Distinct Regions of MAT1 Regulate cdk7 Kinase and TFIIH Transcription Activities*

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The transcription/DNA repair factor TFIIH may be resolved into at least two subcomplexes: the core TFIIH and the cdk-activating kinase (CAK) complex. The CAK complex, which is also found free in the cell, is composed of cdk7, cyclin H, and MAT1. In the present work, we found that the C terminus of MAT1 binds to the cdk7-cyclin H complex and activates the cdk7 kinase activity. The median portion of MAT1, which contains a coiled-coil motif, allows the binding of CAK to the TFIIH core through interactions with both XPD and XPB helicases. Furthermore, using recombinant TFIIH complexes, it is demonstrated that the N-terminal RING finger domain of MAT1 is crucial for transcription activation and participates to the phosphorylation of the C-terminal domain of the largest subunit of the RNA polymerase II.

Cyclin-dependent kinases (cdk) have a central role in the coordination of the eukaryotic cell cycle and participate in the integration of diverse growth regulatory signals. Some members of this protein kinase family are involved not only in cell cycle control but also in other mechanisms that govern cell life, notably transcription. Cdk activities are regulated by several different processes including the binding of an activating cyclin subunit, their phosphorylation, and the association of cdk inhibitors or other stimulatory factors (1). One of them, cdk7, was originally identified as the catalytic subunit of the cdk-activating kinase (CAK) complex that is able to phosphorylate, and thereby activate, several cdkks (2). Besides cdk7, the CAK complex is composed of the regulatory subunit cyclin H (3, 4) and a third partner, MAT1, defined as a stabilizing factor (5, 6).

Yeast genetic as well as biochemical studies demonstrated that MAT1 is also, together with cyclin H and cdk7, part of the general transcription factor TFIIH (7, 8). TFIIH is composed of nine subunits and can be functionally divided into several subcomplexes such as the core TFIIH and the CAK complex (9, 10). TFIIH plays a role not only in transcription but also in DNA repair (11). Although TFIIH factor is absolutely required in nucleotide excision repair (11), the role of the CAK subcomplex in this reaction is not clear (12).

Cdk7 phosphorylates several basal transcription factors (13–15) as well as the C-terminal domain (CTD) of the largest subunit of the RNA polymerase II (RNA pol II) (16). However, cdk7 is not the only kinase responsible for CTD phosphorylation. Indeed, the cdk8-cyclin C complex, which is found associated with the holoenzyme transcription complex, phosphorylates the CTD in vitro (17). Furthermore, the Srb10/Srb11 complex (the yeast counterpart of cdk8-cyclin C) phosphorylates the CTD in vivo prior to the formation of the preinitiation complex (PIC), resulting in an inhibition of the transcription reaction (18). Also, during human immunodeficiency virus infection, the CTD kinase activity of the cdk8-cyclin T complex, characterized as a component of the elongation factor pTEFb (for positive transcription elongation factor b), is required for transactivation (19). One cannot exclude, however, that all three kinases (cdk7, cdk8, and cdk9) could regulate the CTD phosphorylation in concert with other events of gene expression such as splicing, poly-adenylation, capping, and/or dephosphorylation (20–22). Indeed, to engage a new transcription cycle or to reinitiate after pausing, RNA pol II must be dephosphorylated (23); this event involves a CTD phosphatase identified both in human and yeast (24, 25). It thus remains to understand precisely how these various phosphorylation/dephosphorylation events are “milestones of the transcription mechanism.”

As part of the multienzymatic protein complex TFIIH, CAK, in coordination with the XBP and XPD helicases, participates in various events of the transcription reaction. The XBP helicase opens the promoter around the start site to allow the formation of the first phosphodiester bond, the first step of RNA transcript synthesis (26–28). In fact, in vitro RNA synthesis may occur in the absence of either XPD helicase or CAK complex; however, when present, both significantly increase the rate of RNA synthesis (29, 30). Whether transcription stimulation is due either to the XPD enzymatic function itself and/or to its physical presence remains an open question. It is however accepted that XPD anchors CAK to the core TFIIH (9, 10) and promotes the phosphorylation of RNA pol II CTD by CAK (27–29).

Finally, the substrate specificity of the cdk7 kinase varies in accordance to its presence within TFIIH. Indeed, when the CAK complex is free, cdk7 preferentially phosphorlates cdk2, another member of the cyclin-dependent kinase family, whereas when part of TFIIH, a preferential and specific phosphorylation of the CTD of RNA pol II is observed (13, 15). Such
substrate specificity was ascribed to MAT1, the third component of CAK (15). Indeed, the addition of the MAT1 subunit to recombinant cdk7/cyclin H switched its substrate specificity to favor RNA pol II phosphorylation over cdk2. Whether this is connected to the stimulatory function of MAT1 towards cdk7 itself (13) or to its structure also remains to be answered. It should be pointed out that sequence alignments and secondary structure predictions identified two motifs in MAT1 (mass = 34 kDa; 309 amino acids), often involved in protein-protein interactions, an N-terminal RING finger domain, and a central coiled-coil domain (31, 32).

To understand the role of MAT1 both within TFIIF and in the transcription reaction, we generated deletion or point mutations in the RING finger domain. We demonstrate here that MAT1 interacts with the CAX complex through the hydrophobic C-terminal domain and that this region is sufficient to activate cdk7 kinase activity towards synthetic cd4 or cdk2 substrates. Moreover, we demonstrate that, although not required for TFIIF complex formation, the RING finger motif of MAT1 has a crucial role in basal transcription complex activity and in the CTD phosphorylation process.

