The EMBO Journal vol.14 no.22 pp.5608–5617, 1995

In vitro assembly of a functional human CDK7–cyclin H complex requires MAT1, a novel 36 kDa RING finger protein

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It is proposed that the CDK7–cyclin H complex functions in cell cycle progression, basal transcription and DNA repair. Here we report that *in vitro* reconstitution of an active CDK7–cyclin H complex requires stoichiometric amounts of a novel 36 kDa assembly factor termed MAT1 (ménage à trois 1). Sequencing of MAT1 reveals a putative zinc binding motif (a C3HC4 RING finger) in the N-terminus; however, this domain is not required for ternary complex formation with CDK7–cyclin H. MAT1 is associated with nuclear CDK7–cyclin H at all stages of the cell cycle *in vivo*. Ternary complexes of CDK7, cyclin H and MAT1 display kinase activity towards substrates mimicking both the T-loop in CDKs and the C-terminal domain of RNA polymerase II, regardless of whether they are immunoprecipitated from HeLa cells or reconstituted in a reticulocyte lysate. MAT1 constitutes the first example of an assembly factor that appears to be essential for the formation of an active CDK–cyclin complex.

Keywords: CDK/CDK-activating kinase /cell cycle/cyclin/ RING finger

Introduction

Cell cycle progression in all eukaryotes depends on the periodic activation of cyclin-dependent kinases (CDKs). In vertebrates, entry into mitosis is controlled by CDC2, whereas progression through G_1 and S phase requires multiple CDKs, of which CDK4 and CDK2 have been studied most extensively (Norbury and Nurse, 1992; Pines, 1993; Sherr, 1994; Nigg, 1995). In order to be active, CDKs must associate with cyclin subunits (Hunt, 1991; Sherr, 1993). In addition, their activation requires the phosphorylation of a critical threonine residue (Thr161 in human CDC2) located in a region known as the T-loop (Morgan, 1995). This essential phosphorylation event is attributed to CDK-activating kinase (CAK). Early genetic screens have provided no clues as to the identity of CAK, but recent biochemical studies have allowed the identification of a major CAK activity in starfish oocytes (Fesquet *et al.*, 1993), *Xenopus* oocytes and eggs (Poon *et al.*, 1993; Solomon *et al.*, 1993) and cultured mammalian cells (Fisher and Morgan, 1994). Unexpectedly, in all cases the catalytic subunit of CAK was found to be structurally related to the CDK family. Originally designated as MO15 (Shuttleworth *et al.*, 1990), this kinase was subsequently shown to be associated with cyclin H and hence was renamed CDK7 (Fisher and Morgan, 1994; Mäkelä *et al.*, 1994). Thus, these studies suggested an involvement of a CDK–cyclin cascade in cell cycle control.

However, recent findings suggest that CDK7–cyclin H may perform additional or alternative functions. Both CDK7 and cyclin H were in fact found to be associated with TFIIH (Roy *et al.*, 1994; Serizawa *et al.*, 1995; Shiekhattar *et al.*, 1995), a multiprotein complex required for transcription by RNA polymerase II and for nucleotide excision repair (reviewed in Chalut *et al.*, 1994; Drapkin and Reinberg, 1994). Furthermore, CDK7–cyclin H has been implicated in the phosphorylation of the C-terminal domain (CTD) of RNA polymerase II (Roy *et al.*, 1994; Mäkelä *et al.*, 1995; Serizawa *et al.*, 1995; Shiekhattar *et al.*, 1995), a step that has long been recognized as being important for the regulation of transcription (for reviews see Corden, 1990; Dahmus, 1994; Koleske and Young, 1995). Independently, a CDK–cyclin complex structurally related to CDK7–cyclin H has been identified in *Saccharomyces cerevisiae*. This complex, KIN28–CCL1 (Simon *et al.*, 1986, Valay *et al.*, 1993), was also shown to be part of the budding yeast counterpart of TFIIH (Feaver *et al.*, 1994). However, although KIN28–CCL1 is able to phosphorylate the CTD of RNA polymerase II, it does not appear to display CAK activity (Feaver *et al.*, 1994; Cismoswski *et al.*, 1995). These findings suggest that KIN28–CCL1 may function primarily in transcription rather than in cell cycle progression. Although it is by no means proven that KIN28–CCL1 represents a functional homologue of CDK7–cyclin H, these results also strengthen the view that CDK7–cyclin H may perform an important function in regulating transcription in metazoan organisms. Taken together, the properties reported for CDK7–cyclin H indicate that this complex may contribute to integrate several fundamental cellular processes, notably cell cycle progression, transcription and DNA repair. However, the apparent multiplicity of functions of mammalian CDK7–cyclin H constitutes a puzzle, and clarification of the physiological role of this complex is clearly an important issue.

In the course of studying the cell cycle dependent expression of human CDK7, we have previously reported that this kinase associates stoichiometrically with two major subunits, estimated to be 34 and 32 kDa (Tassan *et al.*, 1994). The 34 kDa protein has subsequently been identified as cyclin H (Mäkelä *et al.*, 1994), but the nature of the third putative subunit associated with CDK7–cyclin H had remained mysterious. Here, we identify this component as a novel RING finger protein. Furthermore, we show that this third protein is important for the *in vitro*

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formation of a stable complex between CDK7 and cyclin H, and that the ternary complex is associated with both CAK and CTD kinase activities. Our studies identify the first CDK–cyclin assembly factor, and they point to a novel mechanism for the formation of active CDK–cyclin complexes.

