Substrate specificity of the cdk-activating kinase (CAK) is altered upon association with TFIIF

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The transcription/DNA repair factor TFIIF consists of nine subunits, several exhibiting known functions: helicase/ATPase, kinase activity and DNA binding. Three subunits of TFIIF, cdk7, cyclin H and MAT1, form a ternary complex, cdk-activating kinase (CAK), found either on its own or as part of TFIIF. In the present work, we demonstrate that purified human CAK complex (free CAK) and recombinant CAK (rCAK) produced in insect cells exhibit a strong preference for the cyclin-dependent kinase 2 (cdk2) over a ctd oligopeptide substrate (which mimics the carboxy-terminal domain of the RNA polymerase II). In contrast, TFIIF preferentially phosphorylates the ctd as well as TFIIE α , but not cdk2. TFIIF was resolved into four subcomplexes: the kinase complex composed of cdk7, cyclin H and MAT1; the core TFIIF which contains XPB, p62, p52, p44 and p34; and two other subcomplexes in which XPD is found associated with either the kinase complex or with the core TFIIF. Using these fractions, we demonstrate that TFIIF lacking the CAK subcomplex completely recovers its transcriptional activity in the presence of free CAK. Furthermore, studies examining the interactions between TFIIF subunits provide evidence that CAK is integrated within TFIIF via XPB and XPD.

Keywords: CAK/cdk7/CTD kinase/TFIIF/transcription

Introduction

TFIIF was the first of the basal transcription factors shown to play a role in cellular activities other than expression of protein-coding genes (reviewed in Hoeijmakers *et al.*, 1996; Moncollin *et al.*, 1997). This may be attributed in part to its multisubunit composition of at least nine peptides, XPB, XPD, p62, p52, p44, cdk7, cyclin H, p34 and MAT1. The two helicase subunits (XPB and XPD) of TFIIF are known to play a role in DNA nucleotide excision repair, thereby providing evidence that transcription is intimately coupled to DNA repair (Schaeffer *et al.*, 1993, 1994; Sung *et al.*, 1993). The importance of this link is evident in three transcription/repair syndromes; xeroderma pigmentosum, Cockayne syndrome and trichothiodystrophy, which are due to defects in genes encoding TFIIF subunits (Vermeulen *et al.*, 1994; Broughton *et al.*, 1995; Hoeijmakers *et al.*, 1996).

More recently, cdk7, a kinase known to have a role in the cell cycle, was identified as a component of TFIIF (Roy *et al.*, 1994; Feaver *et al.*, 1994; Shiekhataar *et al.*, 1995; Serizawa *et al.*, 1995). This kinase belongs to a family of cyclin-dependent kinases (cdks) which are key regulatory components that coordinate numerous events such as cell cycle progression, DNA replication and transcription. Cdk activity is controlled by transient association with a specific cyclin, a regulatory subunit or inhibitory protein and activated by phosphorylation in its T-loop domain by a cdk-activating kinase (CAK) (reviewed in Morgan, 1995). Cdk7 forms a ternary complex with cyclin H (Fisher and Morgan, 1994; Mäkelä *et al.*, 1994; Tassan *et al.*, 1994) and MAT1 (Devault *et al.*, 1995; Fisher *et al.*, 1995; Tassan *et al.*, 1995; Adamczewski *et al.*, 1996), and together they form the CAK responsible for activating cdk1, cdk2 and cdk4 (reviewed in Nigg, 1996). The presence of these kinase subunits in TFIIF suggests a role in transcription (Roy *et al.*, 1994; Fisher *et al.*, 1995; Serizawa *et al.*, 1995; Shiekhataar *et al.*, 1995; Adamczewski *et al.*, 1996).

The kinase activity of TFIIF is directed towards the carboxy-terminal domain (CTD) of the largest subunit of the RNA polymerase II (RNA pol II) (Feaver *et al.*, 1991; Lu *et al.*, 1992; Serizawa *et al.*, 1992). This phylogenetically highly conserved domain consists of a heptapeptide sequence (YSPTSPS) tandemly repeated up to 52 times. Phosphorylation of the CTD initiates promoter clearance and transcription elongation (Goodrich and Tjian, 1994; Dahmus, 1995). This includes the dissociation of pre-initiation complex proteins. The fact that both the TATA-binding protein (TBP) and TFIIE (Usheva *et al.*, 1992; Maxon *et al.*, 1994) interact with the non-phosphorylated but not with the phosphorylated form of the RNA pol II supports this idea. The cdk7 kinase can phosphorylate a synthetic ctd oligopeptide (Roy *et al.*, 1994) and is therefore thought to be the kinase responsible for the phosphorylation of the RNA pol II CTD. This is supported by *in vivo* studies in yeast with thermosensitive mutants of Kin28 and Ccl1, the yeast homologue of cdk7 and cyclin H respectively (Feaver *et al.*, 1991; Cismowski *et al.*, 1995; Svejstrup *et al.*, 1996), which demonstrate that these peptides are required for both RNA pol II phosphorylation and transcription (Valay *et al.*, 1995). However, TFIIF containing inactivated CAK complex can support transcription *in vitro* (Mäkelä *et al.*, 1995). Moreover, depending on the promoter, CTD and its phosphorylation are dispensable for transcription *in vitro*, but indispensable *in vivo* (Dahmus, 1995; Gerber *et al.*, 1995).