MATERIALS AND METHODS

Construction of Recombinant Baculoviruses—Baculoviruses allowing the expression of single subunits of the core TFIIF or CAX subunits were described in Refs. 29 and 13, respectively. Baculoviruses allowing the expression of mutant or deletant forms of MAT1 were constructed in the pVL1392 vector (PharMingen). The cDNAs were amplified by polymerase chain reaction using oligonucleotides containing EcoRI and BanHI sites in 5’ and 3’, respectively. After digestion by EcoRI and BanHI (New England Biolabs), polymerase chain reaction fragments were inserted into pVL1392. The vectors were recombined with linearized baculovirus DNA (BaculoGold DNA, Pharmingen) in Spodoptera frugiperda 9 (Sf9) cells (33). The recombinant viruses were plaque-purified, and viral stocks were prepared by a three-step growth amplification.

Protein-Protein Interaction Assays—Pairwise protein interactions were characterized by coimmunoprecipitation in Sf9 cells (typically, 2.5 × 10^7 cells) with the corresponding recombinant baculoviruses at multiplicity of infection of 10 (XPB, XPD, and each MAT1 protein), 5 (p62), and 2 (cdk7 and cyclin H) plaque forming units/cell. Cells were collected 48 h later and washed in 1× phosphate-buffered saline, 30% glycerol. After douncing in 2.5 ml of buffer A (20 mm Tris-HCl, pH 7.5, 20% glycerol, 150 mm NaCl, 5 mm MgCl2, 0.1% Nonidet P-40, 5 mm β-mercaptoethanol, 0.5 mm phenylmethylsulfonyl fluoride, 1× proteinase inhibitor mixture containing 2.5 μg/ml leupeptin, pepstatin, aprotinin, antipain, and chymostatin), clarified lysates were obtained by centrifugation at 12,000 × g for 30 min at 4 °C. 50 μl of clarified lysate were adsorbed on 20 μl of protein G-Sepharose cross-linked with the indicated antibody in buffer B (20 mm Tris-HCl, pH 7.5, 10% glycerol, 0.1 mm EDTA) containing 50 mm KCl. After a 1-h incubation at 4 °C, the beads were washed extensively in buffer B containing 150 mm KCl and resuspended in Laemmli buffer. After SDS-PAGE, the proteins retained on the beads were analyzed by Western blotting using the indicated antibodies.

Purification of Recombinant TFIIF Complexes—Sf9 cells (typically, 1 × 10^8 cells) were infected with combinations of recombinant baculoviruses and were collected 48 h later. Cells were washed in 1× phosphate-buffered saline, 30% glycerol and dounced in 10 ml of buffer A. After centrifugation at 12,000 × g for 45 min at 4 °C, recombinant TFIIF complexes were purified as described previously (29). Briefly, clarified lysates were loaded on an heparin-Ultrogel column (Sepacor, France) pre-equilibrated in buffer A. After extensive washing with buffer A containing 400 mm NaCl, the proteins were eluted with the same buffer containing 500 mm NaCl. The eluted fraction was dialyzed for twice 2 h against buffer C (50 mm Tris-HCl, pH 7.5, 20% glycerol, 300 mm KCl) and was subsequently used as an input material for metal affinity purification. Samples containing recombinant TFIIF were incubated 1 h at 4 °C with metal affinity resin (Talon, ClonTech). After packing the column, the resin was extensively washed with buffer C containing 10 mm imidazole, and proteins were eluted in buffer C containing 400 mm KCl and 100 mm EDTA. The elution fraction was then dialyzed against buffer B containing 50 mm KCl and 0.5 mm dithiothreitol.

Kinase Assays—Kinase assays using either cd4-2 peptide or purified recombinant GST-cdk2 as substrates were performed as described previously (8, 34). Insect cells clarified lysates containing the proteins to be tested (typically 100 μl) were incubated with 40 μl of protein G-Sepharose beads (Amersham Pharmacia Biotech) cross-linked with Ab-ckd7 in buffer B containing 50 mm KCl and 0.1% Nonidet P-40. After a 1-h incubation at 4 °C, the beads were extensively washed in buffer B containing 150 mm KCl plus 0.1% Nonidet P-40. The proteins retained on the beads were analyzed by SDS-PAGE or subjected to a kinase assay.

RNA pol II phosphorylation was carried out in the presence of all the components necessary for standard run-off transcription as well as the four NTPs as described previously (29). Assays were consistent with published reactions except that ATP was added to a final concentration of 5 mm, and 1 μl of purified RNA polymerase II was used/assay. The reaction was stopped by the addition of Laemmli, and samples were loaded on a 5% SDS-PAGE. The polymerase polypeptides were revealed using the T7 antibody (35). Quantification of the kinase activities were done using a Bio-imaging Analyzer FUJIX BAS 2000.

TFIIF Enzymatic Assays—The DNA-dependent ATPase activity was assayed as described previously (8). Briefly, protein fractions were incubated for 2 h at 30 °C in the presence of 1 μCi of [γ-32P]ATP (7000 Ci/mmol, ICN Pharmaceuticals) in a 20 μl reaction volume in 20 mm Tris-HCl, pH 7.9, 4 mm MgCl2, 2 mm cold ATP, 1 mm dithiorthiolate, 50 μg/ml bovine serum albumin, and 4 μg/ml pUC309 plasmid. Reactions were terminated by the addition of EDTA to 50 mm. The reactions were then diluted 5-fold in 10 mm Tris-HCl, 1 mm EDTA, spotted onto polyethylenimine TLC plates (Merck), run in 0.5 m LiCl, 1 m formic acid, and autoradiographed.