Results

The third subunit of CAK is a novel human RING finger protein

In addition to CDK7 and cyclin H, human CAK contains a third, as yet uncharacterized, subunit (Tassan *et al.*, 1994; see also Fisher and Morgan, 1994; Mäkelä *et al.*, 1994). We have now purified this third subunit and used microsequence information obtained from cyanogen bromide cleavage products to clone a corresponding 1.3 kb cDNA. The isolated cDNA codes for a 309 amino acid protein with a calculated molecular weight of 36 kDa. Thus, the protein (hereafter termed p36) is somewhat larger than previously estimated (32 kDa) on the basis of its gel electrophoretic mobility (Tassan *et al.*, 1994). The nucleotide sequence of the cloned cDNA as well as the deduced amino acid sequence of p36 are shown in Figure 1. The two peptide sequences determined from the purified protein (indicated by dashed lines) are encoded by this cDNA, confirming that it codes for the third subunit of CAK. Database searches uncovered no extensive similiarities between p36 and known proteins, but they revealed the presence of a C3HC4 putative zinc binding domain in the N-terminus (bold underlined residues in Figure 1). This particular zinc finger motif is frequently referred to as a RING finger (from the human *ring1* gene; Freemont *et al.*, 1991). RING motifs have been identified in >40 different viral and cellular proteins, many of which have been implicated in gene regulation (for reviews see Freemont, 1993; Schwabe and Klug, 1994).

The nucleotide sequence shown in Figure 1 does not predict an in-frame stop codon upstream of the putative translation initiator AUG. Nevertheless, we are confident that we have cloned a cDNA coding for the entire 36 kDa CAK subunit. First, the sequence context surrounding the proposed initiator AUG matches perfectly the consensus determined for efficient translational initiation (Kozak, 1989). Secondly, SDS–PAGE revealed precise comigration between the 36 kDa protein synthesized from the cloned cDNA *in vitro* (Figure 2A, lane 2) and the third subunit co-immunoprecipitating with CDK7–cyclin H from HeLa cells (Figure 2A, lane 1; see also Figure 2B, lanes 1 and 2).

Subcellular localization and cell cycle expression of p36

Rabbit antibodies were raised against a GST–p36 fusion protein expressed in *Escherichia coli*. When used for immunoblotting, these antibodies recognized endogenous p36 in total HeLa cell protein extracts (Figure 2B, lane 1), as well as p36 proteins translated in a reticulocyte lysate (Figure 2B, lanes 2 and 3). The immunoreactive protein present in HeLa cells comigrated with *in vitro* translated wild-type p36 (compare lanes 1 and 2), whereas a p36 mutant lacking the N-terminal RING domain displayed the expected enhanced electrophoretic mobility (lane 3). As determined by indirect immunofluorescence microscopy, p36 was predominantly nuclear in all interphase cells (Figure 3A), whilst it was diffusely distributed throughout the cell during mitosis, showing no obvious association with condensed chromosomes (data not shown). To determine the expression levels of p36 during the cell cycle, HeLa cells were arrested at the G_1/S phase boundary using a thymidine–aphidicolin double block (Figure 3B) or in prometaphase using nocodazole (Figure 3C). They were then released from these cell cycle blocks for the indicated periods, and the abundance of p36 in each culture was determined by immunoblotting. In parallel, the DNA content of each sample was analysed by flow cytometry (Figure 3B and C). Taken together, these results show that the level of p36 is virtually constant throughout the cell cycle. They fall in line with our previous study showing that immunoprecipitates of CDK7 contained invariant amounts of CDK7, cyclin H and p36 at all stages of the cell cycle (Tassan *et al.*, 1994).

The 36 kDa subunit is required for stabilization of an active CDK7–cyclin H complex

To examine the possible role of p36 in the formation of CDK7–cyclin H complexes, the three proteins were produced separately by coupled transcription–translation in a rabbit reticulocyte lysate (Figure 4A). Proteins were mixed as indicated and incubated for 1 h at 30°C to allow protein–protein interactions to occur. One aliquot of each sample was then loaded onto an SDS–polyacrylamide gel (Figure 4A, upper panel), whereas the remainder was subjected to immunoprecipitation with anti-CDK7 antibodies. In order to visualize protein complex formation, one half of each immunoprecipitate was analysed directly by SDS–PAGE (Figure 4A, middle panel); the other half was used for an *in vitro* kinase assay with GST–CDK2 as a substrate for measuring CAK activity. From samples containing only one CAK subunit, anti-CDK7 antibodies precipitated CDK7, but virtually no cyclin H or p36 (lanes 1–3), as expected. However, when pairwise combinations of CAK subunits were analysed, we were surprised to find that neither cyclin H nor p36 could be co-immunoprecipitated with CDK7 (Figure 4, lanes 4–6). Similarly, no binary complexes could be detected when using anti-cyclin H or anti-p36 antibodies for immunoprecipitation (data not shown). In contrast, a ternary complex consisting of CDK7, cyclin H and p36 could be readily precipitated from the lysate containing all three proteins (lane 7). Similar results were obtained when using a myc epitope-tagged version of CDK7 and anti-myc antibodies for the isolation of CAK complexes (lanes 8 and 9). Furthermore, high levels of CAK activity were observed only when both cyclin H and p36 were present in the reconstituted CAK (bottom panel, lanes 7 and 9), although some background level kinase activity was present in all CDK7 immunoprecipitates (bottom panel, lanes 1–6 and 8). To confirm that the kinase activity measured in reconstituted CAK complexes was due to the catalytic activity of CDK7, the same type of experiment was repeated with a catalytically inactive CDK7 mutant (K41R) carrying an arginine in place of lysine 41. Although the K41R mutant readily participated in the formation of the ternary complex with cyclin H and p36, no specific CAK activity was associated with this complex (data not shown).

The results of the above association studies are corrobor-

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                   GGGCGGGCTGAAACAGGCGCCTGCGAGAGTCTGTAGGAGGGAAACCGCC   49
      ATGGACGATCAGGGTTGCCCTCGGTGTAAGACCACCAAATATCGGAACCCCTCCTTGAAG  109
1      M  D  D  Q  G  C  P  R  C  K  T  T  K  Y  R  N  P  S  L  K

      CTGATGGTGAATGTGTGCGGACACACTCTCTGTGAAAGTTGTGTAGATTTACTGTTTGTG  169
21     L  M  V  N  V  C  G  H  T  L  C  E  S  C  V  D  L  L  F  V
       ------------------------------------------------------------ a
      AGAGGAGCTGGAAACTGCCCTGAGTGTGGTACTCCACTCAGAAAGAGCAACTTCAGGGTA  229
41     R  G  A  G  N  C  P  E  C  G  T  P  L  R  K  S  N  F  R  V
       ---------------------

