

A kinase subunit of the human mediator complex, CDK8, positively regulates transcriptional activation

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The human thyroid hormone receptor-associated proteins (TRAP)/Mediator and related complexes mediate transcription through regulatory factors. To further understand the structural and functional diversity of these complexes we established three HeLa cell lines each expressing one of three epitope-tagged human TRAP/Mediator subunits, MED6, MED7, and CDK8 and isolated the complexes in which these subunits were contained by affinity and HPLC-gel filtration chromatography. The largest complexes from each cell line had a molecular mass of 1.5 MDa and possessed almost identical subunit compositions; we designated these complexes TRAP/Mediator-like complex 1 (TMLC1). Two potential subcomplexes were additionally observed: a 1-MDa complex from the CDK8-cell line (TMLC2) and a 600-kDa complex from the MED6-cell line (TMLC3). All three complexes regulated transcription *in vitro*; TMLC1 and TMLC3 augmented transcriptional activation, whereas TMLC2 repressed it. TMLC1 and TMLC2 phosphorylated RNA polymerase II (Pol II), but TMLC3 did not. Furthermore, TMLC1 predominantly interacted with the general transcription factors TFIIE, TFIIIF, and TFIIH, which function during transcription initiation and the transition to elongation. In a final experiment, knockdown of CDK8 using RNA interference prevented transcriptional activation by Gal4-VP16 in a luciferase-assay. This, together with the effect of TMLC1 on transcription *in vitro*, suggests that CDK8 play positive roles in transcriptional activation.

Introduction

In eukaryotes, RNA polymerase II (Pol II) alone cannot complete mRNA synthesis. Five general transcription factors (TFIIB, TFIID, TFIIE, TFIIIF, and TFIIH) must form a preinitiation complex on the core promoter to initiate accurate transcription and to transition to the stable elongation phase (reviewed in Orphanides *et al.* 1996; Roeder 1998). Various transcriptional activators, cofactors, and mediators, as well as histone modifying enzymes and chromatin remodeling factors, are known to assist in these steps of transcription (reviewed in

Roeder 1998). One of these, the Mediator complex, was first discovered using generics in the budding yeast *Saccharomyces cerevisiae* because its gene products suppress the defects caused by deletion of the C-terminal domain (CTD) heptapeptide repeats of the largest subunit of RNA Pol II, suppressor of RNA polymerase B (SRB) (Nonet & Young 1989). Shortly thereafter, the Mediator complex was discovered to relieve squelching (activator-induced inhibition of transcription) during *in vitro* transcription using crude nuclear extracts (Kelleher *et al.* 1990). The yeast Mediator complex was first purified by Kornberg and colleagues (Kim *et al.* 1994) and has turned out to consist of more than 20 subunits containing nine Med proteins (Med1–Med4, Med6–Med8, Med10, and Med11) and five other subunits (Gal11, Rgr1, Nut1, Sin4, and Rox3) in addition to a set of SRB proteins (Srb2 and Srb4–Srb11) (reviewed in Malik & Roeder 2005). Recently, a novel unified nomenclature

Communicated by: Hiroshi Handa

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DOI: 10.1111/j.1365-2443.2007.01036.x

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for all Mediator subunits was proposed that includes 34 MED proteins (MED1–MED31, MED1L, MED12L and MED13L) as well as CDK8 and Cyclin C (Bourbon *et al.* 2004). Extensive research has revealed that the Mediator complex can function positively and/or negatively in most Pol II transcription (reviewed in Malik & Roeder 2005). In addition to regulating transcriptional activation, this complex affects both basal transcription and phosphorylation of the CTD heptapeptide repeat sequence (YSPTSPS) of the largest subunit of Pol II.

Mediator orthologs are conserved among metazoans. The human Mediator complexes, thyroid hormone receptor-associated proteins (TRAP)/SRB-MED-containing cofactor (SMCC) (Ito *et al.* 1999), vitamin D receptor-interacting proteins (DRIP) (Rachez *et al.* 1999), activator-recruited cofactor (ARC) (Näär *et al.* 1999), cofactor required for *Sp1* activation (CRSP) (Ryu *et al.* 1999), negative regulator of activated transcription (NAT) (Sun *et al.* 1998), positive cofactor 2 (PC2) (Malik *et al.* 2000), and the mouse Mediator complex (Jiang *et al.* 1998), were purified using various methods and turned out to possess essentially similar subunit compositions (Bourbon *et al.* 2004). These complexes fall into two broad classes based mainly on whether or not they contain the MED12–MED13–CDK8–Cyclin C module (reviewed in Malik & Roeder 2005). Biochemical studies indicated further that some complexes (TRAP, DRIP, PC2 and CRSP) positively regulate transcription (Rachez *et al.* 1999; Ryu *et al.* 1999; Malik *et al.* 2000), whereas others (NAT and ARC-L) negatively regulate it (Sun *et al.* 1998; Taatjes *et al.* 2002); SMCC was reported to have both positive and negative effects (Gu *et al.* 1999). Structural studies have demonstrated that there are several structural and conformational subtypes, and the largest Mediator complex in *S. cerevisiae* and in humans, consists of three modules: head, middle, and tail (Taatjes *et al.* 2002). Although this module composition is well-conserved from yeast to human (Malik & Roeder 2005), the Mediator undergoes conformational changes upon binding to different activators and Pol II (Näär *et al.* 2002; Taatjes *et al.* 2002), perhaps to respond to varying environmental situations.

In higher eukaryotes, there are three Cdk/cyclin-containing transcription factors: the general transcription factor TFIID (containing CDK7/Cyclin H), the TRAP/Mediator complex (containing CDK8/Cyclin C), and the transcription elongation factor P-TEFb (containing CDK9/Cyclin T1), all of which are known to phosphorylate the CTD of the largest subunit of Pol II, thereby regulating Pol II function at important stages in transcription as well as at the later steps of gene expression (reviewed in Orphanides & Reinberg 2002). Although

the Mediator complex is known to function at transcription initiation and possibly at the transition to elongation by phosphorylating the CTD of Pol II at the same step in transcription as does TFIID, because of its compositional complexity it remains the least characterized of these three transcription factors.

In this paper, we established three HeLa cell lines, each expressing an N-terminal HA/FLAG-tagged (HF:) human Mediator subunit, hMED6, hMED7, or hCDK8 (the human homolog of SRB10). We examined complexes derived from these cell lines using biochemical approaches to assess if any related novel and/or uncharacterized Mediator subcomplexes that share some Mediator subunits additionally exist in the cell as well as to further elucidate the functional mechanisms of the previously identified 1.5-MDa human complex and CDK8. We especially focused on CDK8, which regulates transcription both positively and negatively in *S. cerevisiae* (Holstege *et al.* 1998; Liu *et al.* 2004). In humans, on the other hand, only a negative role has been reported for hCDK8 in the Mediator complex NAT, which represses transcription *in vitro* by phosphorylation of Cyclin C (Akoulitchev *et al.* 2000). Here, we observed that HF: hCDK8 was present in three complexes with molecular weights of 1.5 MDa, 1 MDa, and 600 kDa that had separate functional activities. To elucidate the mechanisms of these functions, we examined the effects of three Mediator subcomplexes on transcription, CTD phosphorylation of Pol II, and their physical interactions with glutathione S-transferase (GST)-tagged components of the general transcription machinery. In a final study, we examined the effect of knocking down hCDK8 expression with RNA interference on transcriptional activation by Gal4-VP16.