Standard run-off transcription assays were carried out as described previously (36). Briefly, TFIIF fractions were incubated with purified transcription factors (TFB, TFIA, TFIB, TFIE, and TFIF1) and RNA polymerase II in 50 mm Tris-HCl, pH 7.9, 10% glycerol, 1 mm EDTA, 0.5 mm dithiothreitol, 5 mm MgCl2, and 100 ng of DNA template (EcoRI-Sall-digested pUC309). Reaction mixtures (final volume = 20 μl) were preincubated 15 min at 25 °C. The transcription was then carried out for 45 min at 25 °C in the presence of ribonucleotides (250 μM each ATP, GTP, and UTP, 10 μM CTP) including radiolabeled CTP (4 μCi of 400 Ci/mmol [α-32P]CTP; Amersham Pharmacia Biotech). RNA transcripts were resolved by electrophoresis and analyzed by autoradiography. Purification from the dihydrofolate reductase promoter, human TFIID was used instead of TBP in presence of 100 ng of DNA template (EcoRI-NarI-digested pUC18). Quantification of the various TFIIF activities was done using a Bio-imaging Analyzer FUJIX BAS 2000.

Yeast Three-Hybrid System—For the ternary complex studies in the yeast three-hybrid system, we used the L40 strain (MATa, trp1, his3, leu2, ade2, LYS2::(LexAop)4-HIS3, URA3::(LexAop)8-lacZ). The plasmids pLex-cdk7/Met-cyclin H and pVP16-MAT1 have been described previously (37). For the deletion mutants of MAT1, the corresponding cDNAs were amplified by polymerase chain reaction, digested with EcoRI, and cloned in fusion to the activation domain of VP16 into the EcoRI site of pVP16. The correct open reading frames and DNA sequences were verified by sequencing. All the work with yeast including maintenance, transformation, methionine selection, and the liquid β-galactosidase assay using o-nitrophenyl β-D-galactopyranoside (Sigma) as a substrate were performed as described elsewhere (38).

Other Techniques—The purification of TFIIF factor used as a control in our experiments as well as all the basic transcription factors required for RNA pol II kinase and in vitro transcription assays were described previously (30). Monoclonal antibodies raised against XPB, XPD, p52, p62, p44, p34, cdk7, cyclin H, and C-terminal MAT1 were previously described (30). The monoclonal antibody detecting the N-terminal part of MAT1 (1G6) was directed against a MAT1-derived peptide (MD-DQCPRKTKYRNPSSL). For Western blot analysis, we used the ECL chemiluminescent method (Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

RESULTS

The C-terminal Domain of MAT1 Interacts with the cdk7-Cyclin H Complex—Analysis of primary sequence as well as secondary structure predictions defined three putative domains in MAT1 (Fig. 1A): an N-terminal domain containing a canonical C-HC2 RING finger motif (amino acids 1–66), a central coiled-coil domain (amino acids 114–175), and a C-terminal hydrophobic domain (31, 32). We also characterized a “minimal” fragment, which remains associated within the CAX complex after spontaneous degradation of MAT1 (data not
shown). From N-terminal microsequencing, we defined this fragment as starting at amino acid 229.

In a first set of assays, we investigated the interactions between the various domains of MAT1 and the subunits of the CAK. Genetic analysis of Tfb3/Rig2, the yeast homolog of MAT1, revealed that single point mutations in conserved cysteines of the RING finger have detrimental effects on viability. Changing cysteine 16 of yeast Tfb3/Rig2 to tyrosine resulted in temperature-sensitive growth, whereas changing cysteine 34 to serine resulted in unconditional lethality.2 We constructed baculovirus vectors carrying the corresponding point mutations in human MAT1, MAT1-C9Y and MAT1-C26S, respectively, in addition to deletions mutants of MAT1 (MAT1-(1–189), MAT1-(66–309), MAT1-(189–309), and MAT1-(229–309)). The recombinant proteins from the baculovirus-infected cell extracts (as indicated in the legend to Fig. 1) were then immunoprecipitated with antibodies directed against cdk7 or cyclin H (as indicated at the top and at the right of the panel). Recombinant proteins were immunoprecipitated using either an antibody directed against cdk7 or cyclin H (as indicated at the top and at the right of the panel). Recombinant proteins were immunoprecipitated using either an antibody directed against cdk7 or cyclin H (as indicated at the top and at the right of the panel). Recombinant proteins were immunoprecipitated using either an antibody directed against cdk7 or cyclin H (as indicated at the top and at the right of the panel). Recombinant proteins were immunoprecipitated using either an antibody directed against cdk7 or cyclin H (as indicated at the top and at the right of the panel). Recombinant proteins were immunoprecipitated using either an antibody directed against cdk7 or cyclin H (as indicated at the top and at the right of the panel).