      CAACTCTTTGAAGATCCCACTGTTGACAAGGAGGTTGAGATCAGGAAAAAAGTGCTAAAG  289
61     Q  L  F  E  D  P  T  V  D  K  E  V  E  I  R  K  K  V  L  K

      ATATACAATAAAAGGGAAGAAGATTTTCCTAGTCTAAGAGAATACAATGATTTCTTGGAA  349
81     I  Y  N  K  R  E  E  D  F  P  S  L  R  E  Y  N  D  F  L  E

      GAAGTGGAAGAAATTGTTTTCAACTTGACCAACAATGTGGATTTGGACAACACCAAAAAG  409
101    E  V  E  E  I  V  F  N  L  T  N  N  V  D  L  D  N  T  K  K

      AAAATGGAGATATACCAAAAGGAAAACAAAGATGTTATTCAGAAAAATAAATTAAAGCTG  469
121    K  M  E  I  Y  Q  K  E  N  K  D  V  I  Q  K  N  K  L  K  L
           ---------------------------------------------------- b
      ACTCGAGAACAGGAAGAACTGGAAGAAGCTTTAGAAGTGGAACGACAGGAAAATGAACAA  529
141    T  R  E  Q  E  E  L  E  E  A  L  E  V  E  R  Q  E  N  E  Q
       ------------------

      AGAAGATTATTTATACAAAAAGAAGAACAACTGCAGCAGATTCTAAAAAGGAAGAATAAG  589
161    R  R  L  F  I  Q  K  E  E  Q  L  Q  Q  I  L  K  R  K  N  K

      CAGGCTTTTTTAGATGAGCTGGAGAGTTCTGATCTCCCTGTTGCTCTGCTTTTGGCTCAG  649
181    Q  A  F  L  D  E  L  E  S  S  D  L  P  V  A  L  L  L  A  Q

      CATAAAGATAGATCTACCCAATTAGAAATGCAACTTGAGAAACCCAAACCTGTAAAACCA  709
201    H  K  D  R  S  T  Q  L  E  M  Q  L  E  K  P  K  P  V  K  P

      GTGACGTTTTCCACAGGCATCAAAATGGGTCAACATATTTCACTGGCACCTATTCACAAG  769
221    V  T  F  S  T  G  I  K  M  G  Q  H  I  S  L  A  P  I  H  K

      CTTGAAGAAGCTCTGTATGAATACCAGCCACTGCAGATAGAGACATATGGACCACATGTT  829
241    L  E  E  A  L  Y  E  Y  Q  P  L  Q  I  E  T  Y  G  P  H  V

      CCTGAGCTTGAGATGCTAGGAAGACTTGGGTATTTAAACCATGTCAGAGCTGCCTCACCA  889
261    P  E  L  E  M  L  G  R  L  G  Y  L  N  H  V  R  A  A  S  P

      CAGGACCTTGCTGGAGGCTATACTTCTTCTCTTGCTTGTCACAGAGCACTACAGGATGCA  949
281    Q  D  L  A  G  G  Y  T  S  S  L  A  C  H  R  A  L  Q  D  A

      TTCAGTGGGCTTTTCTGGCAGCCCAGTTAACCATTTATAAGATTTGGACCTTGGAGCTGA 1009
301    F  S  G  L  F  W  Q  P  S  * (309)

      ACCAGGGAGCTAGCAAAAGTAAAGCAGACTTATAAAATTATAGCTATGTGCAGCTGCACA 1069
      ACACAGTCCTTCCACTAGCAGCTGTGTTAAAGTATTTATAAGGAGAAAATTTCAGAACTG 1129
      AAGTTGAGTAATATAGGGGATATATATTTGTGAAAAATAATTTTTACTTATATTTTCAGA 1189
      GGATTTGACACGATAGCCTCATCTGATGGAAGAGAGGAATAAATAATTCACCTATATGTG 1249
      TTTGAGGTTGTGACAGACTTATACC                                    1274
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Fig. 1. cDNA and predicted amino acid sequence of human p36. The human p36 cDNA is composed of 1274 nucleotides and encodes a 312 amino acid protein. The dashed lines indicate the two peptides (termed a and b) obtained by microsequence analysis of p36. Amino acids constituting the C3HC4 RING finger motif are indicated by underlined bold letters. This complete sequence was submitted to the EMBL database (Accession No. X87843). Independently, two short partial sequences derived from genome sequencing projects have been deposited (Accession Nos T71380 and Z44069).

ated further by the experiment shown in Figure 4B. A GST–p36 fusion protein was expressed in *E.coli*, and increasing amounts of the purified protein were added to reticulocyte lysates containing equal and constant amounts of ^{35}S-labelled CDK7 and cyclin H. Samples were then incubated for 1 h at 30°C and CDK7 was isolated by immunoprecipitation. As expected, equal amounts of CDK7 were immunoprecipitated from all lysates (Figure 4B). However, virtually no cyclin H was recovered in the absence of GST–p36 (lane 1). Instead, the addition of increasing amounts of GST–p36 caused increasing amounts of cyclin H to associate with CDK7 (lanes 2–8). These results clearly show that *in vitro* formation of a stable CDK7–cyclin H complex is dependent on p36, and that p36 acts as a dose-dependent assembly factor.

In light of a previous study suggesting that the CDK-inhibitor p21 could act as both an inhibitor and an assembly factor of CDK–cyclin complexes, depending on its

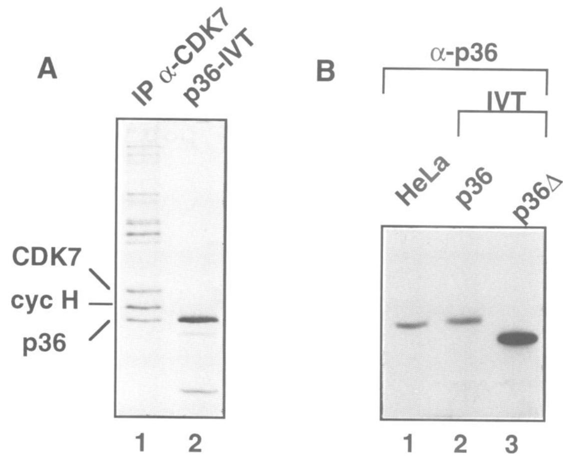

Fig. 2. Detection of p36 in human cells. (**A**) The p36 polypeptide co-immunoprecipitated with CDK7 and cyclin H from ^{35}S-labelled HeLa cells using anti-CDK7 antibodies (lane 1) comigrates in a 10% polyacrylamide gel with p36 produced in reticulocyte lysate (lane 2). This suggests that the p36 cDNA encodes the full-length p36 polypeptide. (**B**) Specificity of rabbit anti-p36 antiserum. p36 (lane 2) and p36Δ (p36 deleted for the RING finger, lane 3) produced *in vitro* as well as total cell lysate prepared from exponentially growing HeLa cells (lane 1) were separated on a 10% polyacrylamide gel and transferred to nitrocellulose. Immunoreactive proteins were detected with anti-p36 antibodies and visualized by enhanced chemiluminescence (ECL).

