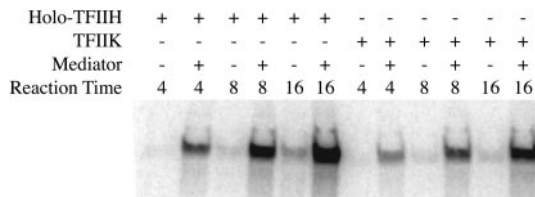


FIG. 3. Mediator enhancement of TFIIF CTD kinase activity is identical to the enhancement of holo-TFIIF CTD kinase activity. Kinase assays were performed with either purified holo-TFIIF or purified TFIIF and purified GST-CTD for 4, 8, and 16 min in the presence or absence of purified Mediator. Reaction products were analyzed by 10% SDS-PAGE and an autoradiogram was taken from the dried gel using a PhosphorImager.

simply a function of the different purification protocols used to isolate them.

**Mediator Exhibits Specificity for Enhancement CTD Phosphorylation by TFIIF**—In addition to the TFIIF kinase Kin28, there are many other kinases that can phosphorylate the CTD. Among the additional kinases in yeast that can phosphorylate the CTD *in vitro* are CTK1, Bur1, and Srb10/11 (5). CTK1 and Bur1 appear to be involved in phosphorylation of the CTD during elongation and promote elongation (49) and polyadenylation (50), respectively. Neither CTK1 nor Bur1 is part of a pre-initiation complex assembled in nuclear extracts (6). Casein kinase 1 will also phosphorylate the CTD *in vitro* with sufficient specificity for Ser-5 to recruit the capping enzyme (51). Kin28 appears to be the primary CTD kinase at initiation of transcription (9, 10). To investigate whether Mediator contributed to this effect by specifically stimulating TFIIF kinase activity, we tested both purified casein kinase 1 and rSrb10/11 for Mediator-enhanced CTD kinase activity. The amounts of the two kinases used were normalized to give an equal amount of CTD phosphorylation as TFIIF in the absence of Mediator. In both cases this amount was approximately stoichiometric to the amount of TFIIF used. Neither rSrb10/11 (Fig. 4A) nor casein kinase 1 (Fig. 4B) showed enhancement of CTD kinase activity upon addition of Mediator. The lack of stimulation suggests that Mediator's role in enhancement of Kin28 kinase activity most likely involves specific interactions with TFIIF rather than presenting the substrate in a favorable orientation for phosphorylation.

**Assembly into the Mediator Complex Is Not a Prerequisite for TFIIF/TFIIF Phosphorylation of Med4**—When performing the Mediator-enhanced kinase assays we observed a band of ~38 kDa that was phosphorylated when Mediator and TFIIF/TFIIF were present, regardless of the presence or absence of

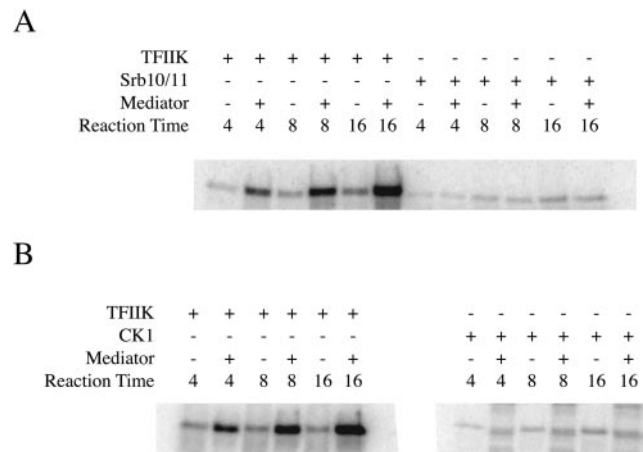
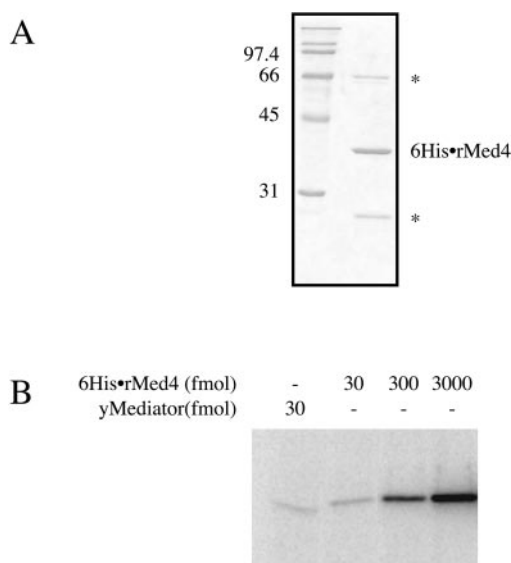