The deletion of the C-terminal part of MAT1 leading to the

2 O. Gileadi, unpublished observations.

FIG. 1. The C-terminal domain of MAT1 interacts with the cdk7-cyclin H complex. A, schematic drawing of MAT1. The RING finger domain (amino acids 1–66), coiled-coil domain (amino acids 114–175), and the hydrophobic C-terminal domain (amino acids 229–309) are shown. N- and C-terminal truncations (MAT1-(1–189), MAT1-(66–309), MAT1-(189–309), and MAT1-(229–309)) and point mutants (MAT1-C9Y and MAT1-C26S) of MAT1 are represented. The sequence of the RING finger of MAT1 is indicated, and the residues involved in zinc-atom ligation are shown in bold. Mutated residues in MAT1-C9Y and MAT1-C26S are denoted by an asterisk. For truncated proteins, the names include the first and the last residue of each deletion mutant. B, SF9 cells were (co)infected with recombinant baculoviruses expressing cdk7 and cyclin H (cycH) individually or in combination with truncated fragments of MAT1 as indicated at the top and at the right of the panel. Recombinant proteins were immunoprecipitated using either an antibody directed against cdk7 (Ab-cdk7, lanes 3–5) or against cyclin H (Ab-cycH, lanes 2 and 6) bound to protein G-Sepharose. After extensive washing, immunoadsorbed proteins were analyzed by Western blotting using a mixture of antibodies generated toward the N and C terminus of MAT1. As a negative control, each deletion mutant of MAT1 was immunoprecipitated with Ab-cdk7 and Ab-cycH (lanes 5 and 6, respectively). Lane 1 (L), SF9 clarified lysate of infected cells expressing each truncated fragment. C, SF9 cells were coinfected with recombinant baculoviruses expressing cdk7 and cyclin H (cdk7/cycH) with either wild-type MAT1 (WT) or point mutants (C9Y or C26S) as indicated above. Recombinant proteins were immunoprecipitated with Ab-cdk7 bound to protein G-Sepharose (lanes 1, 3, and 5). Proteins retained on the beads were analyzed by Western blotting using an antibody directed against the C terminus of MAT1. As a negative control, the human cdk7 and point mutants of MAT1 were immunoprecipitated with Ab-cdk7 (lanes 2, 4, and 6). HC and LC correspond to the heavy chains and the light chains of Ab-cdk7 antibodies, respectively. D, yeast L40 strains were transformed with pLex9-cdk7/Met-cyclin H and either pVP16-MAT1 or the corresponding MAT1 mutants as indicated at the top of each column. These transformants were grown in minimal selective medium in the absence (–, closed bars) or presence (+, hatched bars) of 1 mM methionine leading to the expression or repression of cyclin H, respectively. A liquid β-galactosidase assay was performed, and the values of at least four independent experiments (with MAT1-WT = 100%) are shown with standard deviation bars.

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MAT1-(1–189) protein abolished the interaction with cdk7-cyclin H, whereas progressive N-terminal truncations (MAT1-(66–309), MAT1-(189–309), and MAT1-(229–309)) did not alter it (Fig. 1B, lane 4). This suggests that the RING finger is not involved in CAK formation. Indeed, the point mutations in the RING finger did not prevent the interaction between either MAT1-C9Y or MAT1-C26S with the cdk7-cyclin H complex (Fig. 1C, compare lanes 1 and 3 with lane 5).

To further establish the physiological relevance of the C-terminal interaction with cdk7-cyclin H, we used the previously described inducible three-hybrid system (37). Therefore, MAT1 and the truncated mutants 1–189, 66–309, and 189–309 were fused to the VP16 transcription activator motif giving pVP16-MAT1/WT, pVP16-MAT1-(1–189), pVP16-MAT1-(66–309), and pVP16-MAT1-(189–309) plasmids. Each construct was transformed together with the pLex9-ckd7/Met-cyclin H plasmid, in which cdk7 is fused to the DNA binding motif of LexA and the expression of cyclin H is under the control of the Met25 promoter, into the L40 yeast strain. The Met25 promoter is active in the absence of methionine in the selective minimal medium and repressed when methionine is added. The transcriptionsal activation of the reporter gene lacZ due to the formation of the ternary complex including cdk7, cyclin H, and MAT1 proteins, was measured on yeast cultures grown in the absence or in the presence of methionine according to a liquid β-galactosidase assay. No β-galactosidase activity was observed when cdk7 and cyclin H were expressed with either VP16-MAT1-(1–189) or VP16 alone (Fig. 1D). In contrast, when cdk7-cyclin H was expressed together with either VP16-MAT1-(66–309) or VP16-MAT1-(189–309), we observed an increase in the β-galactosidase activity even higher than with VP16-MAT1/WT. The repression of cyclin H in the presence of methionine completely abolishes the β-galactosidase synthesis. This strongly demonstrates that, in vivo, the C-terminal end of MAT1 interacts with the cdk7-cyclin H complex. Together, these results show that the formation of the CAK complex requires the C-terminal part of MAT1 and that this interaction involves both cdk7 and cyclin H.

The C-terminal Domain of MAT1 Is Sufficient to Stimulate cdk7 Kinase Activity—Previous studies have shown that MAT1 stimulates the kinase activity of the cdk7-cyclin H complex (13, 15). We thus investigated the role of MAT1 fragments on the regulation of CAK kinase activity. Kinase assays were carried out using either the cyclin-dependent kinase cdk2, implicated in cell cycle progression, or the ctd4 peptide, mimicking four repeats of the heptapeptide consensus sequence of the CTD of RNA pol II, as a substrate (8). Sf9 cells were coinfected with combinations of baculoviruses overexpressing cdk7 and cyclin H in addition to MAT1 either wild-type or mutated (as indicated at the top of the Fig. 2). Then, infected Sf9 cell extracts, in which MAT1 proteins were in excess, were immunoprecipitated with antibodies directed against cdk7 (Ab-cdk7) immobilized onto protein G-Sepharose. After extensive washing, the kinase activity of the protein complexes retained on the beads was determined with either cdk2 (Fig. 2, upper and lower panels) or ctd4 (lower panel). The cdk7 kinase activity was then adjusted as a function of the amount of cdk7 immobilized on the beads, according to Western blot analysis (data not shown, and Fig. 2, lower panel). The MAT1-WT as well as MAT1-C9Y and MAT1-C26S point mutants stimulated kinase activity 2–3-fold (Fig. 2, compare lanes 2–4 with lane 1 and lower panel) showing first the stimulatory function of MAT1 and second that the RING finger mutations did not alter either the kinase activity or the substrate specificity toward both cdk2 and the synthetic ctd4 substrates. Thus, in this simple in vitro kinase assay, the RING finger domain of MAT1 does not seem to be required for the CAK kinase activity. We also show that when the recombinant CAK complexes carry any of the C-terminal fragment (MAT1-(66–309), MAT1-(189–309), or MAT1-(229–309)), the phosphorylation of both substrates is still stimulated (2–3-fold) (Fig. 2, lanes 6–8). There was a slight but significant substrate specificity exhibited by the two C-terminal fragments of MAT1 (MAT1-(189–309) and MAT1-(229–309)) resulting in an up to 3-fold increase of cdk2 phosphorylation (Fig. 2, lanes 7 and 8; see also Refs. 13 and 15). The N-terminal fragment of MAT1 (MAT1-(1–189)), which did not bind the cdk7-cyclin H complex (Fig. 1B), did not stimulate cdk2 or ctd4 phosphorylation (Fig. 2, compare lane 5 to lane 1). As a control, a protein complex containing the ATP-binding site-mutated cdk7, cdk7-K41R, in addition to cyclin H and MAT1-WT, was unable to phosphorylate both cdk2 and ctd4 substrates demonstrating that the CAK complex preparations were not contaminated by any Sf9 kinase activity (Fig. 2, lane 9).