We have demonstrated that the TFIIF-associated kinase complex (cdk7, cyclin H and MAT1) is UV irradiation sensitive but that the non-associated CAK complex (hereafter referred to as free CAK, Adamczewski *et al.*, 1996)

is not, suggesting for the first time that cdk7 behaves differently when it is associated with TFIIF. This prompted us to investigate further the possible differences in roles of free CAK versus TFIIF-associated CAK. In this study, we have purified from HeLa cell whole cell extract free CAK composed of cdk7, cyclin H and MAT1, and from baculovirus-infected Sf9 insect cells, a recombinant kinase complex (rCAK). By characterizing their activities, we demonstrate that free CAK, rCAK and TFIIF exhibit different substrate specificities towards a synthetic oligopeptide mimicking the CTD (referred to as ctd) and cdk2, suggesting differential functions dependent on the composition of these complexes. This is supported further in a TFIIF-dependent transcription system, where only the entire TFIIF is capable of phosphorylating the CTD of the RNA pol II and supporting RNA synthesis. As the organization of CAK in TFIIF seems to influence its kinase activity, we characterized several TFIIF subcomplexes and defined the interactions of the kinase complex subunits with the remaining subunits of TFIIF.

Results

Purification of a CAK complex free of TFIIF

To purify the kinase complex (free CAK) composed of cdk7, cyclin H and MAT1, HeLa cell whole cell extract was fractionated on heparin Ultrogel, Sulfopropyl and DEAE columns (see Figure 1A and Materials and methods). The purification of a kinase complex free of TFIIF was followed by Western blot analysis as well as by a ctd kinase assay in which a synthetic peptide (ctd) was used as a substrate (data not shown and Figure 1C). The DEAE-eluted fractions were purified further with antibody against cdk7 (Ab-cdk7) cross-linked to protein A-Sepharose and the elution was performed with an excess of the corresponding epitope peptide (Figure 1A). The eluted fraction contains the three polypeptides, cdk7, cyclin H and MAT1 (Figure 1B and C, WB, lanes 2). The contaminant bands observed at the top of the gel (Figure 1B) are either bovine serum albumin (BSA; 70 kDa), since protein A-Sepharose cross-linked to Ab-cdk7 was saturated with BSA before use, or are artefacts which originate from the silver staining procedure (50–60 kDa). Neither p62 nor XPD was detected in this fraction (Figure 1C, WB, compare lanes 1 and 2) indicating the absence of TFIIF. Furthermore, this complex possesses a ctd kinase activity (Figure 1C, Kinase, lane 2). Together, our results demonstrate that cdk7, cyclin H and MAT1 are associated to form a ternary complex distinct from TFIIF, that possesses a ctd kinase activity. Moreover, the stoichiometry of the three kinase subunits is conserved between TFIIF and the free CAK complex.

Production of a recombinant CAK complex

To study further the properties of the CAK complex and its interaction with the other TFIIF subunits, we reconstituted a recombinant CAK (hereafter referred to as rCAK) composed of cdk7, cyclin H and MAT1, in a baculovirus expression vector system. Sf9 insect cells were co-infected with three recombinant baculoviruses encoding cdk7, MAT1 and a histidine-tagged cyclin H (His-cyclin H). The rCAK was purified from the crude Sf9 cell extract via the histidine-tagged cyclin H, using

Fig. 1. (A) Scheme of purification of human free CAK and TFIIF. WCE, whole cell extract; AS, ammonium sulfate; PO₄, phosphate buffer. The free CAK last step of purification consists of an immunopurification followed by an elution with an excess of cdk7 carboxy-terminal peptide [amino acids 312–329; Pep(312–329)], mimicking the Ab-cdk7 epitope. (B) The eluted fraction was resolved by SDS-PAGE and the gel was silver stained. The position of the molecular weight markers (MW) in kiloDaltons is shown. (C) Free CAK fraction was also analysed by Western blot (WB) and tested for its ctd kinase activity. Cdk7, cyclin H and MAT1 polypeptides are indicated. A purified TFIIF fraction (lane 1) was used as a positive control. Ctd4 indicates the synthetic peptide mimicking four repeats of the CTD of the largest subunit of the RNA polymerase II.

nickel chelate affinity chromatography. The eluted fractions were immunoprecipitated with Ab-cdk7 cross-linked to protein A-Sepharose and the elution was performed with an excess of the corresponding epitope peptide (Figure 2A). The eluted fraction was resolved by SDS-PAGE and analysed by Coomassie staining and Western blot (Figure 2B). As shown in Figure 2B, the eluted fraction contains a highly purified complex composed of the three subunits, cdk7, His-cyclin H and MAT1, which is resolved into two major bands; the first band corresponds to the co-migration of cdk7 (40 kDa) (Figure 2B, lanes 2 and 4) and His-cyclin H (39.8 kDa) (Figure 2B, lanes 2

Fig. 2. (A) Scheme of production and purification of the recombinant CAK (rCAK) from Sf9 cells co-infected with baculovirus encoding cdk7, His-cyclin H and MAT1 respectively. In the last step of the purification, rCAK was eluted with an excess of cdk7 carboxy-terminal peptide [Pep(312–329)]. (B) The purified rCAK was analysed by SDS–PAGE followed by either Coomassie blue staining or Western blot (WB). Since cdk7 and His-cyclin H have the same apparent molecular weight, these two polypeptides were revealed separately. Cdk7, His-cyclin H and MAT1 polypeptides are indicated. The position of the molecular weight markers (MW) in kiloDaltons is shown.

and 3), and the second band corresponds to MAT1 (32 kDa) (lanes 2 and 4).