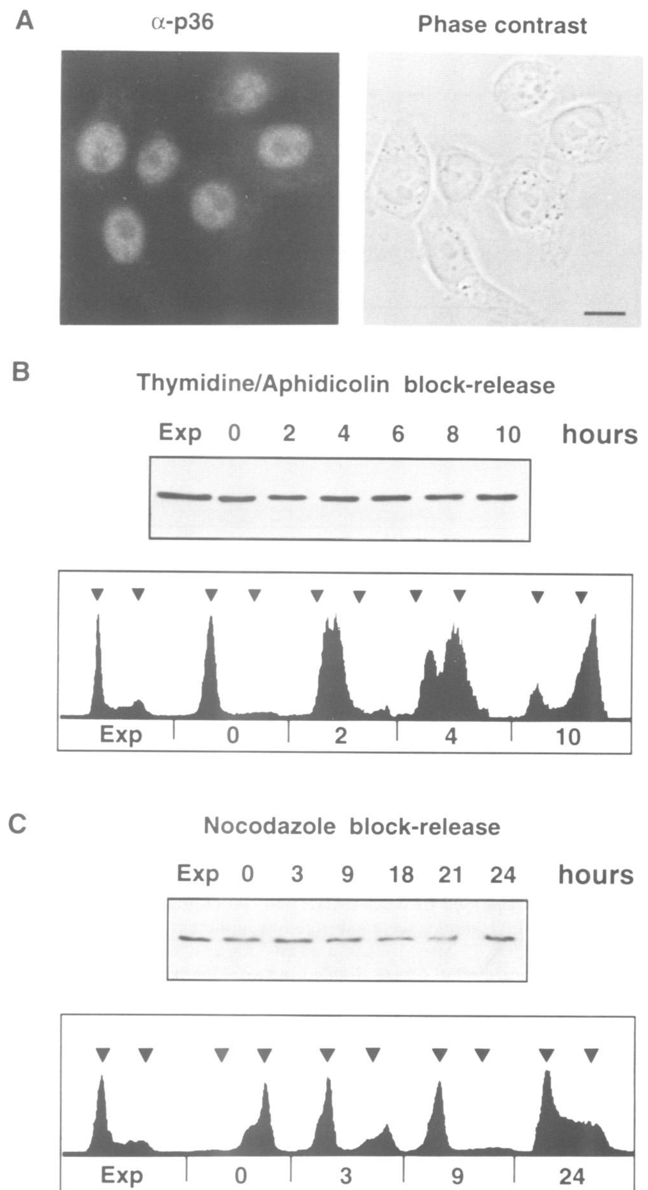

Fig. 3. Subcellular localization and expression of p36 through the human cell cycle. (**A**) p36 is located in the nucleus. Endogenous p36 was detected in HeLa cells by indirect immunofluorescence microscopy using affinity purified anti-p36 antibodies (left panel). Phase microscopy of the same cells is also shown (right panel; bar denotes 10 μm). (**B** and **C**) Levels of p36 protein are constant throughout the cell cycle. HeLa cells were arrested at the G_1/S transition by a thymidine–aphidicolin double block (B) or in prometaphase with nocodazole (C). After the block (0) cells were released for the times indicated and the position of cells in the cell cycle was determined by FACS analysis (shown for the indicated time points; bottom panels in B and C. For each sample, small arrowheads mark the positions of G_1 and G_2/M phase peaks). Equal amounts of proteins were then analysed by immunoblotting with anti-p36 antibodies (upper panels in B and C) and compared with that from exponentially growing cells (Exp).

stoichiometry (Zhang *et al.*, 1994), we asked whether p36 could also function as a dose-dependent inhibitor of CAK activity. To this end, CAK was reconstituted from purified GST–p36 and from *in vitro* translated CDK7 and cyclin H, as described above. Then, CAK activity was measured as a function of GST–p36 levels, using amounts of GST–p36 up to 10-fold higher than those required for maximal formation of the ternary complex. No evidence could be obtained for inhibition of CAK activity by p36 at any concentration tested (data not shown). Also, the addition of large amounts of GST–p36 to CDK7 immunoprecipitates prepared from HeLa cells did not affect CAK activity (data not shown). These results provide no indication that p36 can function as an inhibitor of CDK7–cyclin H-associated CAK activity. However, we caution that they do not exclude the possibility that p36 might act as an inhibitor *in vivo*, for instance in response to post-translational modifications.