Results

Purification of epitope-tagged human TRAP/Mediator complexes from three distinct HeLa cell lines

Biochemical studies have classified human SRB/MED-type Mediator complexes with molecular masses of 1–1.5 MDa into two types: one that augments activator-dependent transcription and one that counteracts it (reviewed in Malik & Roeder 2005). As a first step to further characterize the human Mediator complexes, we established three distinct HeLa cell lines constitutively expressing HF: hMED6, hMED7, or hCDK8 (Fig. 1). We chose these three TRAP/Mediator subunits because (i) hMED6 and hMED7 were reported as core subunits in all the reported SRB/MED-type Mediator complexes,

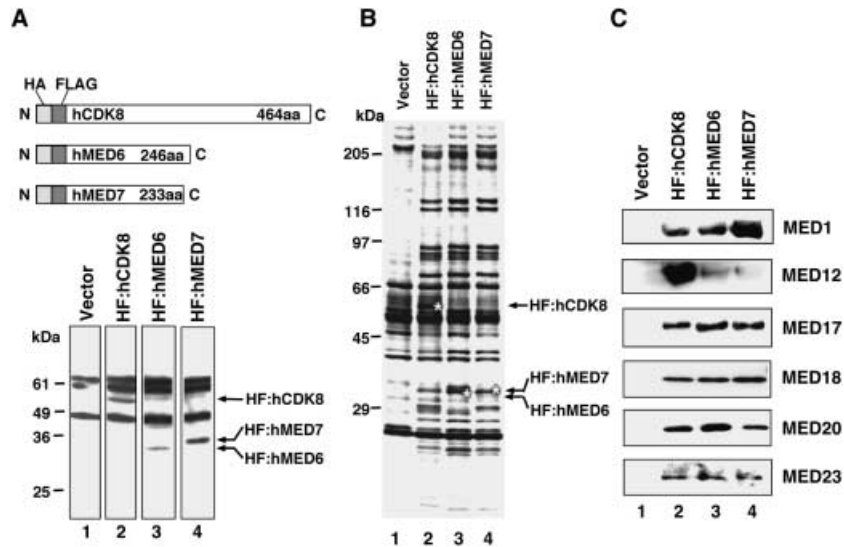


Figure 1 HA/FLAG-tagged human Mediator subunit-expressing cell lines. (A) Three established HeLa cell lines. Top: the HA/FLAG-tagged (HF:) Mediator subunits (hCDK8, hMED6 and hMED7) were subcloned into the pIRESneo2 vector, and the constructed plasmids were transfected into HeLa cells. The pIRESneo2 vector was also transfected as a mock control. Bottom: detection of expressed HF:Mediator subunits. Established cell lysates (50 μ g) were subjected to SDS-PAGE and tagged subunits were detected by Western blotting using mouse anti-HA monoclonal antibody (12CA5). Arrows indicate the positions of each HF:subunit. The sizes of the molecular weight markers are indicated in kDa on the left. (B) SDS-PAGE of anti-FLAG agarose-purified Mediator fractions. Each cell lysate (2 mg) was loaded onto an anti-FLAG (M2) agarose column and eluted with FLAG peptide. Eluates (5 μ g) were subjected to SDS-PAGE and the gel was stained with silver. Arrows and asterisks indicate the positions of each HF:subunit. The sizes of the molecular weight markers are indicated in kDa on the left. (C) Western blot analysis of eluates. Eluates (5 μ g) were subjected to SDS-PAGE and the Mediator subunits were detected using rabbit polyclonal antisera against those subunits.

(ii) genetic and biochemical studies of the *S. cerevisiae* Mediator complex indicate that hMED6 and hMED7 might be located in the head and middle modules, respectively, and (iii) hCDK8 is a kinase subunit that can regulate transcription by phosphorylating the CTD of the largest subunit of Pol II and might also be located in the middle module, but may be a conditional component of the Mediators (Malik & Roeder 2005). hCDK8 has also been reported to negatively regulate transcription in the Mediator complex (Sun *et al.* 1998; Taatjes *et al.* 2002). Each HF: subunit was expressed constitutively in the established cell lines, as identified by Western blotting with an anti-HA antibody (12CA5; Fig. 1A). [Note that although its calculated molecular weight is lower (27 243 Da versus 28 423), hMED7 migrates slower than hMED6 in SDS-PAGE because its estimated pI of hMED6 is much lower than that of hMED7 (5.4 versus 8.8).]

We purified the corresponding HF: complexes on an anti-FLAG M2 monoclonal antibody-agarose column. The N-terminal FLAG-tag of hMED6, hMED7, and hCDK8 bound to the M2-agarose column, and these

subunits were efficiently eluted by the FLAG peptide in complexes with other TRAP/Mediator components (Fig. 1B,C). hMED12 (hTRAP230) was prominently observed in the TRAP/Mediator complex fraction containing tagged hCDK8. It was fainter in the complex fraction containing HF:hMED6, but was very weak in the complex fraction containing HF:hMED7 (Fig. 1C, lane 2 versus lane 3 versus lane 4). However, hMED1 (hTRAP220), hMED17 (hTRAP80), hMED20 (hTRFP), and hMED23 (hTRAP150 β) were observed almost equally in all fractions (Fig. 1C). We further fractionated the TRAP/Mediator complexes on an HPLC-TSK G4000SW gel filtration column (Tosoh) (Fig. 2). The HF:TRAP/Mediator subunits were monitored by Western blotting with an anti-HA antibody. The largest complexes, with molecular mass of about 1.5-MDa, were detected in the fractions from all three cell lines, with peaks in fractions 21 and 22 (Fig. 2A). Western blotting confirmed that these contained more than 20 polypeptide bands that included most of the previously reported SRB/MED-type Mediator subunits (Fig. 2 and data not shown). The overall band profiles in

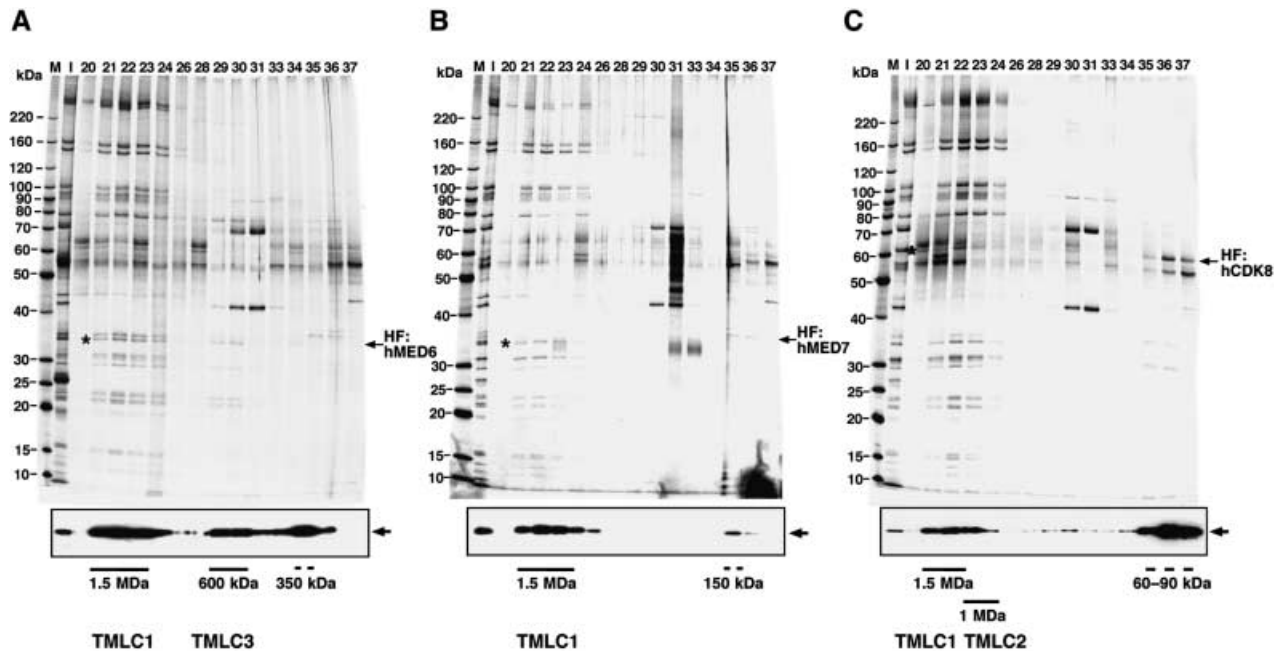


Figure 2 Gel filtration of anti-FLAG column eluates. The eluates (500 μ g) from the three distinct cell lines were subjected to the HPLC TSKgel G4000SW_{XL} gel filtration column (7.5 mm I.D. \times 30 cm, Tosoh). Upper: each fraction was loaded onto a PAG mini 4%–20% acrylamide gradient gel (Daiichi Pure Chemicals), and SDS-PAGE was carried out. The gels were stained with silver. Lower: Western blotting of fractionated HF:Mediator subunits. After SDS-PAGE, separated proteins were electro-transferred to a PVDF membrane instead of staining, and each HF:protein was detected using an anti-HA mouse monoclonal antibody (12CA5). Fractions corresponding to the TRAP/Mediator-like complexes (TMLC1–3) are indicated by straight lines with their molecular masses. (A) The fractions from HF:hMED6 cells. (B) The fractions from HF:hMED7 cells. (C) The fractions from HF:hCDK8 cells. The dotted line below indicate the fractions containing a monomer form of each tagged subunit. Asterisks in the lanes (20 or 21) and arrows on the right side in SDS-gels indicate the positions of each tagged subunit. And the tagged subunits detected by Western blotting were also indicated by arrows.