Free CAK and rCAK phosphorylate TBP

To characterize the kinase activity further, we first compared the behaviour of TFIIF, free CAK and rCAK. The nucleotide specificities of the three kinases are identical: ATP, dATP and GTP all act as cofactors for the kinase activity towards ctd or cdk2 in competition assays, while CTP or cAMP had no effect on the phosphorylation of ctd or cdk2 (Roy *et al.*, 1994; data not shown). All three kinases are similarly inhibited, with IC_{50} values between 10 and 50 μ M, by 5,6 dichloro-1- β -D-ribofuranosylbenzimidazole (DRB), a known inhibitor of the phosphorylation of RNA pol II CTD *in vivo* (Dubois *et al.*, 1994) and TFIIF kinase activity (Yankulov *et al.*, 1995). These results indicate that TFIIF, free CAK and rCAK have the same nucleotide specificity and respond similarly to DRB.

To determine whether free and rCAK were able to phosphorylate known substrates of TFIIF, kinase assays were carried out using the recombinant transcription factors TFIIE α and TBP (Ohkuma and Roeder, 1994; Roy *et al.*, 1994; Yankulov *et al.*, 1995). TFIIF, free CAK and rCAK phosphorylate TBP (Figure 3, Kinase, TBP), whereas TFIIE α is only phosphorylated by TFIIF (Figure 3, Kinase, TFIIE α , lane 1) and not by free CAK and

Fig. 3. Comparison of kinase activities between TFIIF, free CAK and rCAK. The cdk7 concentration of each purified fraction (TFIIF: HAP fraction, free CAK) was estimated by Western blot analysis, and adjusted to equivalent values for the kinase assays. Recombinant substrates TFIIE α (100 ng) and TBP (100 ng), overexpressed and purified from *Escherichia coli*, were tested in the kinase assay (Kinase). The phosphorylation of histone H1 (1 μ g) was tested in a CAK assay (CAK).

rCAK (lanes 2 and 3). To ensure that the three kinase complexes (TFIIF, free CAK and rCAK) were functional, we tested their ability to activate cdk2 in a histone H1 kinase assay. Free CAK (lane 2) and rCAK (lane 3) as well as TFIIF (lane 1) phosphorylate cdk2 and subsequently activate its ability to phosphorylate histone H1 (Figure 3, CAK). Together, these results suggest that CAK gains TFIIE α substrate specificity when associated with TFIIF, without losing its cdk-activating kinase activity. However, we could not exclude the possibility that an unidentified kinase tightly associated with TFIIF could also be responsible for TFIIE α phosphorylation.

CTD versus cdk2 substrate specificity of free or TFIIF-associated CAK

We demonstrated that UV irradiation of living cells partially inhibits TFIIF kinase activity whereas the free CAK is not affected, indicating a potential specificity in the respective enzyme function (Adamczewski *et al.*, 1996). This prompted us to test if, as a function of its state (either free or TFIIF-associated), the kinase complex exhibits a substrate specificity. This study was carried out using an equimolar mixture of cdk2, a substrate implicated in the regulation of the cell cycle, and ctd, a substrate important in transcription. The cdk7 concentration of each purified fraction was estimated by Western blot analysis. The cdk7 concentration of TFIIF and free CAK was adjusted to equivalent values for the following kinase assays. To ensure that the phosphorylated band observed corresponds to cdk2, the gel was Coomassie stained and the radioactive band superimposed with the cdk2 band on the protein gel. Both free CAK and rCAK exhibited a preference for cdk2 over the ctd substrate (Figure 4A, lanes 2 and 3, compare cdk2 with ctd). Quantification of the radioactive signals and calculation of cdk2/ctd ratios illustrate an ~3-fold higher efficiency in cdk2 phosphorylation by both free and rCAK, in our *in vitro* experimental conditions (Figure 4A, see cdk2/ctd4 values at the bottom of the panel). In these conditions, we noticed that TFIIF has a preference for the ctd peptide (Figure 4A, lane 1).

Given the preference of the free CAK towards cdk2, we investigated the ability of the three kinase complexes

Fig. 4. Substrate preferences of CAK versus TFIIF. (A) Differential phosphorylation of cdk2 and ctd4 by TFIIF, free CAK and rCAK. The kinase activity of TFIIF, free CAK and rCAK was assayed using a mixture containing both oligopeptide ctd4 (1 μ g) and GST-cdk2 (50 ng). Quantification of the radioactive signals was performed using a PhosphorImager, and calculations of the cdk2/ctd ratios are indicated at the bottom of the figure. (B) The phosphorylation of RNA pol II (Kinase) was tested in an *in vitro* transcription assay containing all the basal transcription factors and, when indicated (at the top of the figure), TFIIF (HAP fraction), free CAK and rCAK. IIA and IIO are designated as the non-phosphorylated and the hyperphosphorylated forms of RNA pol II respectively. The production of the 309 nucleotide transcript (309 nt) was visualized in a transcription assay (Transcription).