MAT1 Interacts with XPD and XPB Helicases—We then investigated if and how MAT1 binds to the subunits of the core TFIIH subcomplex. Therefore, we coexpressed in Sf9 cells, cdk7-cyclin H, XPD, XPB, or p62 with each of the MAT1 proteins (i.e. MAT1-WT, MAT1-C9Y, MAT1-C26S, MAT1-(1–189), MAT1-(66–309), MAT1-(189–309), and MAT1-(229–309)). The recombinant protein complexes retained by antibodies directed toward some of the above subunits (Fig. 3A) were analyzed by SDS-PAGE followed by Western blotting. Ab-cdk7, Ab-XPB, as well as Ab-XPD were able to immunoprecipitate not only their own antigenic protein (data not shown) but also MAT1-WT (Fig. 3A, lanes 2, 4, and 6, respectively), whereas no significant interaction was observed with p62 (Fig. 3A, lane 8) and with p52, p44, and p34 subunits of the core TFIIH (data not shown and Ref. 13). MAT1-(66–309), as well as MAT1-C9Y and MAT1-C26S, the RING finger-mutated recombinant proteins, still interacted with both helicases (Fig. 3, A–C). However, deletion of the MAT1 C terminus, giving rise to either MAT1-(1–189) (Fig. 3A) or to MAT1-(1–228) (data not shown), abol-
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FIG. 3. MAT1 interacts with XPD and XPB through its median part. A, Sf9 cells were coinoculated either with recombinant baculoviruses expressing the cdk7/cyclin H complex (cdk7/cyclinH), XPD, XPB, or p62 and with recombinant baculoviruses expressing the various truncated MAT1 fragments (MAT1-(1–189), MAT1-(66–309), MAT1-(189–309), and MAT1-(229–309)) as indicated at the top and at the right of the panels. Recombinant proteins were immunoprecipitated using Ab-cdk7 (lanes 2 and 3), Ab-XPD (lanes 4 and 5), Ab-XPB (lanes 6 and 7), or Ab-p62 (lanes 8 and 9) bound to protein G-Sepharose. As a negative control, each deletion mutant of MAT1 was immunoprecipitated with the corresponding antibody (lanes 3, 5, 7, and 9). After extensive washing, adsorbed proteins were analyzed by Western blot using a mixture of antibodies generated against the N and C terminus of MAT1.

B, MAT1-(189–309)) as indicated at the right of the panel. Recombinant proteins were immunoprecipitated with Ab-cdk7 (lanes 2 and 3), Ab-XPD (lanes 4 and 5), Ab-XPB (lanes 6 and 7), or Ab-p62 (lanes 8 and 9) bound to protein G-Sepharose. As a negative control, each deletion mutant of MAT1 was immunoprecipitated with the corresponding antibody (lanes 3, 5, 7, and 9). After extensive washing, adsorbed proteins were analyzed by Western blot using a mixture of antibodies generated against the N and C terminus of MAT1.

FIG. 4. The RING finger domain of MAT1 is involved in transcription. A, recombinant TFIIH complexes containing either wild-type or mutant forms of the MAT1 subunits were purified as described previously (29) and resolved by SDS-PAGE followed by Western blotting using antibodies raised against the different subunits of the core TFIIH (upper panel) or the CAK subcomplex (lower panel). An additional complex, containing a cdk7 subunit that is mutated in its ATP-binding site (rIIIH9-cdk7-K41R) was used as a control. B, equivalent amounts of recombinant complexes purified were tested in an in vitro run-off transcription assay using either the adenovirus 2 major late promoter (MLP) (309 nucleotides) or the DHFR promoter (200 nucleotides). Quantification of three independent in vitro transcription assays were performed using a Bio-imaging Analyzer FUJIX BAS 2000. C, partially purified HeLa TFIIH (36).

The RING finger domain of MAT1 is involved in transcription. Knowing that CAK is part of the TFIIH (7, 8), we wondered which part of MAT1 could allow CAK to be anchored to the core TFIIH complex and if MAT1 could participate in the transcription reaction. To address this question, we designed recombinant TFIIH complexes containing all nine subunits including either wild-type or mutated MAT1. Sf9 cells were coinoculated with baculoviruses harboring genes for the subunits of the core TFIIH (XPB, XPD, p62, p52, p44, and p34) in addition to cdk7 (either wild-type or mutated in its ATP-binding site), cyclin H and either point mutated MAT1 (MAT1-C9Y and MAT1-C26S) or deletion mutants (MAT1-(1–189), MAT1-(66–309), and MAT1-(189–309)). After partial purification (see “Materials and Methods”), the various recombinant TFIIH complexes (rIIIH9), in addition to a complex lacking MAT1 (rIIIH8), were analyzed by Western blotting using antibodies raised against some TFIIH subunits (Fig. 4A). The wild-type MAT1, MAT1-C9Y, MAT1-C26S, and MAT1-(66–309) were able to incorporate the TFIIH factor (Fig. 4A, lanes 3–6) demonstrating that the RING finger is not required for CAK anchoring to the core TFIIH, in agreement with the above results obtained by pairwise interaction assays (Fig. 3). Moreover, using an antibody specifically designed against the C-terminal end of MAT1, we observed that mutations within MAT1 did not modify the stoichiometric composition of the various subunits of rIIIH9 complexes. In contrast, neither MAT1-(1–189) nor MAT1-(189–309) were present in recombinant complexes, even though they were overexpressed in infected Sf9 cells (data not shown). This suggests that these deletion mutants may exhibit some conformational modifications inconsistent with their incorporation within TFIIH.