The RING finger is not necessary for the formation of CAK

The functions of RING domains remain poorly understood, but they have been suggested to bind to nucleic acids and/or to mediate protein–protein or protein–lipid interactions (Freemont, 1993; Schwabe and Klug, 1994). Using p36 alone or p36 reconstituted within a CAK complex, we have been unable to detect any binding of this novel RING finger protein to either single-stranded or double-stranded DNA (data not shown). Hence, we favour the idea that the RING domain of p36 may play a role in mediating protein–protein (or protein–lipid) interactions. To determine whether the RING finger of p36 might be required for the formation of the ternary complex with CDK7 and cyclin H, a p36 mutant (p36Δ) lacking the RING domain was constructed (see Materials and methods). This mutant was then tested for its ability to reconstitute an active CAK complex, following translation of the individual components in a rabbit reticulocyte lysate. As shown in Figure 5 (middle panel), p36Δ was as efficient in forming a ternary complex (lane 2) as was the wild-type p36 (lane 1), indicating that the presence of the

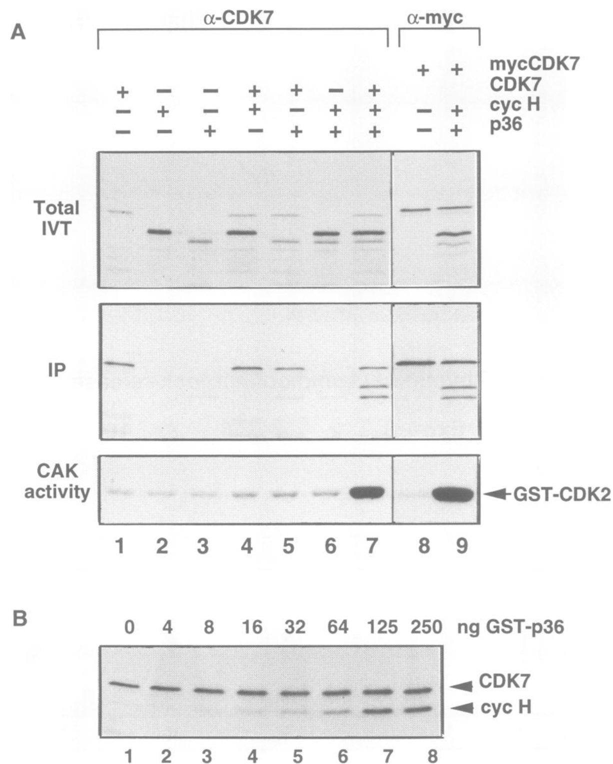

Fig. 4. p36 is required for CDK7–cyclin H association in a dose-dependent manner. (**A**) CDK7 or myc-tagged CDK7, cyclin H and p36 were produced in reticulocyte lysates and mixed as indicated. After incubation at 30°C for 1 h to allow protein association, an aliquot was analysed by SDS–PAGE and autoradiography (total IVT). The remaining samples were subjected to immunoprecipitation with either anti-CDK7 (lanes 1–7) or anti-myc tag antibodies (lanes 8 and 9). Immunoprecipitates were divided in two. One half was analysed by electrophoresis in a polyacrylamide gel followed by autoradiography (IP). The second half of the immunoprecipitate was assayed for CAK activity on the GST–CDK2 fusion protein (CAK activity).
(**B**) Reticulocyte lysates containing equal and constant amounts of ^{35}S-labelled CDK7 and cyclin H were mixed with increasing quantities of purified GST–p36 expressed in *E.coli*. Lysates were then incubated with anti-CDK7 antibodies and immune complexes were analysed by fluorography after separation of the proteins by SDS–PAGE.

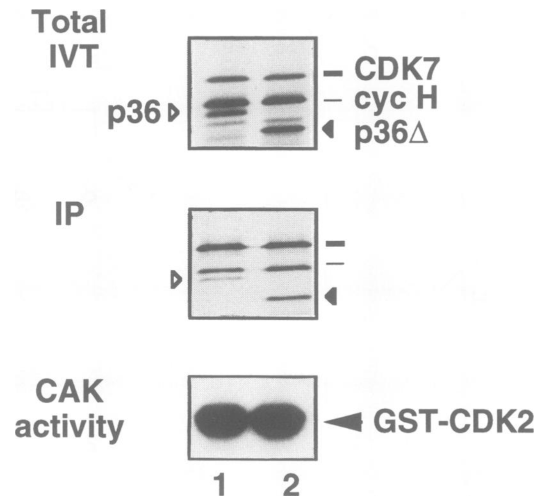

Fig. 5. The RING domain of p36 is not required for the formation of CAK. The CDK7–cyclin H complex was reconstituted in reticulocyte lysates containing CDK7 and cyclin H mixed with either wild-type p36 (lane 1) or p36Δ lacking the RING finger (lane 2) also expressed in reticulocyte lysates. After incubation at 30°C for 1 h proteins were analysed as decribed in the legend to Figure 4A. (In lane 1, the relative amount of p36 is lower than usual. We believe this to be due to proteolysis in this particular experiment.)

RING finger is not required for the association of p36 with CDK7 and cyclin H. Also, the RING finger of p36 was not necessary for conferring CAK activity to CDK7 (Figure 5, bottom panel). These results indicate that the RING finger of p36 remains available for promoting interactions of the ternary complex with other molecules.

p36 is part of both CAK and the CTD kinase

In view of the recent demonstration that the CDK7–cyclin H complex copurifies with the basal transcription factor TFIIH (Roy *et al.*, 1994; Mäkelä *et al.*, 1995; Serizawa *et al.*, 1995; Shiekhattar *et al.*, 1995), it was of interest to determine whether the ternary complex identified here would also display kinase activity towards the CTD of RNA polymerase II. To address this issue, two experiments were performed. First, CDK7 was isolated from HeLa cells, using either anti-p36 or anti-CDK7 antibodies for immunoprecipitation. Then, GST–CTD and GST–CDK2 fusion proteins were used as substrates to measure CTD kinase and CAK activities associated with these immunoprecipitates. As shown in Figure 6A, both p36 and CDK7 immunoprecipitates contained CTD kinase as well as CAK activities (compare lanes 2 and 5 with lanes 3 and 6). No phosphorylation of GST alone could be detected (data not shown) and no significant kinase activities were seen in control immunoprecipitates prepared with pre-immune sera (lanes 1 and 4). In the second experiment, CDK7–cyclin H–MAT1 complexes were reconstituted in a reticulocyte lysate, using myc-epitope tagged versions of either wild-type CDK7 or the catalytically inactive (K41R) mutant. After immunoprecipitation of the complexes with anti-myc antibodies, kinase activities were monitored using both GST–CDK2 and GST–CTD as substrates. For control, kinase assays were also performed using immunoprecipitates prepared from lysates that had not been primed with mRNA. As shown in Figure 6B, reconstituted complexes containing wild-type CDK7 readily phosphorylated both GST–CDK2 and GST–CTD (lanes 1 and 4), whereas only background activities were associated with complexes containing the K41R mutant CDK7 (compare lanes 2 and 5 with lanes 3 and 6). These results indicate that the ternary complex of CDK7, cyclin H and p36 displays both CAK and CTD kinase activities, consistent with the possibility that this complex may perform a dual function.