to phosphorylate the CTD of the RNA pol II, a natural substrate of TFIIF (Lu *et al.*, 1992; Serizawa *et al.*, 1992). Knowing that the CTD phosphorylation was greatly enhanced when RNA pol II was integrated in the transcription complex, we set up the following assay. The phosphorylation of RNA pol II was investigated in an *in vitro* reconstituted transcription assay containing, in addition to RNA pol II, the general transcription factors TFIIA, TFIIB, TBP, TFIIE and TFIIIF, a linear DNA template containing the adenovirus major late promoter (AdMLP) and (as indicated at the top of Figure 4B) either TFIIF, free CAK or rCAK. The phosphorylation of RNA pol II was visualized by Western blot analysis using an antibody raised against its largest subunit (Dubois *et al.*, 1994, see also Materials and methods). Only the TFIIF fraction resulted in the shift from the lower molecular weight RNA pol IIA (IIA, the non-phosphorylated form), to the higher molecular weight RNA pol IIO (IIO, the hyperphosphorylated form, Figure 4B, lane 1). This shift is due exclusively to TFIIF and not to some contamination present in the other transcription factors (lane 4). Although free CAK and rCAK use ctd synthetic peptide as a substrate (Figure 4A), neither of them lead to this shift (Figure 4B, lanes 2 and 3). When the phosphorylation of the RNA pol II was

analysed under the same conditions but in the presence of radiolabelled ATP, incorporation of 32 P was observed only in the presence of TFIIF. The labelled band corresponded to the IIO form of the RNA pol II (data not shown). To investigate whether the presence of transcription factors and DNA template could inhibit the free CAK and rCAK CTD kinase activity, the RNA pol II phosphorylation was tested in a simple kinase assay devoid of any basal transcription factors and DNA template. Under these conditions, only incubation with TFIIF leads to the IIO form of the RNA pol II, although at a very low level, whereas incubation with free CAK and rCAK do not (data not shown). Production of the AdMLP-specific transcript was detected only in the presence of TFIIF (lane 1) but not with the free CAK (lane 2) or rCAK fractions (lane 3) (Figure 4B, Transcription). These results demonstrate that CAK does not support transcription in the absence of the other TFIIF subunits. Together, our data show that CAK requires an association with TFIIF to phosphorylate the CTD of the RNA pol II and to play a role in transcription.

TFIIF can be resolved into different subcomplexes

Glycerol gradient analysis performed under high salt conditions indicated that the ternary kinase complex containing cdk7, cyclin H and MAT1 could be resolved from TFIIF (Adamczewski *et al.*, 1996). We have also observed that XPD could be partially dissociated from the other TFIIF subunits (Roy *et al.*, 1994; Schaeffer *et al.*, 1994). To characterize the organization of TFIIF further, we set up the following assay according to the scheme outlined in Figure 5A. A purified TFIIF fraction was treated with high salt (1.2 M KCl) and then immunoprecipitated in a buffer containing 1.2 M KCl, with an excess of either Ab-cdk7, Ab-p62 or Ab-XPD (see Materials and methods). The corresponding supernatant (Sn) and the proteins adsorbed on the three immunoadsorbents (Bd) were analysed by Western blot. The supernatant from a fraction immunoprecipitated with Ab-cdk7 (Sn/Ab-cdk7) contained all the TFIIF subunits, XPB, some XPD, p62, p52, p44 and p34, but not cdk7, cyclin H or MAT1 (Figure 5B, compare lanes 1 and 2). Analysis of the proteins bound to Ab-cdk7 cross-linked to protein A-Sepharose (Bd/Ab-cdk7) demonstrated that Ab-cdk7 immunoprecipitates not only cdk7, cyclin H and MAT1 but also some XPD (Figure 5C, lane 2). This indicates that Ab-cdk7 is able to immunoprecipitate two subcomplexes: one containing, in addition to cdk7, the two subunits of the CAK complex, cyclin H and MAT1, and a second complex, CAK-XPD, which contains the three CAK subunits in addition to a fourth subunit XPD. Analysis of the Sn/Ab-p62 fraction revealed the absence of not only p62 but also p52, p44, p34 and the majority of XPB (Figure 5B, lane 3), thus demonstrating that these five subunits strongly interact with each other. It should be noted that part of XPD also immunoprecipitates with the five other subunits (XPB, p62, p52, p44 and p34; see Figure 5C, lane 3). These results indicate a tight association between XPB, p62, p52, p44 and p34, making up a large complex also called core TFIIF. In the Sn/Ab-XPD fraction, only XPD was missing (Figure 5B, lane 4). Furthermore, Ab-XPD immunoprecipitates cdk7, a subunit belonging to the CAK complex, as well as p62, a subunit of the core TFIIF

Fig. 5. Analysis of TFIIH subcomplexes. (A) Scheme of TFIIH dissociation by 1.2 M KCl treatment and immunoprecipitation with Ab-cdk7, Ab-p62 or Ab-XPD. (B) The supernatants (Sn) were collected and analysed by Western blot for the presence of all TFIIH subunits. (C) The proteins remaining on the beads (Bd) after extensive washing were analysed by Western blot using Ab-XPD, Ab-p62 (subunit of the core TFIIH) and Ab-cdk7 (subunit of the kinase complex).

(Figure 5C, lane 4). Together, these results indicate that TFIIH can be resolved into two major complexes: the core TFIIH, which contains XPB, p62, p52, p44 and p34, and the CAK complex, which contains cdk7, cyclin H and MAT1. XPD is found in two subcomplexes, associated with the core of TFIIH (core TFIIH–XPD) and with the CAK complex (CAK–XPD).