The various recombinant TFIIH complexes containing or lacking MAT1 (rIIIH9 and rIIIH8, respectively) were then tested in an in vitro transcription system containing RNA pol II as well as all the basal transcription factors in addition to either the adenovirus 2 major late promoter (309 nucleotides) or the DHFR promoter (200 nucleotides) as templates. First, in the absence of MAT1, rIIIH8 was transcriptionally active, although to a very low level (Fig. 4B, compare lanes 2 and 3); when present, MAT1 strongly stimulated (8–10-fold) the transcription reaction independently of the nature of the promoter. The rate of transcription stimulation is similar to what was observed when the entire CAK complex was added to
Complexes were tested for ATPase activity. Pi generated upon ATP hydrolysis is indicated at the right of the panel. Quantification of the ATPase activity from three independent experiments was done using a Bio-imaging Analyzer FUJIX BAS 2000, and the relative activity P/(ATP + P) of each complex is shown. The value of autohydrolysis of ATP (lane 10) was subtracted for all values obtained. rIIH6, rIIH6XPD-K48R, and rIIH6XBP-K346R are recombinant complexes containing the six subunits of core TFIIH including the XPD or XBP subunits mutated in their ATP-binding site when indicated. B, the phosphorylation of RNA pol II was tested on the major late promoter (MLP) or DHFR promoter, as indicated, in an in vitro transcription assay containing all the basal transcription factors as well as the four NTPs and equivalent amounts of the various recombinant TFIIH complexes, as indicated at the top of the figure. The hyperphosphorylated (IIO) and the nonphosphorylated (IIA) forms of the RNA polymerase II were detected by Western blotting after SDS-PAGE separation. Quantification of the hyperphosphorylated form of RNA pol II phosphorylation assay from independent experiments was performed using Molecular Analyst software (Bio-Rad), and the values corresponding to the IIO/IIA ratio are shown.

(a) ATPase and kinase activities of the mutated MAT1-containing complexes. A, equivalent amounts of the various recombinant complexes were tested for ATPase activity. Pi generated upon ATP hydrolysis is indicated at the right of the panel. Quantification of the ATPase activity from three independent experiments was done using a Bio-imaging Analyzer FUJIX BAS 2000, and the relative activity P/(ATP + P) of each complex is shown. The value of autohydrolysis of ATP (lane 10) was subtracted for all values obtained. rIIH6, rIIH6XPD-K48R, and rIIH6XBP-K346R are recombinant complexes containing the six subunits of core TFIIH including the XPD or XBP subunits mutated in their ATP-binding site when indicated. B, the phosphorylation of RNA pol II was tested on the major late promoter (MLP) or DHFR promoter, as indicated, in an in vitro transcription assay containing all the basal transcription factors as well as the four NTPs and equivalent amounts of the various recombinant TFIIH complexes, as indicated at the top of the figure. The hyperphosphorylated (IIO) and the nonphosphorylated (IIA) forms of the RNA polymerase II were detected by Western blotting after SDS-PAGE separation. Quantification of the hyperphosphorylated form of RNA pol II phosphorylation assay from independent experiments was performed using Molecular Analyst software (Bio-Rad), and the values corresponding to the IIO/IIA ratio are shown.

A recombinant TFIIH containing the six core subunits (29). We also observed that transcription from the DHFR promoter was not only dependent on MAT1 but also of a fully active CAK (Fig. 4B, compare lanes 7 and 3), a point which was already observed previously (29, 39). Second, although associated with TFIIH, neither the N-terminal deleted MAT1 (MAT1-(66–309)) nor the RING-finger-mutated MAT1 (MAT1-C9Y and MAT1-C26S) stimulated RNA synthesis (Fig. 4B, compare lanes 4–6 with lane 3). We even noticed a slight inhibition of the transcription reaction, which probably reflects some difficulties of the mutated recombinant rIIH9 factor to associate with the transcription PIC. Finally, the addition of the various purified recombinant CAK harboring MAT1 mutations in transcription reaction containing an endogenous HeLa core TFIIH complex (13) reproduced to the same order of magnitude the transcription defects described above (data not shown). Altogether, these results highlight the crucial role of MAT1, and more specifically of its RING finger domain, in the transcription reaction.

The RING Finger of MAT1 Is Involved in CTD Phosphorylation of RNA pol II—Having observed the transcription defect due to MAT1 RING finger mutations, we then wondered if such mutations would affect XPD and XBP activities. Already knowing that MAT1 did not modulate either XPD or XBP helicase activities (data not shown and Ref. 30), we thus evaluated the ATPase activities of the various TFIIH complexes in the presence of DNA template (8).