the SDS-PAGE gels were similar between the fractions of approximately 1-MDa molecular mass (fractions 23 and 24) and those of the 1.5-MDa fractions (fractions 21 and 22). However, some additional bands were seen in the 1-MDa fractions whereas others were absent. As expected, the peak fractions with low molecular masses, which contained free forms of the tagged subunits, were also detected as major components (at about 300 kDa in the hMED6-cell line, at 150 kDa in the hMED7-cell line, and at 70 kDa in the hCDK8-cell line). Most intriguingly, a novel 600-kDa peak was observed only in the column fractions derived from the hMED6-cell line (fractions 29 and 30).

Three human TRAP/Mediator-like complexes (TMLCs) regulate transcription

We next tested the effects of the human TMLCs on *in vitro* transcriptional activation. We examined the 1-MDa fractions in addition to the 1.5-MDa and 600-kDa

complex fractions (Fig. 3) because the band profiles were slightly different between the 1.5-MDa and 1-MDa complexes, as described above, and because several laboratories reported that there are transcriptionally positive and negative types of human Mediator complexes (reviewed in Malik & Roeder 2005). Although no complexes affected basal transcription in the absence of the potent transcriptional activator protein Gal4-VP16, the 1.5-MDa and 600-kDa complexes augmented Gal4-VP16-dependent transcriptional activation by about 1.4- to 2.1-fold in its presence (Fig. 3A, lanes 9, 10, 13, and 14 versus lane 2; Fig. 3B, lanes 7, 8, 15, and 16 versus lane 2). Strikingly, however, the 1-MDa complex from the hCDK8-cell line clearly reduced both basal (from the template that gives 290 nt transcripts) and Gal4-VP16-activated transcription (from the template that gives 390 nt transcripts) to an almost undetectable level in the presence of Gal4-VP16. However, the corresponding fractions from the hMED6 and hMED7-cell lines still augmented transcriptional activation, albeit to a reduced

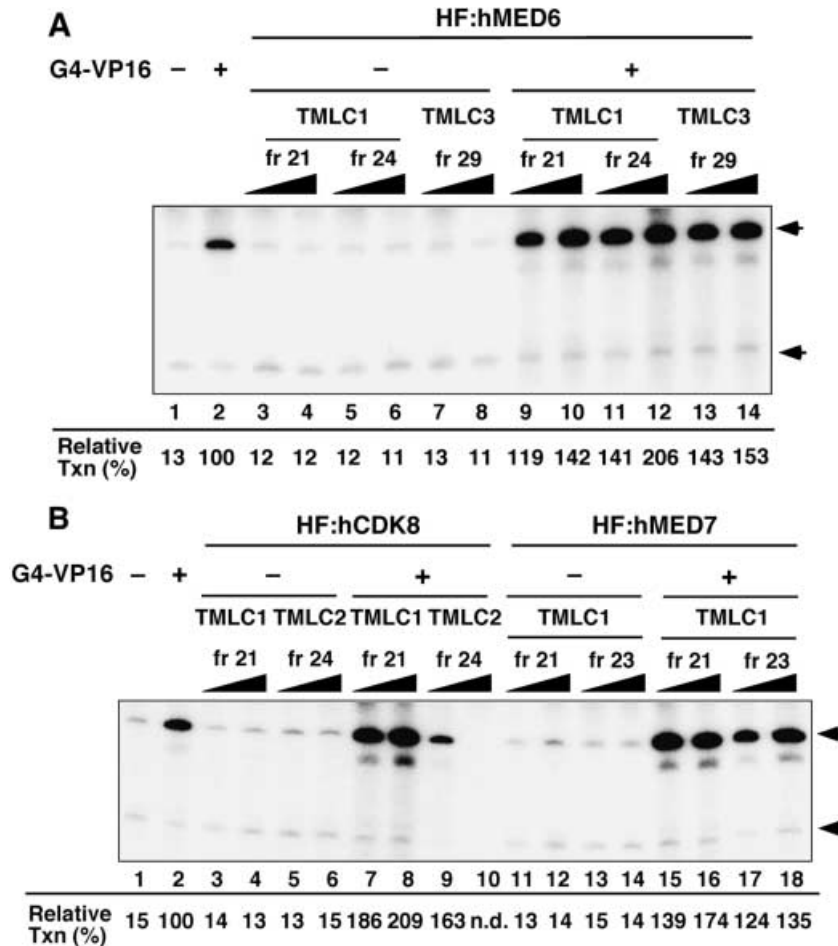


Figure 3 *In vitro* transcription activities of gel filtration column fractions. (A) HF:hMED6 fractions with or without GAL4-VP16 activation. Reaction mixtures (25 μ L) contained 100 ng of pG5HM(C₂AT), 50 ng of pML(C₂AT) Δ -53Sh, and all general transcription factors together with Pol II as described (Ohkuma *et al.* 1990), except that 20 ng of purified flag-tagged TFIID (f:TFIID) was used in place of native TFIID (Chiang *et al.* 1993). Increasing amounts of the fractions (100 and 200 ng) were used for transcription as indicated at the top of the panel. The fractions 21 and 24 contain TMLC1 and the fraction 29 contains TMLC3 as indicated. The upper arrow (390 nt) indicates transcripts of pG5HM(C₂AT) and the lower arrow (290 nt) indicates transcripts of pML(C₂AT) Δ -53Sh. Lanes 1 and 2, transcription in the absence of the Mediator fraction. Relative transcription activity was calculated by comparing transcription levels in the absence (-) and presence (+) of 30 ng of GAL4-VP16 (G4-VP16) measured by a Fuji BAS2500 Bio-Imaging analyzer. The transcription activity of lane 2 was defined as 100%. (B) HF:hCDK8 (left) and HF:hMED7 (right) fractions with or without GAL4-VP16. The fractions 21 and 24 from HF:hCDK8 cells contain TMLC1 and TMLC2, respectively. And the fractions 21 and 23 from HF:hMED7 cells contain TMLC1. Transcription was carried out as described in Fig. 3A. In lane 10, n.d. means "not detectable."

extent (about 1.5-fold) (Fig. 3B, lanes 9 and 10 versus Fig. 3A, lanes 11 and 12 and Fig. 3B, lanes 17 and 18). In contrast, this 1-MDa complex did not affect transcription when no Gal4-VP16 was added, regardless of the presence and the absence of the Gal4 binding sites (Fig. 3B, lane 1 versus lanes 5 and 6). Since we have observed three distinct functional complexes, we designated the 1.5-MDa, 1-MDa, and 600-kDa complexes TMLC 1, 2, and 3, respectively.