Protein–protein interactions between the kinase complex and other subunits of TFIIH

To understand how the CAK complex is integrated into TFIIH, we identified the interactions between the kinase complex subunits and the other subunits of TFIIH. The cDNAs encoding XPB, XPD, p62, p44, p34, cdk7, cyclin H and MAT1 were inserted into baculovirus expression vectors (see Materials and methods). Each subunit of TFIIH, alone or in combination with each of the kinase complex subunits, was co-expressed in Sf9 cells. Sf9 cell crude extracts were made and protein expression tested by Western blot analysis using the appropriate antibodies raised against TFIIH polypeptides (data not shown). Immunoprecipitation with Ab-cdk7 bound to protein A–Sepharose was performed on crude extracts containing either cdk7 with the TFIIH subunit being tested (XPB, XPD, p62, p44, p34, cyclin H and MAT1; Figure 6, lanes 2, 4, 6, 8, 10, 12, 14 and 15) or, as a negative control, the subunit alone (lanes 1, 3, 5, 7, 9, 11 and 13). The interactions between cyclin H or MAT1 and the TFIIH subunits were also carried out according to the same scheme presented for cdk7 in Figure 6, and the results obtained are summarized in Table I. Data obtained from at least four sets of independent experiments allow us to distinguish three categories of interaction: +, – and +/- . A (+) indicates an interaction reflecting similar levels of each protein tested on a Western blot (for example see Figure 6, lane 2), a (–) indicates no detectable interaction (lane 10) and a (+/-) reflects a weak interaction, indicated by the presence of a strong signal for one partner and a weak signal for the second protein (lane 6). These experi-

ments demonstrate that cdk7 interacts not only with cyclin H as previously observed (Fisher and Morgan, 1994; Tassan *et al.*, 1995; Adamczewski *et al.*, 1996) but also with MAT1 (Figure 6, lanes 13 and 15), whereas no interaction was detected between cyclin H and MAT1. Interactions were also detected between cdk7 and XPB, MAT1 and XPB, and MAT1 and XPD (Figure 6, lanes 2 and Table I). We also detected a weak interaction between cdk7 and p62, cdk7 and p44, and cyclin H and XPB (Figure 6, lanes 6 and 8 and Table I). Since the level of human recombinant TFIIH subunits overexpressed in Sf9 cells greatly exceeds the concentration of endogenously expressed TFIIH subunits (data not shown), the interactions observed are most likely not mediated by insect cell proteins and thus could be considered as direct interactions. Note that the same interactions were observed when individually expressed TFIIH subunits were incubated together before immunoprecipitation (data not shown).

Knowing that cdk7, cyclin H and MAT1 form a ternary complex which is part of TFIIH, we also investigated the interactions between this ternary complex and the other subunits of TFIIH *in vitro*. The kinase complex (cdk7, cyclin H and MAT1) was produced in Sf9 cells as was either XPB, XPD, p62, p44 or p34. Crude cell extracts containing approximately equal amounts of the kinase complex and of the additional subunit being tested were mixed, pre-incubated and immunoprecipitated with Ab-cdk7 (Table I). Similar results were also obtained when the immunoprecipitations were performed with the antibodies raised against the other subunits being tested (Table I). The interaction between the kinase complex and the other TFIIH subunits takes place via the two helicases, XPB and XPD, and also, but at a very low level, via two subunits of the core TFIIH, p62 and p44.

Free CAK and rCAK restore the transcription activity of TFIIH devoid of CAK

We subsequently investigated the effect of the CAK complex on TFIIH transcription activity. A highly purified

Fig. 6. Interaction of cdk7 with the other subunits of TFIIH. Sf9 cells were (co)infected with recombinant baculoviruses expressing TFIIH subunits individually or in combination with cdk7 as indicated at the top of the figure. Sf9 crude extracts were made and immunoprecipitated using Ab-cdk7 cross-linked to protein A–Sepharose. After extensive washing, the proteins absorbed were then analysed by Western blot with antibodies generated towards the various TFIIH subunits. TFIIH fractions (M) were run in parallel in order to determine the expected migration of the various TFIIH subunits. Since His-cyclin H (lane 14) and cdk7 (lane 15) have the same apparent molecular weight, these two polypeptides were immunoblotted separately.

reconstituted transcription assay lacking TFIIH, in which a DNA containing the AdMLP serves as template, was used. Transcription was performed with a complete TFIIH or with a TFIIH lacking CAK (Sn/Ab-cdk7; Figure 5B, lane 2). The transcription activity of TFIIH lacking the CAK complex was decreased compared with the activity of the complete TFIIH (Figure 7A, compare lanes 1 and 2). Interestingly, when either free CAK or rCAK is added to the TFIIH lacking the CAK, the transcription activity is almost completely restored (Figure 7A, compare lane 1 with lanes 5 and 6). Quantification of the radioactive signals demonstrates that TFIIH devoid of CAK possesses 30% of the normal TFIIH transcription activity, and addition of either free CAK or rCAK allows the recovery of 80% of this activity. The residual transcription activity observed with TFIIH lacking CAK is not due to the presence of residual CAK since the Sn/Ab-cdk7 containing the TFIIH devoid of CAK does not exhibit any kinase activity towards the ctd oligopeptide (Figure 7B, compare lanes 1 and 2). Together, these results demonstrate: first, that a TFIIH lacking CAK can support *in vitro* transcription from the AdMLP; second, that although not absolutely necessary for the transcriptional activity of TFIIH, CAK enhances this activity; third, that both free CAK and rCAK could restore the TFIIH activity; and, finally, since the addition of either free CAK and rCAK to the entire TFIIH does not affect its transcription activity (data not shown), the activation observed is certainly due to the integration of the CAK complex in TFIIH.