FIG. 4. Mediator does not enhance the CTD kinase activity of Srb10/11 or casein kinase 1. A, kinase assays were performed with either TFIIF or rSrb10/11 and GST-CTD for 4, 8, and 16 min in the presence or absence of Mediator. The amount of rSrb10/11 used was adjusted to produce comparable phosphorylation of the CTD in the absence of Mediator. B, kinase assays were performed with either TFIIF or casein kinase 1 and GST-CTD for 4, 8, and 16 min in the presence or absence of Mediator. The amount of casein kinase 1 used was adjusted to produce comparable phosphorylation of the CTD in the absence of Mediator. Reaction products for the above experiments were analyzed by 10% SDS-PAGE, and an autoradiogram was taken from the dried gel using a PhosphorImager.

the CTD substrate (Fig. 5B). Neither rSrb10/11 nor casein kinase 1 phosphorylated a protein corresponding to this molecular weight (data not shown). The above data indicated that TFIIF is directly phosphorylating a subunit of Mediator. The molecular weight of this band and an earlier report (39) that Med4 was a phosphoprotein led us to suspect that Med4 was the subunit being phosphorylated. A report by Hahn and colleagues, while this work was in progress, supported this hypothesis by demonstrating that Kin28 could phosphorylate Med4 in a purified pre-initiation complex (6). This same report demonstrated that Kin28 also phosphorylated the Rgr1 subunit of Mediator. Interestingly we have not observed TFIIF/TFIIF phosphorylation of a band of the molecular weight of Rgr1 in our system composed only of purified TFIIF and Mediator (data not shown), suggesting perhaps that an alternative Mediator conformation (45) or additional factor may be required for Rgr1 phosphorylation by Kin28. To determine whether TFIIF could phosphorylate Med4 only in the context of Mediator, or whether the other members of the complex were dispensable for phosphorylation of Med4 by TFIIF, we expressed and purified recombinant yeast Med4 protein from *Escherichia coli*. By placing a 6-histidine tag on the N terminus of the complete Med4 open reading frame (His<sub>6</sub>rMed4) we were able to express and isolate milligram quantities of soluble Med4 using a one-step purification on nickel-agarose (Fig. 5A). Med4, when isolated from yeast, is present in a 1:1 molar ratio with the rest of the Mediator complex (22). Using an equimolar amount of His<sub>6</sub>rMed4 and Mediator as substrates, we examined TFIIF phosphorylation of Med4. Regardless of whether it is present as a subunit in Mediator or not, TFIIF phosphorylates Med4 to an equal degree (Fig. 5B). Adding increasing amounts of the His<sub>6</sub>rMed4 in the kinase reactions shows that TFIIF is not limiting in these reactions.