First, the ATPase activity of the recombinant core TFIIH complex (rIIH6) is neither CAK- nor MAT1-dependent (Fig. 5A, compare lanes 4 and 5 with lane 1). Mutations or deletion of the RING finger of MAT1 did not modulate the ATPase activity of rIIH9 (Fig. 5A, compare lanes 6–8 with lane 5). As expected, the rIIH6XPD-K48R and rIIH6XBP-K346R complexes, harboring a mutation in the ATP-binding site of either XPD or XPB (40, 41), displayed a 50% inhibition in each case (Fig. 5A, compare lanes 2 and 3 with lane 1). It is worthwhile to note that the ATP-binding site mutation in XBP completely abolishes its promoter opening capability (29); similarly ATP-binding site mutation of XPD prevents its unwinding capacity (26). Moreover, mutation in cdk7 did not affect the ATPase activity (lane 9), a result which is in fact not surprising because transcription of rIIH9-cdk7-K41R occurred almost normally (Fig. 4). Thus, we could conclude that MAT1 is not involved in the ATP hydrolysis, a step which was suggested to furnish energy to the transcription reaction.

The above results suggested a role of MAT1 in stimulating basal transcription. Furthermore, mutations in its RING finger diminished the overall transcription activity of TFIIH. We then examined if this transcriptional deficiency could be correlated with the phosphorylation of the CTD of the largest subunit of RNA pol II. Therefore, the ability of rIIH6, wild-type rIIH9, or mutated rIIH9 to phosphorylate the CTD was investigated in an in vitro transcription system containing, in addition to RNA pol II, all the basal transcription factors and the major late promoter or DHFR as a linear template (Fig. 5B). All the components of the transcription machinery as well as the four NTPs are absolutely required for in vitro phosphorylation of RNA pol II CTD (16). Indeed, when TBP, which is essential for the assembly of the PIC, was omitted from the reaction mixture, CTD remained unphosphorylated (data not shown and Ref. 42). After classical in vitro transcription reaction performed in the presence of all the basal transcription factors as well as the four NTPs to allow the formation of the elongation complex, the phosphorylation of RNA pol II was visualized using an antibody raised against its large subunit (13). The presence of MAT1 increased the CTD phosphorylation independently of the promoter used (Fig. 5B, compare lanes 1 and 2 with lane 7). However, recombinant TFIIHs carrying deletions of the N terminus and mutations in the RING finger of MAT1 still promoted CTD phosphorylation, although to a lower level than with rIIH6 (Fig. 5B, compare lanes 3–5 with lanes 1 and 7). This is likely the result of some conformational changes that do not allow an accurate integration of these mutants within the PIC. It therefore seems clear that MAT1 modulates CTD phosphorylation, and this likely involves the RING finger.
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motif that could allow optimal positioning of cdk7 in the vicinity of the CTD tail of RNA pol II.

**DISCUSSION**

In the course of this study, we delineated the various functions of the MAT1 domains. As part of CAK, MAT1 stimulates the kinase activity through its C-terminal end. It anchors CAK to the core TFIIH likely through interactions with XPD and XPB helicases. When associated with TFIIH, MAT1 allows optimal transcription and RNA pol II phosphorylation through its N-terminal RING finger motif (Fig. 6A).

The cdk7 Activation Domain of MAT1 Resides in Its C Terminus—MAT1 was able to interact separately with either cdk7 or cyclin H and much more strongly when it was coexpressed with cdk7 and cyclin H. This situation is reminiscent of what was observed in the three-dimensional structure of the cdk2-cyclin Ap27Rip ternary complex in which the inhibitor binds both cdk2 and cyclin A (43). Keeping this in account, as well as the structure of the cdk2-cyclin A complex (44) and the x-ray structure of the cyclin H, we built a structural model containing both cyclin H and cdk7 (34). The model showed that a cleft was left vacant on one side of the binary cdk7-cyclin H complex and could be filled by MAT1 or any other regulatory subunit. We also observed that, in this model, MAT1 could bridge and stabilize the binding of the two subunits. The present data show that this binding mainly involves the C-terminal end of MAT1, in vitro and in vivo. However, the observation that the C-terminal domain was not able to interact separately with either cdk7 or cyclin H, whereas the entire MAT1 molecule could, suggests the participation of the N-terminal domain of MAT1. The N-terminal deletions may change the overall folding of the MAT1 protein, thus weakening the interaction. Because neither point mutation in the RING finger domain (MAT1-C9Y and MAT1-C26S) nor the N-terminal deletion (MAT1-66–309) altered the interaction between MAT1 and the cdk7-cyclin H complex, we concluded that the RING finger domain of MAT1 was not responsible for CAK assembly. Furthermore, the C-terminal portion of MAT1, which interacts with the cdk7-cyclin H binary complex, is responsible for the stimulation of the cdk7 kinase activity when cdk2 and ctd4 were used as substrates. This advises that, within CAK, the C-terminal domain of MAT1 is responsible for cdk7 activation (Fig. 6A), in agreement with previous in vitro studies demonstrating that abrogation of MAT1 RING finger does not affect the CAK kinase activity (6). This, in addition to previous studies that demonstrated that truncation of the 22 C-terminal amino acids of Tb3/Rig2 (MAT1 yeast counterpart) was lethal (45), suggests a crucial role of MAT1 in the regulation of the cdk7 kinase and related activities.

The Coiled-Coil Domain of MAT1 Interacts with XPD and XPB—Because CAK exists also as part of the transcription/DNA repair factor TFIIH, the next question concerned the mechanism, which allows the CAK complex to anchor the core TFIIH. Our results show that this binding is mediated by XPD and XPB, the two largest subunits of TFIIH. Moreover, the characterization of the CAKXPD subcomplex (9, 10), as well as the fact that MAT1 could not stimulate the transcription activity of the core TFIIH in the absence of XPD, suggested that the anchoring of CAK in TFIIH is likely to be mediated by the XPD helicase. This assumption was backed up by the observation that a purified TFIIH factor, from an XPD patient cell line, harboring a natural mutation in XPD that alters the interaction with p44, another subunit of the core, was found to partially lack the CAKXPD complex (30). In light of the present data, it is then possible to draw a model in which CAK binds the core of TFIIH through an interaction with XPD. On one hand, CAK would bind XPD through the median portion of MAT1 (Fig. 6A), whereas XPD would carry CAK to the core TFIIH through an interaction with the p44 subunit (46). It is not excluded that, in such a model (Fig. 6B), the stabilization of CAK with the core of TFIIH might also be enhanced by XPB-MAT1 (the present study) or XPB-cdk7 (13) interactions as suggested by the fact that a recombinant rIIH8 complex, lacking MAT1, could be isolated (Fig. 4).