Discussion

Purification of free CAK

As indicated by previous studies (Adamczewski *et al.*, 1996; Drapkin *et al.*, 1996; Yankulov and Bentley, 1997), CAK exists in at least two distinct forms, either free or as part of the transcription/DNA repair factor TFIIH. We show here the purification of free CAK which contains the three subunits cdk7, cyclin H and MAT1 and possesses a kinase activity towards substrates such as cdk2 and the synthetic oligopeptide ctd4. No other TFIIH subunits were shown to be present in this fraction. Because of the relatively low levels of recovery of free CAK from HeLa cell extracts, we have designed a protocol for overexpression and purification of a recombinant CAK

Table I. Qualitative summary of the interactions between the kinase complex subunits themselves and the subunits of TFIIH

	Cdk7	Cyclin H	MAT1	rCAK
XPB	+	+/-	+ ^a	+
XPD	-	-	+	+
p62	+/-	-	-	+/-
p44	+/-	-	-	+/-
Cdk7	nt	+ ^a	+	nt
Cyclin H	+ ^a	nt	-	nt
p34	-	-	-	-
MAT1	+	-	nt	nt

The averages of at least four independent experiments, similar to the one presented in Figure 6, are shown. +, +/- and - correspond to detected, very weak (but detectable) and no interaction between the kinase subunits and TFIIH subunits. nt, indicates that the interaction was not tested.

^aThe interaction was also positive in the two-hybrid system.

Fig. 7. Free CAK and rCAK restore the transcription activity of a TFIIH devoid of CAK. The p62 concentration of the TFIIH fraction and of the Sn/Ab-cdk7 fraction was estimated by Western blot analysis, and adjusted to equivalent values. (A) Transcription reactions were carried out in the presence of complete TFIIH, TFIIH lacking CAK (Sn/Ab-cdk7), free CAK or rCAK or in the absence of TFIIH (-). The AdMLP-specific transcript is indicated (309 nt). (B) The kinase activity of TFIIH and of the Sn/Ab-cdk7 fraction was assayed using the oligopeptide ctd4 (1 µg).

complex. Overexpression was performed by co-infecting insect Sf9 cells with baculoviruses encoding each of the subunits of the CAK complex; one of them cyclin H, was His tagged to facilitate purification. Under these conditions, we were able to overproduce a highly purified rCAK complex that was stoichiometrically identical to TFIIH and free CAK and also exhibited kinase activity towards ctd and cdk2 substrates. Characterization of free

CAK and rCAK kinase activities demonstrates that both complexes exhibit the same nucleotide specificity as TFIIF; they preferentially use ATP and are similarly inhibited by DRB, an inhibitor of RNA pol II phosphorylation.

Tentative 'design' of the TFIIF complex

Using normal chromatographic procedures, we and others have isolated several TFIIF-derived complexes (Gérard *et al.*, 1991; Adamczewski *et al.*, 1996; Drapkin *et al.*, 1996; Reardon *et al.*, 1996; Yankulov and Bentley, 1997; this study). These include CAK [which contains three polypeptides: cyclin H, the regulatory subunit, and MAT1, the stimulatory subunit (Adamczewski *et al.*, 1996), bound to cdk7], a CAK–XPD complex, the core TFIIF (also named TFIIF*, Reardon *et al.*, 1996) containing the five subunits: XPB, p62, p52 (previously named p41; Marinoni *et al.*, 1997), p44 and p34, and the whole TFIIF containing all nine subunits (Roy *et al.*, 1994). It cannot be excluded that some of these complexes could be generated by the purification process itself. Indeed, under high salt concentration, TFIIF can be dissociated into several subcomplexes: CAK, CAK–XPD, core TFIIF and core TFIIF–XPD (see also Schaeffer *et al.*, 1994).

TFIIF subcomplexes have also been observed in yeast. One, identified as TFIIFK, contains only the two subunits Kin28 and Ccl1, the homologues of the human cdk7 and cyclin H respectively, but lacks Tfb3, the yeast homologue of MAT1 (Svejstrup *et al.*, 1996; W.J.Feaver and R.D.Kornberg, personal communication). Furthermore, in early descriptions of yeast TFIIF, there was some controversy concerning the presence of Ssl2/XPB as a component (Feaver *et al.*, 1994). This subunit subsequently was confirmed as being part of TFIIF (Svejstrup *et al.*, 1995) but illustrates the difficulty in defining a multiprotein complex using various purification steps. Together, these studies led us to hypothesize that there are at least two CAK-containing complexes that play a role in the cell: free CAK and TFIIF. The others (CAK–XPD, core TFIIF–XPD and core TFIIF) could be either functional subcomplexes, transient subcomplexes and/or breakdown subcomplexes.

How does CAK, which is found free in the cell, interact with the other TFIIF subunits? It seems that XPD could be one of the bridging subunits between the two main subcomplexes of TFIIF. XPD is found associated with CAK and with the core of TFIIF according to purification and salt dissociation experiments (this study; see also Adamczewski *et al.*, 1996; Drapkin *et al.*, 1996; Reardon *et al.*, 1996). Baculovirus co-infection experiments demonstrate a direct interaction between XPD and CAK through the MAT1 subunit. This interaction could be stabilized by other TFIIF subunits, such as XPB which contacts the CAK complex, as evidenced by our baculovirus co-infection experiments (Table I) and the yeast two-hybrid system (C.Malaguti, F.Tirode and J.-M.Egly unpublished data).

CAK for the cell cycle, TFIIF for transcription

Free CAK and rCAK complexes show a stronger preference for the cdk2 substrate versus the ctd oligopeptide. CAK is thus most likely involved in regulation of the cell cycle through cdk phosphorylation (Morgan, 1995).

Although free CAK is able to use the ctd oligopeptide as a substrate, it cannot phosphorylate the CTD of RNA pol II alone or when added to an *in vitro* transcription system lacking TFIIF. On the contrary, TFIIF which contains CAK, is able to phosphorylate the CTD of RNA pol II, in addition to TBP and TFIIE α , two polypeptides absolutely required for basal transcription of protein-coding genes.