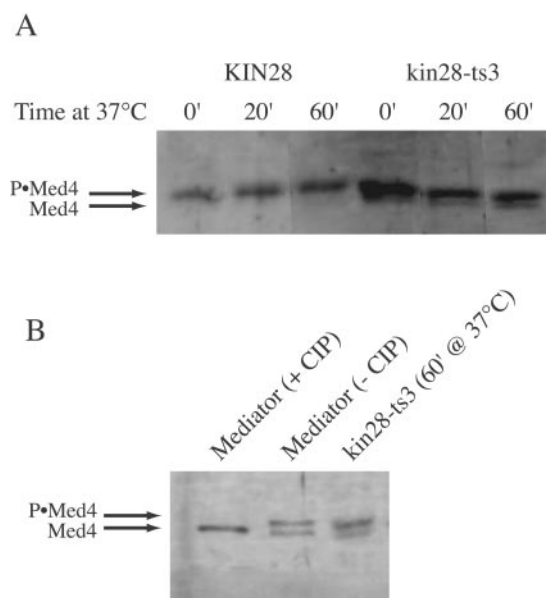
**Kin28 Phosphorylates Med4 *In Vivo***—Even though a recent study demonstrated that Kin28 can phosphorylate Med4 in purified pre-initiation complexes, it was still unclear whether Kin28 phosphorylates Med4 *in vivo*. Med4 has been shown to exist mostly in a phosphopeptide form when Mediator is isolated under conditions that prevent dephosphorylation (39).



**FIG. 5. TFIIF phosphorylates Med4 in the presence or absence of the other subunits of Mediator.** *A*, His<sub>6</sub>-rMed4 was expressed in *E. coli*, purified on nickel-agarose, and analyzed by 10% SDS-PAGE and staining with Coomassie Blue. Bands marked with an asterisk are contaminants. *B*, kinase assays were performed with holo-TFIIF and Mediator or varying amounts of purified His<sub>6</sub>-rMed4. Reactions were allowed to proceed for 16 min, the products were analyzed by 10% SDS-PAGE, and an autoradiogram was taken from the dried gel using a PhosphorImager.

This same work also established that the two bands observed in  $\alpha$ -Med4 Western blots represented the phosphorylated (low mobility) and non-phosphorylated (high mobility) forms of the protein. To investigate Kin28 phosphorylation of Med4 *in vivo*, we adapted the methodology used to monitor CTD phosphorylation by Kin28 *in vivo* (8). Using the mobility shift of Med4 (39) as a diagnostic, we monitored the phosphorylation of Med4 in wild-type cells and in mutant *kin28-ts3* cells grown at 24 °C or shifted to the non-permissive temperature (37 °C) for up to 60 min. At the point of the temperature shift we observe that virtually all of Med4 is present in the lower mobility phosphoprotein form in both strains (Fig. 6A). Upon shifting to the non-permissive temperature, the higher mobility form of the protein begins to accumulate in the mutant and its amount increases with time (Fig. 6A). In contrast, the wild-type cells maintain a constant level of the Med4 phosphoprotein and the higher mobility form cannot be detected. We conclude that Kin28 is, at least in part, responsible for maintaining Med4 phosphorylation *in vivo*. It is unclear whether the remaining phospho-Med4 in the *kin28-ts3* cells after an hour at the non-permissive temperature reflects slow dephosphorylation and/or turnover of Med4, or whether there are additional kinases contributing to its phosphorylation. It was observed earlier that purified Mediator often contains two bands recognized by  $\alpha$ -Med4 antibodies, corresponding to the phosphorylated and the non-phosphorylated forms of the protein. To confirm the assignment of the two forms of the protein detected in extracts (Fig. 6A), we compared samples of purified Mediator and purified Mediator treated with phosphatase side by side with a mutant extract sample. The data indicate that the low mobility form of Med4 and the high mobility form, which results from phosphatase treatment, correspond to the two forms of the protein identified in extracts (Fig. 6B).

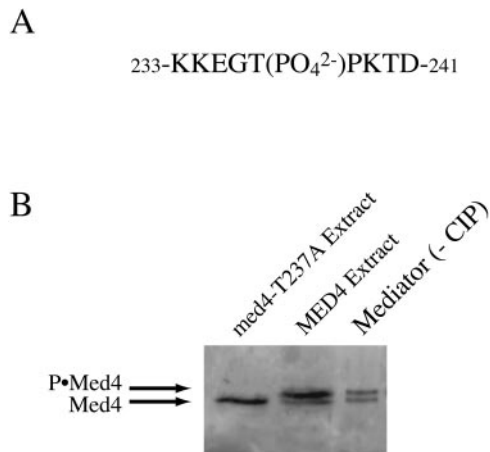
**Kin28 Phosphorylates Med4 on Thr-237**—To identify the TFIIF phosphorylation site(s) on Med4 we performed a preparative scale phosphorylation of His<sub>6</sub>-rMed4 to use for phosphopeptide mapping. To prepare the phospho-Med4, we used the purified components and the conditions (leaving out the