Discussion

Many CDK–cyclin complexes have previously been reconstituted in an active form *in vitro*, without the apparent need for the stable association of additional proteins (e.g. Desai *et al.*, 1992; Atherton-Fessler *et al.*, 1993; Connell-Crowley *et al.*, 1993; Maridor *et al.*, 1993; Pan and Hurwitz, 1993; Peeper *et al.*, 1993; Matsuoka *et al.*, 1994). Interestingly, however, Sherr and coworkers recently reported that assembly of CDK4–cyclin D complexes occurred rather inefficiently in insect cells, leading them to speculate on the existence of a hypothetical assembly

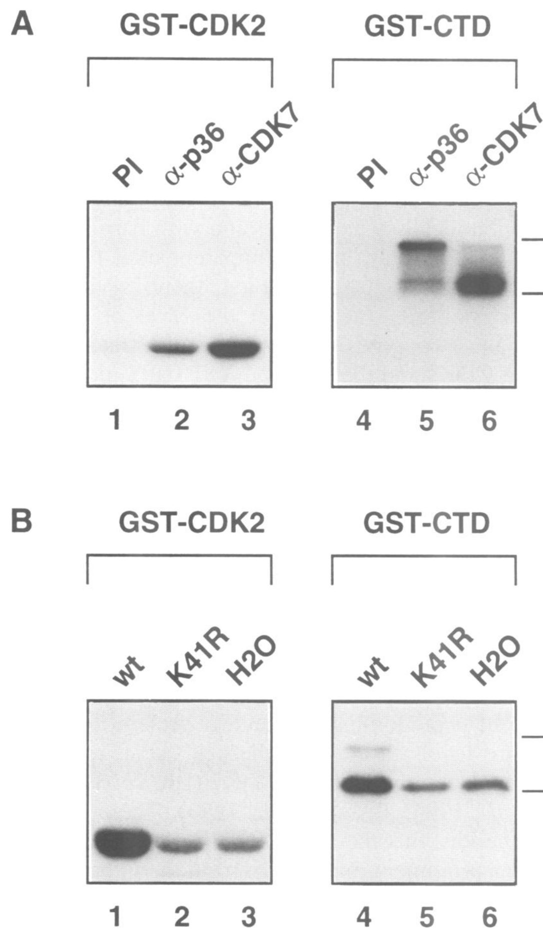

Fig. 6. p36-containing CDK7 complexes are associated with both CAK and CTD kinase activities. (**A**) Immunoprecipitations with anti-CDK7 (lanes 3 and 6) and anti-p36 (lanes 2 and 5) sera were carried out from exponentially growing HeLa cells and kinase activity was assayed on GST–CDK2 (lanes 2 and 3) and GST–CTD (lanes 5 and 6). Lanes 1 and 4 correspond to kinase assays carried out on GST–CDK2 and GST–CTD, respectively, using pre-immune sera (PI). As expected, phosphorylation of the GST–CTD causes an upshift in the apparent molecular weight (e.g. Dahmus, 1994). This upshift (indicated by bracket) is particularly pronouced with the anti-p36 immunoprecipitate, suggesting a higher stoichiometry of phosphorylation than that obtained with the anti-CDK7 immunoprecipitate. As anti-p36 antibodies were prepared using GST–p36 for immunization, this is most likely due to anti-GST antibodies, resulting in stabilization of substrate–kinase interaction. (**B**) Ternary complexes containing p36, cyclin H and myc-epitope tagged versions of either wild-type CDK7 (lanes 1 and 4) or the inactive K41R CDK7 mutant (lanes 2 and 5) were reconstituted in a reticulocyte lysate and immunoprecipitated with anti-myc antibodies. For control, immunoprecipitates were also carried out on lysates to which H_2O had been added in place of RNA (lanes 3 and 6). Kinase activities were then measured on GST–CDK2 (lanes 1–3) and GST–CTD (lanes 4–6), as described above for (A).

factor (Kato *et al.*, 1994). In this study we show that the efficient formation of an active kinase complex composed of CDK7 and cyclin H requires the presence of a third protein, p36. Importantly, p36 is readily detected in a ternary complex with CDK7 and cyclin H *in vivo* (Tassan *et al.*, 1994), indicating that this third protein functions not merely as a chaperone in a catalytic fashion but remains associated with CDK7 and cyclin H as a bona fide subunit of a stable complex. Hence, we propose to designate this novel CDK–cyclin associated protein as MAT1 (ménage à trois 1).

MAT1 points to a novel mechanism for assembling CDK–cyclin complexes

One mechanism previously recognized as contributing to the stability of several CDK–cyclin complexes is the phosphorylation of the CAK site within the T-loop of CDKs (e.g. Ducommun *et al.*, 1991; Gould *et al.*, 1991; Solomon *et al.*, 1992; Kato *et al.*, 1994; for a review see Morgan, 1995). Considering that CDK7–cyclin H was first identified by virtue of its ability to phosphorylate such sites *in vitro*, it is intriguing that CDK7 itself contains a threonine phosphorylation site (Thr170 in human CDK7) within its T-loop, suggesting that there may be an additional level in the proposed CDK–cyclin cascade. Phosphorylation of Thr170 is important for the activity of CDK7 (Labbé *et al.*, 1994; Poon *et al.*, 1994), and it is possible that this modification may contribute to stabilization of the CDK7–cyclin H complex (for further discussion see Devault *et al.*, 1995; Fisher *et al.*, 1995).