TMLC1 and TMLC2 phosphorylate Pol II, but TMLC3 does not

CDK8-containing TRAP/Mediator complexes phosphorylate the CTD of the largest subunit of Pol II, particularly at Ser2 and Ser5 of the heptapeptide repeat sequence (YSPTSPS) (Sun *et al.* 1998; Park *et al.* 2001). We tested whether the three complexes we isolated (TMLC1, TMLC2, and TMLC3) also had this activity

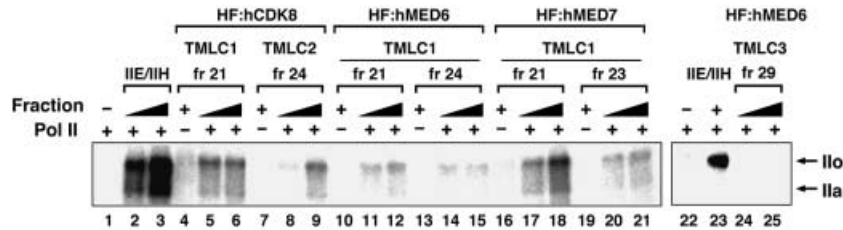


Figure 4 Effects of gel-filtration column fractions on CTD phosphorylation. Kinase assays (25 μ L) were carried out as described in the Experimental procedures. Lanes 1 and 22 contain reactions without a gel-filtration fraction. Lanes 2 and 3, 40 ng of TFIIE and 5 and 20 ng, respectively, of TFIIH were added. Lane 23, 40 ng of TFIIE and 20 ng of TFIIH were added. Lanes 4–21, 24 and 25, each fraction was added as indicated on the top. Two doses (100 and 200 ng) of each Mediator fraction were used. Phosphorylated proteins were separated in a 5.5% acrylamide-SDS gel and detected by autoradiography. Arrows indicate the positions of the phosphorylated form of the largest subunit of Pol II (Ilo) and the unphosphorylated form (Ila).

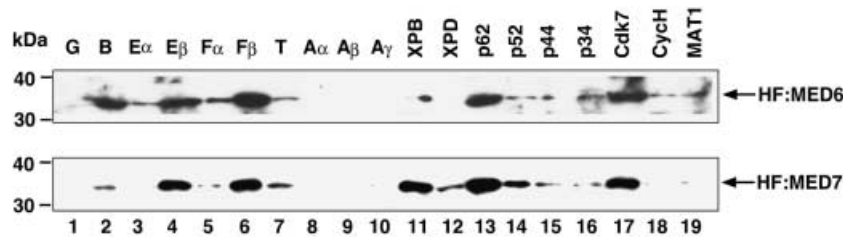


Figure 5 Assays of Mediator binding to general transcription factors. GST-pull down assay of human general transcription factor subunits with intact human TMLC1 fractions. All of the GST-tagged human general transcription factors (300 ng each) except for the TAF subunits of TFIID were studied. The assay was carried out with 400 ng of the hTMLC1 fractions. Upper panel: hTMLC1 fraction (fraction 21) from HF:hMED6 cells. Lower panel: hTMLC1 fraction (fraction 21) from HF:hMED7 cells. After SDS-PAGE in a 5.5% polyacrylamide gel and Western blotting, bound Mediator was detected with a mouse anti-HA monoclonal antibody (12CA5). Lane 1, GST alone (G); lane 2, GST-TFIIB (B); lane 3, GST-TFIIE α (E α); lane 4, GST-TFIIE β (E β); lane 5, GST-TFIIF α (F α); lane 6, GST-TFIIF β (F β); lane 7, GST-TBP (T); lane 8, GST-TFIIA α (A α); lane 9, GST-TFIIA β (A β); lane 10, GST-TFIIA γ (A γ); lane 11, GST-XPB (XPB); lane 12, GST-XPD (XPD); lane 13, GST-p62 (p62); lane 14, GST-p52 (p52); lane 15, GST-p44 (p44); lane 16, GST-p34 (p34); lane 17, GST-Cdk7 (Cdk7); lane 18, GST-CyclinH (CycH); and lane 19, GST-MAT1 (MAT1). The positions of 40- and 30-kDa molecular weight markers are indicated at left of each panel. Arrows indicate the position of HF:hMED6 and HF:hMED7.

in vitro (Fig. 4). Among the three complexes, TMLC1 showed the CTD kinase activity on the largest subunit of Pol II (Fig. 4, lanes 5, 6, 11, 12, 17, and 18). TMLC2 also showed similar kinase activity (Fig. 4, lanes 8 and 9). The 1-MDa fractions from the hMED6 and hMED7-cell lines, which would correspond to TMLC2, phosphorylated Pol II despite much more weakly (Fig. 4, lanes 14, 15, 20, and 21 compared to lanes 8 and 9). In clear contrast to TMLC1 and TMLC2, the TMLC3 fractions did not show any kinase activity (Fig. 4, lanes 24 and 25).

TMLC1 predominantly interacts with the general transcription factors essential for the transition from initiation to elongation

Although Pol II is reported to associate with the human Mediator complex (reviewed in Malik & Roeder 2005), no systematic study of the binding of the Mediator

complexes to the other components of the general transcription machinery has been carried out. We therefore performed a GST-pull down analysis of TMLC1 from the HF:hMED6 and hMED7-cell lines using GST-fused general transcription factor subunits (Fig. 5, upper and lower panels). Bound TMLC1 was detected by Western blotting. TMLC1 predominantly bound to human TFIIB, TFIIE β , TFIIF β (Rap30), and the XPB, p62 and CDK7 subunits of TFIIH (Fig. 5, lanes 2, 4, 6, 11, 13, and 17). Faint binding to TBP, and the XPD and p52 subunits of TFIIH was also observed (Fig. 5, lanes 7, 12, and 14). Previous results by Y. J. Kim and his colleagues on the binding of the *Drosophila* Mediator complex to the GST-tagged general transcription components (except for TFIIH subunits) correlate with our result except for the binding to TFIIF subunits (Park *et al.* 2001). We observed that TMLC1 bound the smaller subunit TFIIF β , but the *Drosophila* Mediator complex bound to the

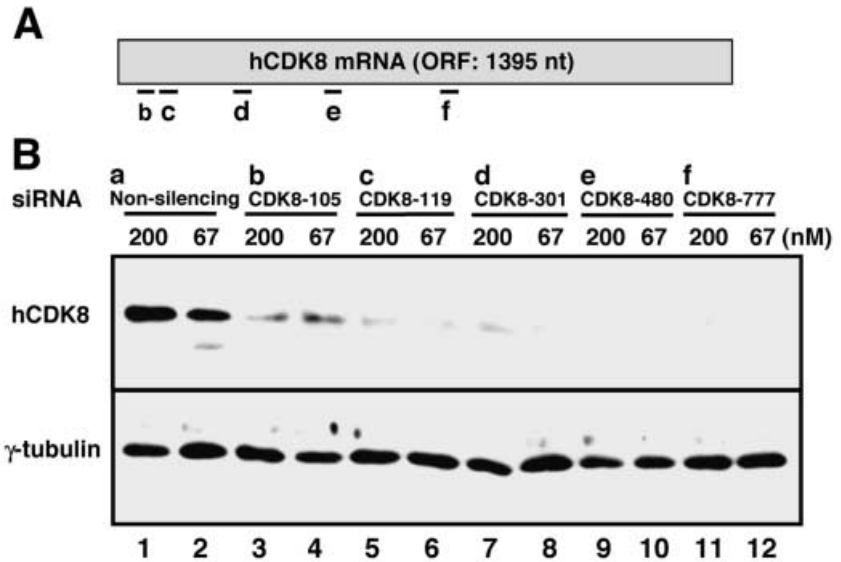


Figure 6 Effect of siRNAs on hCDK8 expression. The sequences of the five siRNAs (b–f) and one non-silencing RNA (a) are described in the Experimental procedures. (A) Location of the targets of the five siRNAs in the hCDK8 mRNA. b: CDK8-105, c: CDK8-119, d: CDK8-301, e: CDK8-480, and f: CDK8-777. (B) Knockdown of hCDK8 expression by siRNAs. Two doses (67 and 200 nM) of each RNA were used for transfection into HeLa cells. hCDK8 expression was checked by Western blotting. Expression of γ -tubulin was checked at the same time as a negative control for siRNA effects.

larger TFIIF α . Considering this discrepancy, we will compare the binding specificities of the Mediator sub-complexes (TMLC1, 2, and 3) to the components of the general transcription machinery in future experiments.