**FIG. 6. Phosphorylation of Med4 decreases *in vivo* upon inactivation of Kin28.** *A*, cells were grown 0, 20, or 60 min at 37 °C, and crude extracts were prepared from strains KIN28 and *kin28-ts3*. Similar amounts of total protein were electrophoresed on a 8% SDS-PAGE gel and the phosphorylation state of Med4 analyzed by Western blotting, utilizing a polyclonal antibody against Med4. The relative amount of phosphorylated and non-phosphorylated Med4 was monitored by following the ratio of the two bands representing these forms. Comparison of silver stain analysis and Western blot analysis of pure Mediator indicates that the  $\alpha$ -Med4 antibody recognizes the unphosphorylated and phosphorylated forms of the protein equally well (data not shown). *B*, to confirm the assignment of the slower migrating band (*upper*) to the phosphorylated form of Med4 (*P-Med4*) and the faster migrating band (*lower*) to the unphosphorylated form of Med4 (*Med4*) we incubated purified Mediator for 1 h at 37 °C in the presence or absence of calf intestine phosphatase (*CIP*). These two samples and the *kin28-ts3* extract sample (after 60 min at the non-permissive temperature) were analyzed by 8% SDS-PAGE and Western blotting with  $\alpha$ -Med4 serum.

<sup>32</sup>P-labeled ATP) described in Fig. 5B for the 3000-fmol reaction and increased the reaction time to 10 h to ensure as complete phosphorylation as possible. An analytical scale reaction (using <sup>32</sup>P-labeled ATP) run in parallel estimated that ~75% of the substrate was phosphorylated (assuming phosphorylation at a single site). The bands corresponding to phosphorylated and non-phosphorylated rMed4 were excised from the gel, digested with trypsin, and analyzed by MALDI-TOF mass spectrometry. Peptide patterns served to confirm identity of the proteins and were then compared for differences. Two *m/z* peaks, at 1793.79 and 1921.90 atomic mass units, were observed in the spectra of “rMed4-PO<sub>4</sub>” that were absent from the “rMed4.” The *m/z* values mapped to predicted, largely overlapping, monophosphorylated fragments of the Med4 sequence (EGTPKTDTSFIFDGTAK and KEGTPKTDTSFIFDGTAK) with mass discrepancies of less than 20 ppm. Next, the same two peptides were selectively retrieved by immobilized gallium(III) affinity chromatography (44) and reanalyzed by MALDI-TOF/TOF MS/MS sequencing. The presence of several unique fragment ions confirmed the peptide identities and allowed us to map the single phosphate group to Thr-237 (Fig. 7A).

**Phosphorylation of Med4 on Thr-237 Is Not Required for Its Essential Function *in Vivo***—The *S. cerevisiae* MED4 gene is essential for viability (22). To determine whether phosphorylation on Thr-237 was required for its essential function we mutated Thr-237 to alanine (T237A). Alignment of Med4 orthologues from diverse eukaryotic species (34) showed that Thr-237 falls in a largely non-conserved region from residue 210 to the C terminus at residue 284. To ascertain if the C



**FIG. 7. *med4-T237A* is not phosphorylated *in vivo*.** *A*, amino acid sequence of *S. cerevisiae* Med4 surrounding the phosphorylation site on Thr-237. *B*, cells were grown at 30 °C, and crude extracts were prepared from strains *MED4* and *med4-T237A*. The samples were analyzed by 8% SDS-PAGE and Western blotting with  $\alpha$ -Med4 serum. To confirm the assignment of the slower migrating band (*upper*) to the phosphorylated form of Med4 (*P-Med4*) and the faster migrating band (*lower*) to the unphosphorylated form of Med4 (*Med4*) we also loaded a lane of purified Mediator that contained both forms (see Fig. 6*B*).