The Crucial Role of MAT1 RING finger in Transcription—The present study also points out the role of MAT1 in transcription. A role of MAT1 in transcription regulation had already been suggested by previous studies showing that, in yeast, antisera against Tb3/Rig2 partially inhibits RNA pol II transcription in vitro (12) and that a temperature-sensitive mutant of Tb3/Rig2 is defective for transcription at the non-permissive temperature (47). We observed that MAT1 mutations in the RING finger domain completely abolish its function in transcription. Indeed, TFIIH complexes containing mutated MAT1, or in which MAT1 is absent, exhibit weak transcription activities. The presence of MAT1 within TFIIH increased 8–10-fold the transcription activity independently of the nature of the promoter. This situation is reminiscent of the stimulation of the transcription activity of the recombinant core TFIIH (containing the six subunits: XPB, XPD, p62, p52, p44, and p34) by CAK, when added in an in vitro transcription system (29).

It was tempting to attribute the weak transcription activity of TFIIH complexes carrying point mutations in the RING finger of MAT1 to a weak kinase activity toward CTD of the largest subunit of RNA pol II. However, this seems to be unlikely. Indeed, mutations in the RING finger drastically decreases the transcription activity of the corresponding TFIIH factor, whereas the CTD phosphorylation is reduced to a much lesser extent (compare histograms of Figs. 4B and 5B). Moreover, the reduction of RNA pol II phosphorylation observed in the presence of all the basal transcription factors, in addition to TFIIHs either carrying RING finger mutations or lacking MAT1, is not because of a drop of cdk7 kinase activity itself. In
our simple enzymatic assay, the RING finger mutations do not affect the activity of cdk7 to phosphorylate the synthetic cdtd oligopeptide or cdkt2, whether cdkt7 is part of CAK (this study) or TFIIF (data not shown). This suggests an additional role of MAT1, and more particularly of its RING finger, in the transcription reaction. It should be kept in mind, however, that phosphorylation of the CTD could also occur in the absence of promoter opening, which is a prerequisite for RNA synthesis (29).

One can hypothesize that the MAT1 RING finger, as part of TFIIF, would position the transcription factors around the promoter to allow optimal phosphorylation of RNA pol II CTD to regulate some events of the transcription reaction. This model is consistent with the current idea that RING fingers would mediate protein-protein interactions and function in formation of large macromolecular scaffolds (48). At the present state of our investigations, we failed to show interactions between the RING finger of MAT1, when part of either CAK or TFIIF, and the other basal transcription factors and RNA pol II in baculovirus coinfection experiments.3

**MAT1 Has Diverged through Evolution—Advances in the understanding of the role of TFIIF in both transcription and DNA repair result from parallel studies performed in yeast as well as in human (11). These studies nevertheless point out some discrepancies mainly concerning the kinase complex associated with TFIIF. In *Saccharomyces cerevisiae*, TFIIF phosphorylates the CTD and this phosphorylation is required for *in vivo* transcription of the majority of protein encoding genes (49–51). This kinase activity is mediated by Kin28 (the homologue of cdkt7), which interacts with Ccl1 (the homologue of the cyclin H to form the TFIIF complex (49, 52). However, in contrast to mammalian cells, the TFIIF complex does not contain Tbd3/Rig2 (the homologue of MAT1), which was found to be preferentially associated with the core TFIIF rather than with the kinase subunit.4 Furthermore, the TFIIF complex has no kinase activity toward cdks. Careful analysis of the primary sequence of MAT1 homologues (Table I) points out that the C-terminal portion of human MAT1, which interacts with the cdkt7/cyclin H binary complex, is poorly conserved in *S. cerevisiae* (25% similarity) and in Schizosaccharomyces pombe (28%) as compared with Xenopus laevis (76%) and Drosophila melanogaster (64%). In contrast, the central portion, which interacts with the core TFIIF, seems more conserved between these five species (38% and 35% similarity between human and both yeasts). This may explain why Tbd3/Rig2 is on one hand poorly associated to the TFIIF complex and on the other hand a subunit of the core TFIIF. These data, in addition to the large conservation of the RING finger sequence between *S. cerevisiae* and human (70% similarity), suggest that Tbd3/Rig2 might have the same functions as MAT1 within the TFIIF factor. It seems possible that during evolution, the C-terminal portion of MAT1 has diverged, resulting in CAK activities. It cannot be excluded; however, that the addition of an excess of Tbd3/Rig2 may restore the “CAK activity” to TFIIF, a point that was not kept in account in previous studies.

The striking feature of this study is that MAT1 is not only a CAK assembly factor, as originally mentioned, but also plays an important role in the transcription reaction. We have characterized the different domains of MAT1 implicated in the architecture of the TFIIF complex, which regulate some enzymatic activities of the PIC. In conclusion, we propose that the C-terminal end of MAT1 regulates cdkt7 kinase activity itself within the CAK complex, whereas the RING finger motif will preferably play a role within the transcription complex. To complete functional analysis of MAT1 RING finger domain, resolution of its solution structure by NMR methods is under way. It will be interesting to compare these structural data with those obtained for the two point mutants in order to establish structure-function relationships.

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