Here we have shown that the stable association of *in vitro* translated CDK7 and cyclin H requires the presence of MAT1. This novel protein acts as an assembly factor in a dose-dependent manner *in vitro*, and it is associated with CDK7–cyclin H in a stable ternary complex *in vivo*. Whilst MAT1 is necessary for the generation of CDK7 activity, there is no evidence so far to indicate that MAT1 might also act as an inhibitor. Thus, the mode of action of MAT1 is clearly distinct from that proposed for p21, which readily inhibits preformed CDK–cyclin complexes *in vitro* (Zhang *et al.*, 1994). On the other hand, it would be premature to exclude the possibility that MAT1 might negatively control the activity of CDK7 *in vivo*. It is possible, for instance, that MAT1 might function as a relay for as yet unidentified signals, and that conformational changes in MAT1 might produce changes in the level of CDK7 activity. A precedent for such a hypothetical role is set by PHO81, a protein which appears to be bound permanently to the budding yeast CDK–cyclin complex PHO85/PHO80, but inhibits this complex only under conditions of high extracellular phosphate levels (Schneider *et al.*, 1994).

The question arises as to how the proposed critical role of MAT1 in CDK7–cyclin H assembly can be reconciled with a recent study on the purification and reconstitution of supposedly binary CDK7–cyclin H complexes (Fisher and Morgan, 1994). Specifically, it has been reported that an active CDK7–cyclin H complex could be formed using subunits expressed from recombinant baculoviruses in insect cells (Fisher and Morgan, 1994). We would argue, however, that an insect homologue of MAT1 might have allowed the formation of a comparatively small amount of highly active complex. In support of this interpretation, recent work confirms that an efficient interaction between the bulk of the expressed CDK7 and cyclin H does in fact require MAT1 in insect cells (Fisher *et al.*, 1995). It has also previously been reported that highly purified mammalian CDK7–cyclin H complexes displayed CAK activity in the apparent absence of MAT1 (Fisher and Morgan, 1994). However, one could argue that MAT1 might have been lost or degraded proteolytically in the course of purification. In the latter case, MAT1-derived

peptides might have persisted on the CDK7–cyclin H complexes in quantities sufficient to perform a stabilizing function. Alternatively, and perhaps more likely, additional mechanisms such as phosphorylation of Thr170 might contribute to stabilization of CDK7–cyclin H complexes (Fisher *et al.*, 1995). Thus, once CDK7–cyclin H complexes were formed *in vivo* in the presence of MAT1, they might then have persisted even under experimental conditions that led to a loss of MAT1. In any event, we emphasize that no significant levels of binary CDK7–cyclin H complexes have so far been observed in any somatic cell type studied. In particular, MAT1 was found to be associated with CDK7 and cyclin H in near-stoichiometric amounts in all cultured human cells analysed (Tassan *et al.*, 1994), and anti-MAT1 antibodies immunoprecipitated most if not all CDK7 from HeLa cells (J.P.Tassan and E.A.Nigg, unpublished results).

MAT1 is a novel RING finger protein

Interestingly, sequence analysis of MAT1 revealed the presence of an N-terminal RING finger, a specialized form of zinc finger defined by a C3HC4 sequence motif. The precise functions of such RING domains remain to be elucidated, but many RING finger proteins appear to be involved in the regulation of gene activity (Freemont, 1993). Also, mutations affecting genes coding for RING finger proteins were shown to contribute to malignant transformation, examples being provided by the *pml* proto-oncogene implicated in acute promyelocytic leukaemia (Grignani *et al.*, 1994) and the breast cancer susceptibility gene *BRCA1* (Futreal *et al.*, 1994; Miki *et al.*, 1994). Recently, solution structures have been obtained for the RING domains of the IE110 protein of herpes virus (Barlow *et al.*, 1994) and the PML proto-oncoprotein (Borden *et al.*, 1995). The core of the RING domain was found to be similar in the two proteins, with two independent zinc binding sites forming a single structural domain. However, significant differences could also be observed, indicating that the RING motif can adopt appropriate three-dimensional structures to reflect a diversity in function.

The first proteins utilizing tetrahedral co-ordination of Zn^{2+} by cysteine and histidine residues for structural stabilization were exclusively nucleic acid binding proteins, but binding of RING domains to DNA–cellulose has been reported for only very few RING finger proteins, and no specificity for particular DNA sequences has yet been observed (Freemont, 1993). Also, it has become apparent that stabilization by Zn^{2+} may be used to confer structural features important for protein–protein or protein–lipid interactions (Berg, 1993; Schwabe and Klug, 1994). In the case of MAT1, zinc binding has only been inferred from the sequence but not yet been demonstrated biochemically, and the functional significance of the putative zinc binding domain remains to be explored. Here, we have shown that the RING finger of MAT1 is not necessary for the assembly of ternary complexes with CDK7 and cyclin H. Hence, this domain remains available for interactions with other, as yet unidentified, molecules. We have been unable to detect any binding of MAT1 to DNA–cellulose, suggesting that MAT1 is unlikely to make direct contact with DNA. Instead, the MAT1 RING finger may be important for mediating the interaction of CDK7 and cyclin H with other cellular proteins. In view of the observed association of CDK7 and cyclin H with TFIIH, subunits of this basal transcription factor constitute prime candidates for binding to the RING domain of MAT1.

CDK7: the CAK, the CTD kinase or both?

It is tempting to speculate that CDK7 may play a pivotal role in linking cell cycle progression with basal transcription and DNA repair, but the apparent dual function of CDK7 constitutes a paradox. First, there is virtually no structural similarity (at least at the level of sequence) between the two types of purported substrates, i.e. the T-loop of CDKs and the repeated YSPTSPS motifs of the CTD of RNA polymerase II. Secondly, it remains to be explained how the same protein kinase complex might function both as an essential upstream element of cell cycle regulatory CDKs and as a downstream CDK responsible for promoter clearance during transcription by RNA polymerase II. To understand the physiological role of CDK7 better, it is clearly important to establish a complete inventory of all the components of the CAK–CTD kinase complex. Here, we have shown that MAT1 functions as an assembly factor and permanent subunit of a stable ternary complex with CDK7 and cyclin H. MAT1 was found to be a predominantly nuclear protein and present at constant levels throughout the cell cycle, reminiscent of previous data obtained for CDK7 (Brown *et al.*, 1994; Poon *et al.*, 1994; Tassan *et al.*, 1994). These properties fit with the idea that the CDK7–cyclin H–MAT1 complex may play a prominent role in transcription, without excluding a participation in cell cycle regulation. We have also shown that CDK7–cyclin H–MAT1 complexes are associated with kinase activity towards both the T-loop of CDK2 and the CTD of RNA polymerase II, at least as measured using GST–CDK2 and GST–CTD as substrates. Similar results were obtained regardless of whether these ternary complexes were immunoprecipitated from HeLa cells or reconstituted in a reticulocyte lysate. Hence, it seems unlikely that the apparent dual specificity of the CDK7 complex could be explained by spurious kinases contaminating the immunoprecipitates, although this possibility cannot be formally excluded.