Free CAK and rCAK are not able to substitute for TFIIF in transcription. TFIIF lacking CAK complex allows RNA synthesis when added to an *in vitro* transcription system that contains all the components of the basal transcription machinery. However, when a CAK subcomplex (free CAK or rCAK) is added, the level of RNA synthesis is significantly increased. TFIIF may thus incorporate CAK to become fully active in transcription. In yeast, a comparable effect was also observed by Svejstrup *et al.* (1995). The association/integration of CAK may be mediated by the other subunits of TFIIF and/or the other components of the transcription machinery.

The above *in vitro* transcription study demonstrates the requirement for CAK for optimal basal transcription. Such a requirement was also shown *in vivo*. Indeed, microinjection of Ab-cdk7 in normal fibroblasts reduced RNA synthesis (Roy *et al.*, 1994). This inhibition was also observed with antibodies directed towards the other subunits of TFIIF, thus suggesting that the presence of each of the nine components of TFIIF is necessary for the transcription reaction (Vermeulen *et al.*, 1994). Together, our results demonstrate that the physical presence of CAK in TFIIF is necessary for transcription. This is also in agreement with the work of Mäkelä *et al.* (1995), who show that a TFIIF containing an inactive mutant of cdk7 kinase is still active in transcription.

The present work illuminates the roles of CAK in the transcription reaction. We have to distinguish between the presence of CAK as a component of TFIIF and its kinase function. First, *in vitro* transcription may occur without CAK. Its physical presence stimulates the transcription reaction, whether or not cdk7 kinase is active. Second, free CAK does not phosphorylate the CTD of RNA pol II. Phosphorylation of the CTD is optimal when CAK is presented in the context of TFIIF. Optimal basal transcription as well as CTD phosphorylation are thus a function of the presence of CAK in TFIIF and of the positioning of TFIIF in the transcription complex. Our results and those of Mäkelä *et al.* (1995) suggest that CTD phosphorylation is not related to the initiation process and may serve a regulatory function (Jiang and Gralla, 1995). Current understanding of the function of TFIIF in transcription is defined by its enzymatic activities. XPB and XPD helicases may promote the opening of the DNA at the promoter (Holstege *et al.*, 1996) and/or, in the case of DNA damage, catalyse the excision reaction for DNA repair (Mu *et al.*, 1996; Sung *et al.*, 1996). Cdk7 could accelerate the release of RNA pol II that is anchored to the transcription initiation complex (Usheva *et al.*, 1992) and aid in the elongation process (Yankulov *et al.*, 1996) and/or in the switch from non-activated transcription to the activated mode (Akhtar *et al.*, 1996).

In conclusion, our results strongly suggest that free CAK (and rCAK), which exhibits a preference for phosphorylating the cdk2 substrate, switches its substrate specificity to the CTD of RNA pol II upon integration

with TFIIF. Based upon these observations, the following hypothesis could be proposed in which free CAK plays a role in regulation of the cell cycle, whereas, upon association with TFIIF, its role is mainly in transcription. Since we observed that free CAK, which also exists on its own in the cell, could be re-incorporated into TFIIF, leading to a stimulated level of transcription, these results suggest that free CAK may be in equilibrium with TFIIF and provide an interesting mechanism for the functional regulation of TFIIF in the cell cycle, transcription and DNA repair.

Materials and methods

Free CAK purification

A whole cell extract was prepared from 9×10^{10} HeLa cells as described previously (Gérard *et al.*, 1991). The extract was applied onto a heparin Ultrogel column (Sepracor, France) (7.5 × 9 cm, flow rate 6 ml/min) equilibrated with buffer A [10 mM Tris-HCl pH 7.9, 20% glycerol, 5 mM MgCl₂, 0.5 mM dithiothreitol (DTT)] containing 100 mM KCl. The step gradient elution was performed with three column volumes of buffer A containing 0.22, 0.40 and 1 M KCl. The heparin 0.22 M KCl-eluted fractions which contained free CAK were dialysed against buffer B (50 mM Tris-HCl pH 7.9, 10% glycerol, 0.1 mM EDTA, 0.5 mM DTT) containing 50 mM KCl and were applied onto a Sulfopropyl-5PW column (Toso-Haas, Germany) (2.15 × 15 cm, flow rate 2 ml/min) equilibrated with buffer B containing 50 mM KCl. The flow through was then loaded onto a DEAE-5PW column (Toso-Haas, Germany) (2.15 × 15 cm, flow rate 2 ml/min). Proteins were eluted with a 100 ml linear gradient from 50 to 600 mM KCl in buffer B. The free CAK-containing fractions of the DEAE-5PW (4 ml, peak at 0.15 M KCl) were pooled and incubated for 3 h at 4°C with 350 µl of protein A-Sepharose cross-linked with 350 µg of Ab-cdk7. The beads were washed three times in buffer B containing 500 mM KCl, and elution was performed for 12 h at 4°C with 350 µl of buffer B containing 50 mM KCl and a 10-fold molar excess of epitope peptide. The eluate was collected together with a 350 µl wash of buffer B containing 50 mM KCl.