Endogenous expression of the hCDK8 protein was efficiently reduced by RNA interference

One of the Mediator subunits, CDK8, is known to be a component of the labile Mediator submodule consisting of CDK8, Cyclin C, MED12, and MED13. *S. cerevisiae* SRB10 (the yeast homolog of hCDK8) regulates transcription both positively and negatively (Holstege *et al.* 1998; Liu *et al.* 2004). In humans, however, the only functions that have been reported for hCDK8 have been negative; hCDK8 phosphorylates the Cyclin H subunit of TFIIF (Akoulitchev *et al.* 2000) and inactivates it and phosphorylates Hes-1 after recruitment by Mastermind and causes its degradation (Fryer *et al.* 2004). To further elucidate the mechanisms of hCDK8 function, we knocked it down using RNA interference. Five siRNA oligos were designed on hCDK8 mRNA (Fig. 6A and Experimental procedures), and their effects on hCDK8 expression were examined (Fig. 6B). Four of the five efficiently reduced hCDK8 expression, (Fig. 6B lanes 3 and 4 versus lanes 5–12) but CDK8-105 was less effective than the others.

Knockdown of hCDK8 counteracted transcriptional activation by Gal4-VP16

We selected two siRNAs (CDK8-119 and CDK8-480) to further elucidate the hCDK8 function. To judge the

effectiveness and specificity of each siRNA, siRNA-treated HeLa cells were co-transfected with either of two hCDK8 cDNA plasmids, one of which expresses hCDK8 that is wild type except for a point mutation in the sequence corresponding to the siRNA that makes it resistant to the siRNA-mediated degradation (CDK8^{si^r}) and the other of which expresses hCDK8 with a mutation in the kinase domain, D151A, in addition to the siRNA-resistance mutation (CDK8^{si^r} D151A). The level of transcription in Gal4-VP16-stimulated control cells (treated with non-silencing siRNA or the control vector) was defined as “1.” When the cells were treated with siRNA CDK8-119, the transcription activity was reduced to 40% of the control and siRNA CDK8-480 further reduced the transcription activity to 20% of the control (Fig. 7A). Transcription was not repressed when the mutant plasmid CDK8^{si^r} D151A was co-transfected; instead, transcription was repressed to as low as 40% of control. Treatment with siRNA CDK8-480 gave similar results, but transcription was reduced to a greater extent, to 20% of the level in the cells co-transfected with control vector. Treatment with CDK8-480 and the siRNA-resistant plasmids (CDK8^{si^r} and CDK8^{si^r} D151A) also gave results similar to those obtained with siRNA CDK8-119 and the siRNA-resistant plasmids.

Western blotting indicated that siRNA treatment efficiently reduced hCDK8 expression (Fig. 7B, CDK8, lanes 2 and 5). Co-transfected siRNA resistant hCDK8 cDNA plasmids (both wild type and mutant) efficiently expressed hCDK8 proteins (Fig. 7B, CDK8, lanes 3, 4, 6, and 7). The siRNA clearly repressed both Ser2 and Ser5 phosphorylation of the CTD of Pol II (Fig. 7B,

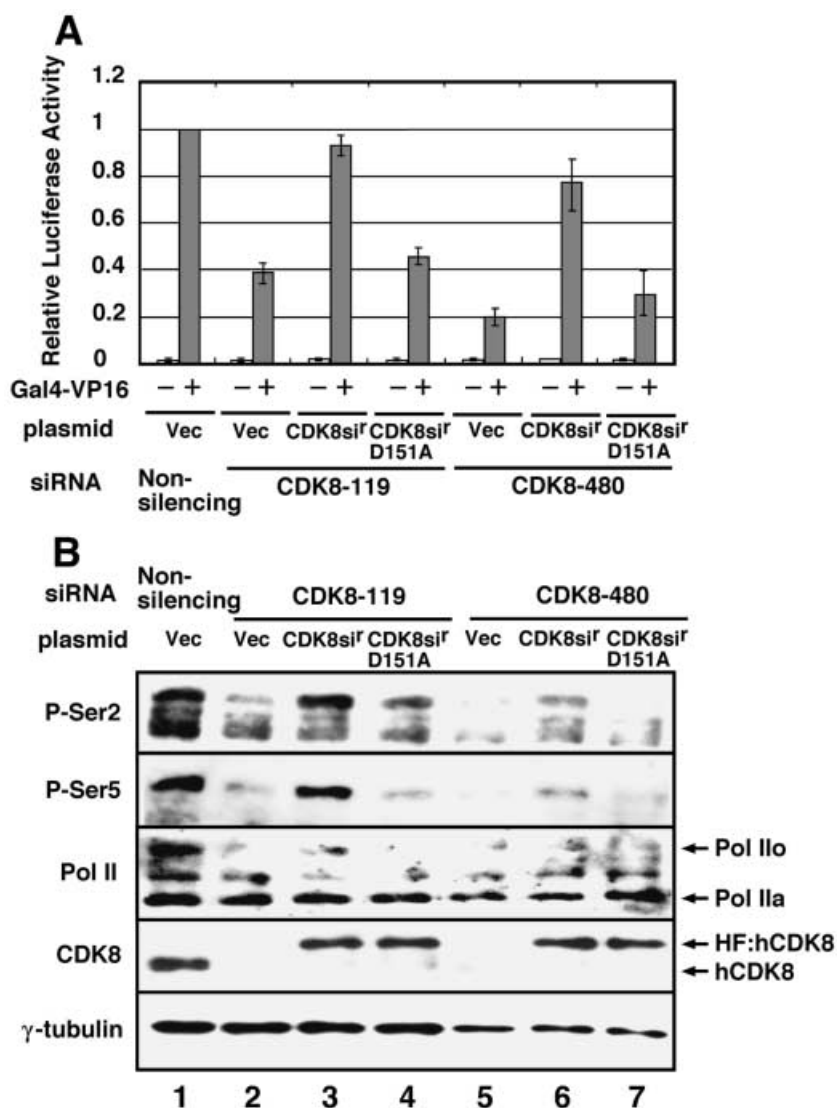


Figure 7 Knockdown of hCDK8 counteracts transcriptional activation by Gal4-VP16. (A) Luciferase assay of siRNA-treated cells. HeLa cells were treated with two specific siRNAs (CDK8-119 and CDK8-480) together with siRNA-resistant CDK8 cDNAs (CDK8si^r and CDK8si^r D151A). D151A mutation abolishes a kinase activity of hCDK8. Transcription activities were measured with luciferase assays in the presence or absence of Gal4-VP16. Transcription activity in HeLa cells treated with the non-silencing control RNA in the presence of Gal4-VP16 was defined as "1." Error bars denote the standard deviations of repeated experiments. (B) Levels of hCDK8 protein expression and CTD phosphorylation of Pol II. Protein amounts were examined by Western blotting. The antibodies used were as described in the Experimental procedures. Arrows on the right side indicate phosphorylated form (Pol Ilo) and unphosphorylated form (Pol Ila) of the largest subunit of Pol II, HF-tagged hCDK8 (HF:hCDK8), and non-tagged hCDK8 (hCDK8).

Ser2 and Ser5, lanes 2 and 5 versus lane 1). Repressed phosphorylation at either site was resumed almost completely by the CDK8si^r plasmid (Fig. 7B, Ser2 and Ser5, lanes 3 and 6) but not by the CDK8si^r D151A plasmid (Fig. 7B, Ser2 and Ser5, lanes 4 and 7). Consistent with the effects of the siRNAs on transcription, CDK8-119 repressed CTD phosphorylation more weakly than CDK8-480 (Fig. 7B, Ser2 and Ser5, lanes 2–4 versus lanes 5–7). Contrary to the previously reported conclusions, our findings indicate that Cdk8 is necessary for Gal4-VP16-dependent transcriptional activation.