terminus of Med4, regardless of its phosphorylation state, was required for the essential function of Med4 we also mutated Thr-237 to a stop codon. In haploid strains in which the only source of Med4 was supplied by the mutant gene, both the T237A and Thr-237 to stop codon mutant strains were identical to wild-type for growth on rich media at 30 °C (data not shown). To determine if Thr-237 was the site of Med4 phosphorylation *in vivo*, we grew the T237A strain and a wild-type control strain, and prepared extracts to examine Med4 phosphorylation state by SDS-PAGE. Fig. 7*B* shows that the Med4 T237A mutant protein migrates solely as the non-phosphorylated form, whereas the wild-type Med4 control migrates almost entirely as the low mobility phosphorylated form.

#### DISCUSSION

New biochemical and structural studies are leading to an increased understanding of the assembly of transcription initiation complexes. It is becoming readily apparent that the interactions between these large protein complexes most likely involve multiple contacts among many subunits over large surface areas, combined with substantial conformational changes. From structure alone, it may be difficult to identify the critical contacts that enable these complexes to function together. The kinase assays in this report help identify the most critical functional interactions between Mediator, RNA pol II, and TFIIF. The finding that the residues of TFIIF and the CTD alone are sufficient for Mediator to enhance the Kin28 kinase activity substantially reduces the critical target areas for understanding Mediator function. The finding that Mediator enhances the phosphorylation of a full-length CTD, but not a synthetic CTD peptide of three repeats, implies that there is a critical number of repeats necessary for functional interactions between TFIIF, the CTD, and Mediator. The specificity of the enhancement for Kin28-containing complexes also suggests a finely tuned mechanism to assure that a Kin28-containing complex phosphorylates the CTD predominantly at transcription initiation.

The demonstration that Kin28 phosphorylates Med4, both in and out of the context of the 20-subunit Mediator complex, most likely places Med4 as a largely surface exposed subunit of the complex. Med4 may also be an important contact point between Mediator and TFIIF, and studies are underway to

investigate physical interactions between these proteins. In addition, the identification of the phosphorylated residue in Med4 helps to further define the recognition motif used by Kin28. Earlier work had shown that, within a single CTD heptapeptide repeat, a proline at the *n*-2 position is critical for efficient TFIIF phosphorylation of the substrate (52). Surprisingly, aligning the Ser-5 of the CTD and Thr-237 of Med4 shows no other identical or similar residues, except the proline in the *n*+1 position, immediately adjacent to the phosphorylated residue (Fig. 7*A*). The finding that the Med4 phosphorylation site *in vivo* corresponds to the site phosphorylated by TFIIF *in vitro* further supports the conclusion that Kin28 is the Med4 kinase *in vivo*. Immunoprecipitations using  $\alpha$ -lexA antibody in extracts from lexA-Med1 or lexA-Srb7-tagged strains show that the phosphorylated form of Med4 selectively co-precipitates with the tagged Mediator and led to the suggestion that Med4 phosphorylation may affect Mediator assembly (39). Although we can not rule out this possibility, two of our results suggest that Med4 phosphorylation does not have a strong effect on assembly. First, because a functional Mediator complex is essential for the transcription of virtually all genes in yeast, the finding that a T237A Med4 mutant is viable and healthy suggests that Mediator assembly is not substantially hindered when Med4 cannot be phosphorylated. Second, the assignment of Kin28 as the Med4 kinase suggests that Med4 is phosphorylated at a post-assembly step. This is based on the assumption that Mediator and TFIIF primarily interact as part of a pre-initiation or re-initiation complex, where Mediator is almost certainly already assembled into a complex. An alternative explanation for the selective co-immunoprecipitation of the phosphorylated Med4 could be that, upon arrival at an initiation complex, the phosphorylation of Med4 is accompanied by a change in Mediator conformation that places the LexA epitope in a more accessible position.

It is interesting that Kin28 is specific for phosphorylation of Med4 and that Mediator, in turn, specifically enhances the CTD kinase activity of Kin28. Additional studies will be required to determine if these events are coordinated.

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