Recombinant CAK production and purification

S.frugiperda 9 cells (typically 2.5×10^8 cells) were infected with cdk7-, His-cyclin H- or MAT1-recombinant baculoviruses at a multiplicity of infection (m.o.i.) of 2, 2 and 10 p.f.u./cell respectively. The cells were collected 48 h post-infection and dounced in buffer L [20 mM Tris-HCl pH 7.9, 20% glycerol, 150 mM NaCl, 0.1% NP-40, 5 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 × protease inhibitor cocktail] (Adamczewski *et al.*, 1996). The extract was clarified by centrifugation (20 000 g, 30 min) at 4°C and loaded onto 1 ml of Ni-chelate His-Bind resin (Novagen, USA) previously saturated with 50 mM NiSO₄. The column was then washed with six column volumes of Ni buffer (20 mM Tris-HCl pH 7.9, 250 mM NaCl) containing 40 mM imidazole, and elution was performed with six column volumes of Ni buffer containing 100 mM imidazole. The eluted fractions were pooled, dialysed against buffer B containing 50 mM KCl and incubated for 3 h at 4°C with 250 µl of protein A-Sepharose cross-linked with 250 µg of antibody against cdk7. The beads were washed three times in buffer B containing 500 mM KCl, equilibrated in buffer B containing 50 mM KCl and elution was performed for 12 h at 4°C in 250 µl of buffer B containing 50 mM KCl and a 10-fold molar excess of peptide competitor. The eluate was collected together with 125 µl of wash of buffer B containing 50 mM KCl. The protein concentration was estimated by the Bradford technique (Bio-Rad) and rCAK was stored at -80°C. Typically, 1 l of culture (10^9 cells) yielded 250–350 µg of kinase complex.

TFIIF dissociation

The TFIIF heparin-5PW fraction (250 µg, Gérard *et al.*, 1991) was dissociated in buffer B at a final concentration of 1.2 M KCl and immediately incubated for 4 h at 4°C with 150 µl of protein A-Sepharose cross-linked with 150 µg of either Ab-cdk7 (2F8), Ab-p62 (3C9) or Ab-XPB (2F6). The supernatants were collected and dialysed against buffer B containing 50 mM KCl. The beads were washed three times with buffer B containing 1.2 M KCl and 0.05% NP-40, equilibrated in buffer B containing 50 mM KCl, and finally resuspended in 150 µl of Laemmli

buffer. After SDS-PAGE and transfer to nitrocellulose, the various polypeptides contained in each supernatant or on the beads were detected with their corresponding antibodies.

Production of recombinant baculoviruses

The following restriction endonuclease fragments of the cDNAs encoding the TFIIF subunits were subcloned into pVL1392 transfer vector (PharMingen, USA): XPB/*Bam*HI, XPD/*Eco*RI, p62/*Pst*II, p44/*Eco*RI, p34/*Eco*RI, cdk7/*Eco*RI and MAT1/*Eco*RI. The cDNA open reading frame encoding cyclin H was inserted in-phase into the *Bam*HI site of a modified pVL1392 containing a hexa-histidine tag. Production of the different recombinant baculoviruses was performed as described in O'Reilly *et al.* (1992).

Protein-protein interaction assays

Protein-protein interactions between two subunits of TFIIF were characterized by co-infection in Sf9 cells (3×10^6 cells) with either cdk7-, His-cyclin H- or MAT1-recombinant baculoviruses and either XPB-, XPD-, p62-, p44- or p34-recombinant baculoviruses at an m.o.i. of 10, 10, 5, 10 or 10 p.f.u./cell, respectively. To characterize the interaction between CAK and the other subunits of TFIIF, Sf9 cells (3×10^6 cells) were co-infected with cdk7-, His-cyclin H- and MAT1-recombinant baculoviruses. Sf9 cells were also infected with either XPB-, XPD-, p62-, p44- or p34-recombinant baculoviruses at an m.o.i. of 10, 10, 5, 10 or 10 p.f.u./cell respectively. Cell extracts were prepared 48 h post-infection as described above. One-tenth of each extract was pre-adsorbed on 20 µl of protein A-Sepharose pre-treated with 1 mg/ml BSA, for 1 h at room temperature in buffer B containing 50 mM KCl, 5 mM MgCl₂, 0.05% NP-40. Each supernatant was then incubated for 1 h at room temperature with 20 µl of protein A-Sepharose cross-linked with the indicated antibodies. The beads were washed with buffer B containing 150 mM KCl and resuspended in Laemmli buffer. After SDS-PAGE and transfer to nitrocellulose, the various polypeptides were detected with the corresponding antibodies.

Kinase assays

Kinase assays using either ctd4 peptide or recombinant GST-cdk2, His-TFIIFα and human TBP (Chalut *et al.*, 1994) as substrates were performed as described in Roy *et al.* (1994). The histone H1 phosphorylation was performed as described in Poon *et al.* (1993). RNA pol II phosphorylation was assayed in a 20–40 µl reaction containing 50 mM Tris-HCl pH 7.9, 6.5 mM MgCl₂, 0.1 mM EDTA, 50 mM KCl, 10% glycerol, 50 ng of an AdMLP *Eco*RI-*Sal*I DNA template, transcription factors as described in Chalut *et al.* (1994) and, as indicated, the various CAK complexes. After a pre-incubation period of 15 min at 25°C, CTP, GTP and UTP were added to a final concentration of 250 µM and ATP to a final concentration of 5 mM. Reactions were stopped after 45 min at 25°C by adding Laemmli buffer and resolved on an SDS-5% polyacrylamide gel. After electrotransfer, the different forms of the polymerase were detected with antibody (pol3/3) raised against the largest subunit of the RNA pol II (Dubois *et al.*, 1994).

Other techniques

The purification of TFIIF and all the basic transcription factors required for RNA pol II kinase and *in vitro* transcription assays were described previously (Humbert *et al.*, 1994). Monoclonal antibodies raised against XPB, XPD, p62, p52, p44, cdk7, cyclin H, p34 and MAT1 were as described in Marinoni *et al.* (1997).

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