Discussion

In this study, we identified and characterized three distinct TMLC1–3 using HeLa cell lines expressing epitope-

tagged human Mediator subunits (hMED6, hMED7, and hCDK8). All three complexes shared a subset of Mediator-specific subunits, yet the complexes functioned differently in transcription: TMLC1 augmented both transcriptional activation and CTD phosphorylation of Pol II, TMLC2 counteracted transcriptional activation but augmented CTD phosphorylation, and TMLC3 augmented transcription but failed to stimulate phosphorylation. A GST-pull down assay to evaluate the interaction with general transcription factors revealed that TMLC1 predominantly bound to TFIIB, TFIIE β , TFIIF β , and the XPB, p62, and Cdk7 subunits of TFIIH. Our binding profiles strengthen the previous interpretations that Mediator function in transcription initiation and the transition from initiation to elongation. We are convinced that our approach makes it possible to better

define the functions of the human Mediator complexes (Sun *et al.* 1998; Näär *et al.* 1999; Rachez *et al.* 1999; Ryu *et al.* 1999; Malik *et al.* 2000, 2005; Taatjes *et al.* 2002) and to further elucidate the structures and functions of these complexes.

Three human TMLCs function distinctly in transcription

Recently, extensive studies have identified several important structural and functional features of Mediator and its related complexes. First, the structures and functions of the Mediator complexes are more conserved from yeast to human than we expected (Bourbon *et al.* 2004; Sato *et al.* 2004; Malik & Roeder 2005); for example, recent progress in sequencing of the genomes of various species led to the identification of orthologs of Sin4 and Gal11 in higher eukaryotes, hMED23 (hSur2) and hMED16 (TRAP95), respectively (Boyer *et al.* 1999; Ito *et al.* 1999). Second, two closely related Mediator complexes consisting of more than 20 SRB/MED-related subunits are conserved among eukaryotes; one positively regulates and the other negatively regulates transcriptional activation (reviewed in Malik & Roeder 2005). Third, another Mediator-related complex with a molecular weight around 600 kDa was reported as Mediator core (Medc) in *S. cerevisiae* (Liu *et al.* 2001), and as Mediator complex C1 in *Drosophila* (Gu *et al.* 2002). Indeed, Kim and coworkers identified three *Drosophila* Mediator complexes altogether (Gu *et al.* 2002), but their systematic structural and functional studies of these complexes have just begun.

In this study, we identified three distinct human Mediator-like complexes, TMLC1, TMLC2, and TMLC3, after purification of the nuclear extracts of three HeLa cell lines expressing epitope-tagged hMED6, hMED7, and hCDK8. The 1.5-MDa TMLC1 complex was isolated from all three cell lines, the 1-MDa TMLC2 complex was isolated solely from the hCDK8-cell line, and the 600-kDa TMLC3 complex was isolated solely from the hMED6-cell line. Functional studies of the transcriptional activation and CTD phosphorylation of Pol II demonstrated that the three complexes were functionally distinct. Previous models suggested that Mediator complexes containing hCDK8 negatively regulate transcription (Sun *et al.* 1998; Taatjes *et al.* 2002), but TMLC1 and TMLC2, which both contain hCDK8, behave differently (Fig. 2); TMLC1 augments transcriptional activation despite being isolated from the hCDK8-cell line, whereas TMLC2 represses transcription, consistent with the previous models. We observed that the TMLC1 fractions from the hCDK8-cell line

contained higher amount of hMED12 than those from the other two cell lines (Fig. 1C, lane 2 versus lanes 3 and 4). This is likely to be the case because the TMLC1 fractions from the hCDK8 cell-line might consist of nearly 100% of the hCDK8 submodule-containing Mediator complexes whereas the TMLC1 fractions from the other cell line may mostly consist of the hCDK8 submodule-lacking Mediator complexes and less amount of the submodule-containing Mediator complexes. The important observation is that they both confer positive effects on Gal4-VP16-mediated transcriptional activation (Fig. 3A,B). Therefore, it is highly possible that as we purified it further, TMLC1 will be found to be consisting of two subtypes. The activity of human TMLC1 was similar to that of the major form of the yeast Mediator complex (Pol II-Med) identified by Liu *et al.* (2001), which also augments transcriptional activation despite containing SRB10 (yeast CDK8). The human ARC-L and PC2/CRSP complexes possess molecular masses of 2- and 1.25-MDa, respectively, which are similar to those of TMLC1 and TMLC2 (Taatjes *et al.* 2002), but their activities were opposite to those of TMLC1 and TMLC2; ARC-L, which contains hCDK8, negatively regulates transcription, and PC2/CRSP, which does not contain hCDK8 but does contain hMED26 (CRSP70), positively regulates transcriptional activation.

Our isolated TMLC3 does not resemble any of the previously identified Mediator complexes. Although like PC2, which has been described as an active complex of TRAP/Mediator (Malik *et al.* 2000, 2005), TMLC3 lacks CDK-Cyclin submodules (CDK8-Cyclin C-MED12-MED13; Fig. 2A), and it displays a distinct subunit structure based on the SDS-PAGE band pattern. The inability of TMLC3 to phosphorylate the Pol II CTD correlates well with the absence of this module (Fig. 4). Thus, TMLC3 effects on Pol II are likely to be indirect; that is, it stimulates transcriptional activation without modulating Pol II. Therefore, overall, we conclude that TMLC1 and TMLC3 affect transcriptional activation through distinct mechanisms.

The human Mediator subunit hCDK8 plays a positive role in transcriptional activation

Human Mediator complexes containing hCDK8 are reported to repress transcription by phosphorylating the CTD of Pol II and the Cyclin H subunit of TFIIF (Sun *et al.* 1998; Akoulitchev *et al.* 2000). When the Pol II CTD is phosphorylated, it cannot form a transcription preinitiation complex (Lu *et al.* 1991). To gain further insight into the functions of CDK8, we knocked it down using siRNA and studied its activity with a luciferase

assay. Transcriptional activation by Gal4-VP16 was suppressed by both CDK8-119 and CDK8-480 siRNAs (Fig. 7A), indicating that hCDK8 functions positively in transcription *in vivo*. Co-transfection studies with the siRNA-resistant hCDK8 expression plasmid (either wild type or the kinase mutant D151A) confirmed this positive role because restoration of normal levels of wild-type hCDK8 restored the transcription activity, but introduction of the kinase mutant did not. This positive role perfectly corresponds to the CTD phosphorylation of both Ser2 and Ser5 (Fig. 7B, lanes 1, 3, and 6 versus lanes 2, 4, 5, and 7). Considering these results, we can imagine that it is the CTD kinase activity of hCDK8 of the Mediator complex that positively regulates transcription. It is still not known why Ser2 phosphorylation was suppressed by hCDK8 siRNA as much as Ser5 phosphorylation, because yeast CDK8 (SRB10) has been reported to predominantly phosphorylates Ser5 (Hengartner *et al.* 1998).

Although the positive effect of hCDK8 on transcription was clearly demonstrated *in vivo*, hCDK8 might have a negative regulatory function as well. For instance, we observed both positive and negative effects on *in vitro* transcriptional activation using two distinct hCDK8-containing subcomplexes (Fig. 3B). These contradictory functions are supported by various yeast studies. It is widely accepted that the CDK8 subunit in the Mediator complex negatively regulates transcription *in vivo* by phosphorylating the CTD of Pol II prior to formation of the preinitiation complex, or other transcription factors like Ste12 and the transcriptional co-repressor Tup1 (Hengartner *et al.* 1998; Nelson *et al.* 2003; Green & Johnson 2004). In contrast, it was also reported that CDK8 is required for efficient transcriptional activation by Gal4 and Sip4 (Vincent *et al.* 2001). It is clear that the subunit components of the TMLC1 and TMLC2 complexes are different; in particular, the lower band of the doublet band just above 220 kDa (possibly hMED12) and several subunits smaller than 30 kDa (especially those smaller than 15 kDa) display a lower stoichiometry in TMLC2 relative to TMLC1 (Fig. 3C). Since CDK8 is reported to form the so-called CDK8 submodule with three other Mediator subunits (Cyclin C, MED12, and MED13), our observation that the content of MED12 is not stoichiometric in TMLC2 might be noteworthy. Thus, an interesting possibility is that the Mediator complex possessing an intact CDK8 submodule has a positive role in transcriptional activation, but a partial CDK8 submodule that contains only CDK8 and Cyclin C has a negative role. At present, it is difficult to consider how the lower stoichiometry of the smaller subunits would directly convert the transcriptionally positive TMLC1 to

the negative form TMLC2. In humans, at least two Mediator subcomplexes with distinct molecular masses have been reported so far (Ito *et al.* 1999; Malik *et al.* 2000, 2005; Taatjes *et al.* 2002) but extensive studies to elucidate their biological role are still underway. We need to further purify all three TMLCs to identify their constituent subunits by mass spectrometry and to elucidate their different functional mechanisms.

Experimental procedures

DNA templates

For the basal transcription assays, the plasmid pML(C₂AT)Δ-50 containing the adenovirus type 2 major late (AdML) promoter was used as a template (Sawadogo & Roeder 1985). To study transcriptional activation, the plasmid pG5HM(C₂AT) was used as the test template (Chiang *et al.* 1993), with the plasmid pML(C₂AT)Δ-53Sh as the baseline control (Ohkuma *et al.* 1995). pG5HM(C₂AT) contains five GAL4-binding sites and the core promoter, as described previously (Ohkuma *et al.* 1995). The two templates pML(C₂AT)Δ-50 and pG5HM(C₂AT) give 390-nucleotide (nt) transcripts, and pML(C₂AT)Δ-53Sh gives a 290-nt transcript.

Antibodies

We used the following antibodies for Western blotting and immunoprecipitation assays of the human TRAP/Mediator complex subunits. Rabbit polyclonal antibodies against hMED1 (C-19; sc-5334), hMED6 (C-16; sc-9434), hMED12 (A-18; sc-5374), hMED13 (E-20; sc-12013), hMED23 (L-20; sc-9431), γ -tubulin (C-20; sc-7396), and the largest subunit of Pol II (N-20; sc-899) were purchased from Santa Cruz Biotechnology, the rabbit polyclonal antibody against hCDK8 was purchased from BioSource International Inc., and we prepared the rabbit polyclonal antibody against hMED18 was prepared by ourselves (Furumoto & Ohkuma, in preparation). The rabbit anti-hMED7, anti-hMED17 and anti-hMED20 polyclonal antibodies, and the mouse anti-HA monoclonal antibody (12CA5) are as described elsewhere (Malik *et al.* 2000; Watanabe *et al.* 2000). The mouse monoclonal antibodies (IgM) against phosphorylated Ser2 (H5) and Ser5 (H14) of the CTD heptapeptide repeat sequence of Pol II were purchased from Berkeley Antibody Company.

Construction of bacterial expression plasmids

To construct the plasmids expressing the human Mediator subunits, the cDNA sequences of hMED6, hMED7, and hCDK8 were retrieved from the Entrez DNA sequence database system. The GENBANK accession number of each cDNA was as follows: hMED6, NM_005466; hMED7, AF031383; and hCDK8, NM_001260. The full-length cDNAs were isolated by PCR using the following combinations of PCR primers. For hMED6, the oligonucleotide HMED6-1T (5'-GAGCCTCATATGGCG-GCGGTGGATATC-3') was designed to create an *Nde*I site

(underlined) at the first methionine codon of hMed6 cDNA, and the oligonucleotide HMED6-1B (5'-GGACACTCGAGTACTCACTGAAGTCTCATC-3') was designed to create an *XhoI* site (underlined) after the stop codon. For hMED7, the oligonucleotide HMED7-1T (5'-CTCAGGCATATGGGTGAACCACAGCAAG-3') was used to create an *NdeI* site (underlined) at the first methionine codon of hMED7 cDNA, and the oligonucleotide HMED7-1B (5'-GTCTTGGATCCTTCATGGTCTTTCATTCATC-3') was used to create a *BamHI* site (underlined) after the stop codon. For hCDK8, the oligonucleotide HSR10-1T (5'-GACACCATATGGACTATGACTTTAAAGTG-3') was used to create an *NdeI* site (underlined) at the first methionine codon of hMED6 cDNA, and the oligonucleotide HSR10-1B (5'-CGTTCTCTCGAGTGCAGCTCAGTACCGATG-3') was used to create an *XhoI* site (underlined) after the stop codon. An *NdeI-XhoI* fragment of hMED6 cDNA, an *NdeI-BamHI* fragment of hMED7 cDNA, and an *NdeI-XhoI* fragment of hCDK8 cDNA were subcloned into the 6His-pET11d vector (Merck Biosciences). All PCR products were checked by sequencing using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Expression and purification of recombinant proteins

Recombinant proteins were expressed in *Escherichia coli* BL21(DE3)pLysS by induction with isopropyl- β -D-thiogalactopyranoside (IPTG). The *E. coli* cells were harvested and resuspended with buffer B (BB500; 20 mM Tris-HCl [pH 7.9 at 4 °C], 0.5 mM EGTA, 10% [vol/vol] glycerol, 1 mM phenylethylsulfonyle fluoride [PMSF], 2 μ g/mL antipain, 2 μ g/mL aprotinin, 1 μ g/mL leupeptin, 0.8 μ g/mL pepstatin, 10 mM 2-mercaptoethanol, and 500 mM NaCl) (Ohkuma *et al.* 1995). After sonication, soluble bacterial lysates or the bacterial pellet solubilized with 2 M guanidine-HCl (pH 7.9) were used for purification depending on the protein solubilities (Watanabe *et al.* 2000). Six histidine (6His)-tagged proteins were purified through Ni-nitrilotriacetic acid (NTA) Sepharose (GE Healthcare Bio-Sciences) by eluting with buffer D (BD500; 20 mM Tris-HCl [pH 7.9 at 4 °C], 20% [vol/vol] glycerol, 1 mM PMSF, 10 mM 2-mercaptoethanol, and 500 mM KCl) containing 100 mM imidazole-HCl (pH 7.9). HA-tagged and GST-fusion proteins were also similarly expressed in *E. coli* BL21(DE3)pLysS by IPTG induction. *E. coli* cells from 50 to 100 mL of culture were harvested, resuspended in 1 mL of BB500, and sonicated. Soluble lysates were separated from insoluble debris by ultracentrifugation and stored at -80 °C until use.

Construction of eukaryotic expression plasmids and establishment of HeLa cell lines

We used two steps to construct the HF: hMED6, hMED7, and hCDK8 plasmids for establishing HeLa cell lines constitutively expressing these tagged subunits. First, the coding regions of hMED6, hMED7, and hCDK8 were digested from the above-described bacterial expression plasmids with *NdeI* and *EcoRI* and subcloned into the corresponding sites of the HA/FLAG(AS)-pGEM7 vector (Chiang *et al.* 1993). Second, these subunit coding

regions with HA/FLAG-tags at the N-termini were cut out with *EcoRV* and *EcoRI* (New England Biolabs), and subcloned into the *SmaI/EcoRI* fragment of the pIRESneo2 mammalian expression vector (BD Biosciences).

Human HeLa S3 cells were transfected with the HF: hMED6, hMED7, and hCDK8 plasmids using the PolyFect mammalian cell transfection reagent (Qiagen). Stable cell lines were screened in the presence of 800 μ g/mL G418. We selected two cell lines of the tagged subunits and established them in suspension culture.

Purification of Mediator and its related complexes

HeLa cells lines were grown in about 100 L of RPMI 1640 medium containing 10% calf serum, and about 100 mL of nuclear extracts (10 mg/mL protein) were prepared as described (Dignam *et al.* 1983). Each nuclear extract (100 mL) was adjusted to the same buffer composition as buffer C (20 mM Tris-HCl [pH 7.9 at 4 °C], 20% [vol/vol] glycerol, 0.5 mM EDTA [pH 8.0], 0.5 mM PMSF, 2 μ g/mL antipain, 2 μ g/mL aprotinin, 1 μ g/mL leupeptin, 0.8 μ g/mL pepstatin, 0.05% [vol/vol] NP40, and 10 mM 2-mercaptoethanol) containing 300 mM KCl (BC300) and loaded onto a 2-mL column of anti-FLAG M2 monoclonal antibody agarose (Sigma-Aldrich) at 4 °C. After washes with 10 mL BC300, the column was incubated overnight with 3 mL of 300 μ g/mL FLAG peptide (Sigma-Aldrich) and the HF:TRAP/Mediator complexes were efficiently eluted. To fractionate the various Mediator subtypes and remove the free form of the tagged subunits, the eluted peak fractions were subjected to a TSKgel G4000SW high-performance liquid chromatography (HPLC) gel filtration column (2.15 \times 30 cm; Tosoh) equilibrated with BC300. The HF:Mediator complexes were monitored by Western blotting with the anti-HA (12CA5) antibody. After SDS-PAGE in a PAG Mini 4%-20% acrylamide gradient gel (Daiichi Pure Chemicals), the separated proteins were transferred to an Immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore) as described previously (Ohkuma *et al.* 1995). Chemiluminescent signals were detected using the SuperSignal detection system (PIERCE) and RX-U film (Fuji Film).

In vitro transcription assays

TFIIH was purified from HeLa nuclear extracts as previously described (Watanabe *et al.* 2000). Pol II was purified from HeLa nuclear pellets by DE52, A25, P11, and HPLC-DEAE 5PW chromatography as described elsewhere (Watanabe *et al.* 2000). FLAG-tagged TBP (f-TBP)-containing TFIID (f-TFIID) was purified from nuclear extracts of f-TBP-expressing HeLa cells (cell line #3-10) through phosphocellulose (P11, Whatman) and anti-FLAG M2 monoclonal antibody agarose (Sigma-Aldrich) column chromatographies as described (Chiang *et al.* 1993). *In vitro* transcription was carried out as described (Ohkuma *et al.* 1995). To observe transcriptional activation, 20 ng of f-TFIID was used instead of 20 ng of 6His-TBP. As an activator, we used 40 ng of GAL4-VP16 containing the C-terminal acidic activation domain of VP16 (residues 413-490) fused to GAL4(1-94) (residues 1-94). Autoradiography was performed at -80 °C with Fuji RX-U X-ray film.

The incorporation of [α - 32 P] CTP (GE Healthcare Bio-Sciences) into transcripts was quantified using the Fuji BAS2500 Bio-Imaging analyzer.

Kinase assays

Assays were carried out essentially as described (Ohkuma & Roeder 1994) in the presence of Pol II. Phosphorylation reactions were performed at 30 °C for 1 h and stopped by the addition of 75 μ L of phosphorylation stop solution (10 mM EDTA, 0.1% NP40, and 0.05% SDS). Phosphorylated proteins were TCA-precipitated, analyzed by SDS-PAGE (5.5% acrylamide), and detected by autoradiography performed at -80 °C with Fuji RX-U X-ray film.

GST pull-down assays

GST-fusion proteins were used for protein interaction assays. Each TRAP/Mediator complex (500 ng) was incubated with lysates containing 300 ng of GST-proteins together with 5 μ L (packed volume) of Glutathione-Sepharose 4B (GE Healthcare Bio-Sciences) in a 500- μ L reaction volume of buffer C with 100 mM KCl (BC100) containing 200 μ g/mL BSA for 4 h at 4 °C with rotation. The glutathione-Sepharose resin was washed twice with 500 μ L of buffer C with 200 mM KCl (BC200), and once with 500 μ L of BC100, boiled in SDS sample buffer, and analyzed by SDS-PAGE (12% acrylamide). Pulled down proteins were detected by Western blotting with the anti-HA antibody (12CA5).

siRNA oligos

Five double-stranded RNA oligonucleotides were designed by B-Bridge International and generated by Dharmacon. The sequences of each strand of the five siRNA are as follows:

Cdk8-105: sense strand: 5'-CUACAAAGCCAAGAG-GAAAdTdT-3'

antisense strand: 5'-UUUCCUCUUGGCUUUGUAGdTdT-3'

Cdk8-119: sense strand: 5'-GGAAAGAUGGGAAGGAU-GAdTdT-3'

antisense strand: 5'-UCAUCCUCCCAUCUUUCCdTdT-3'

Cdk8-301: sense strand: 5'-GAACAUGACCUCUG-GCAUAdTdT-3'

antisense strand: 5'-UAUGCCAGAGGUCAUGUUCdTdT-3'

Cdk8-480: sense strand: 5'-GGGUGAAGGUCCU-GAGCGAdTdT-3'

antisense strand: 5'-UCGCUCAGGACCUUCACCCdTdT-3'

Cdk8-777: sense strand: 5'-GGGAUUUCCUGCA-GAUAAAAdTdT-3'

antisense strand: 5'-GGGAUUUCCUGCAGAUAAAAdTdT-3'

The sequence of the non-silencing siRNA was 5'-AUUCUAU-CACUAGCGUGACUU-3' (Dharmacon).

siRNA-resistant hCDK8 cDNAs

Two siRNA-resistant cDNAs were designed. For CDK8si^r against Cdk8-119 siRNA, hCDK8 cDNA in the pBluescript cloning

vector (Stratagene) was mutated at three sequential codons simultaneously corresponding to the amino acid residues 124D (GAT to GAC), 125G (GGG to GGA), and 126K (AAG to AAA) by using the site-directed mutagenesis kit (Molecular Biology Laboratory, Japan) together with the mutant oligo 1 (5'-AAAGCCAA-GAGGAAA GACGGAAAAGATGATAAAGACTAT-3'). In the sequence, three changed nucleotides were underlined. For CDK8si^r against Cdk8-480 siRNA, hCDK8 cDNA in the pBlue-script cloning vector was similarly mutated at three sequential codons simultaneously corresponding to the amino acid residues 484E (GAA to GAG), 485G (GGT to GGC), and 486P (CCT to CCA) by using the site-directed mutagenesis kit together with the mutant oligo 2 (5'-ATTTTAGTTATGGGTGAGGGCCCA-GAGCGAGGAAGAGTA-3'). In the sequence, three changed nucleotides were underlined. Similarly to the construction of the above-described wild-type hCDK8 expression plasmid, those mutated hCDK8 cDNAs were subcloned into the HA/FLAG(AS)-pGEM7 vector (Chiang *et al.* 1993), and further into the pIRESneo2 mammalian expression vector (BD Biosciences).

CTD kinase mutation in hCDK8

A point mutation was created in the pBluescript cloning vector (Stratagene) at the site essential for the CTD kinase activity of hCDK8 (D151A) by using the site-directed mutagenesis kit (Medical & Biological Laboratories) together with a mutant oligonucleotide (5'-TGGGTGTTGCACAGAGCTTTGAAAC-CTGCTAAT-3'). In the sequence, one mutated nucleotide is underlined. Similarly to the construction of the above-described hCDK8 expression plasmid, those mutated hCDK8 cDNAs were subcloned into the HA/FLAG(AS)-pGEM7 vector (Chiang *et al.* 1993), and further into the pIRESneo2 mammalian expression vector (BD Biosciences).

Luciferase assay

Luciferase assays were performed based on the method of Osada *et al.* (1999). HeLa S3 cells (2×10^4) were seeded into each well of 24-well plates. After 1 day, siRNA oligos (final concentration 33 nM) were transfected into cells using Lipofectamine 2000 (Invitrogen). Cells were cultured for 2.5 days and, after washing with PBS, were cotransfected with 100 ng of pE1b-TATA-luciferase reporter plasmid, 0.5 ng of pRL-TK (Renilla luciferase used as an internal control), and 0.5 ng of pM-VP16 (expression vector for Gal4-VP16). After 1 day, the cells were lysed and their transcription activities were quantitated using a PicaGene Dual SeaPansy Luminescence kit (Toyo Ink).

Acknowledgements

We thank Tetsuro Kokubo for critical reading of the manuscript, Chikahide Masutani and Akio Uchida for advice on establishing the epitope-tagged HeLa cell lines, and Shigehiro Osada for the luciferase assay. We also thank our colleagues in University of Toyama and Osaka University for helpful discussions. This work was supported in part by grants from the Ministry of Education,

Culture, Sports, Science and Technology of Japan (Y.O. and F.H.), the Japan Society for the Promotion of Science (Y.O. and A.T.) and the Solution Oriented Research for Science and Technology (SORST) (F.H.), and in part by National Institutes of Health grants (S.M.).

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Accepted: 12 October 2006

Received: 4 September 2006