Regardless of the precise physiological function of the CDK7–cyclin H–MAT1 complex, we emphasize that CDK7 and cyclin H constitute the first example of a CDK–cyclin complex that cannot form *in vitro* in the absence of a third polypeptide component. In turn, MAT1 is the first example of a ternary complex factor shown to be necessary for the formation of an active CDK–cyclin complex *in vitro*. Considering the structural and functional diversity of the rapidly growing CDK family, it would not be surprising if additional proteins functionally resembling MAT1 should await discovery.

Materials and methods

Purification of the 36 kDa CAK subunit

For purification of CAK, HeLa cells were grown on 70 15-cm tissue culture dishes and lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP40, 1% deoxycholate, 0.1% SDS, 1 mM PMSF and 10 μg/ml each of leupeptin, aprotinin and pepstatin) containing phosphatase inhibitors (Tassan *et al.*, 1994). Protein complexes containing CDK7 were then immunoprecipitated by incubation of lysates for 2 h at 4°C with MO-1.1 immunoglobulin (Tassan *et al.*, 1994) covalently attached

to protein G–Sepharose beads with dimethyl pimelimidate (Harlow and Lane, 1988). After washing with RIPA and PBS, immune complexes were released from the beads by boiling in 3% SDS, 5% β-mercaptoethanol, 5 mM Tris–HCl, pH 6.8. Proteins were concentrated by precipitation for 1 h at –70°C with 7 vol of acetone, and pellets were resuspended in 3× gel sample buffer. The p36 (formerly p32; Tassan *et al.*, 1994) polypeptide (100–200 pmol) coprecipitating with CDK7 was separated on a mini-SDS gel (10%, 1.5 mm thickness) and visualized by negative staining as described by Ortiz *et al.* (1992). Bands were cut out, sliced into small pieces, dried by rotary evaporation, and reswollen in a solution of 70% formic acid containing 100 mg/ml CNBr. Digestions were performed for 48 h at room temperature with occasional shaking. Gel pieces were then extracted twice for 1 h with 300–500 μl of 0.1% trifluoroacetic acid/acetonitrile (4:6, v/v). Supernatants were pooled and dried, and the extracted peptides were separated on a Tris–tricine SDS gel as described by Schägger and Von Jagow (1987) before blotting onto a PVDF (polyvinylidene difluoride) membrane. After staining with 0.1% amidoblack in water, the blot was destained extensively with water and the bands were cut out. N-terminal sequence determinations were performed using either a model 473A or 477A microsequencer (Applied Biosystems, Foster City, CA) equipped with Problott reaction cartridges.

Degenerate oligonucleotides were designed on the basis of two peptide seqences. First, a 25mer peptide (peptide b in Figure 1) was used to design two PCR primers corresponding to its N- and C-terminal sequences (EIYQKEN and TREQEE, respectively). Using these oligonucleotides for RT-PCR on HeLa poly(A)$^+$ RNA, as described by Schultz and Nigg (1993), a 72 bp cDNA encoding peptide b was isolated. Subsequently, a homologous oligonucleotide corresponding to the internal sequence KDVIQK was synthesized. In combination with a degenerate primer corresponding to the sequence FVRGAGN in peptide a (VNVXGHTLXESXVDLLFVRGAGNXPE, X representing unidentified residues; Figure 1) a longer PCR product of 280 bp could be obtained. This 280 bp cDNA fragment was then used as a probe for screening of a cDNA library, as described below.

Cloning and sequencing of human MAT1 cDNA

Plaque forming units (10^6) from a human placenta λgt10 cDNA library (Clontech) were screened by DNA hybridization as described by Tassan *et al.* (1994). Inserts were excised from λgt10 by *Eco*RI digestion and subcloned into the *Eco*RI site of pBluescript IIKS (Stratagene, La Jolla, CA). Sequencing of the MAT1 cDNA was carried out for both strands by the dideoxynucleotide method as described previously (Maridor *et al.*, 1993). Fortuitously, the initiator ATG was found to be contained within an *Nco*I restriction site. This allowed us to delete the N-terminal RING finger by excising an *Nco*I–*Bgl*II fragment (encompassing the RING domain) and replacing it with a PCR fragment spanning Gly50 to the *Bgl*II site of MAT1. This fragment was amplified using a PCR primer introducing an *Nco*I site upstream of Gly50. Hence, p36Δ codes for a polypeptide corresponding to the fusion of the initiator codon with the sequence C-terminal to Gly50.

To create a catalytically inactive mutant of CDK7 (K41R), lysine 41 was substituted by arginine, using the Transformer^{TM} Site-Directed Mutagenesis Kit (Clontech), as described by the manufacturer. Myc-epitope tagged versions of wild-type and K41R mutant CDK7 were constructed as described previously (Tassan *et al.*, 1994).

Expression of human MAT1 in E.coli and production of anti-MAT1 antibodies

A cDNA spanning the entire coding sequence of MAT1 was cloned into the plasmid pGEX-KG (Guan and Dixon, 1991), and the GST–MAT1 fusion protein was expressed in *E.coli* strain BL21 (DE3). The expressed protein was highly insoluble and was therefore solubilized under denaturing conditions, before being renatured and subjected to purification (Sambrook *et al.*, 1989). To this end, 200 ml cultures were grown to an OD_{600} of 1.0, and production of GST–p36 protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside (0.1 mM). After centrifugation, cells were resuspended and lysed in 15 ml of 50 mM Tris–HCl pH 7.5, 2 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin and 2 mg/ml lysozyme. The lysate was sonicated and centrifuged at 10 000 *g* for 15 min. The pellet was resuspended in 2.5 ml of 0.1 M Tris–HCl, pH 8.5, containing 6 M urea, and the sample was centrifuged again for 15 min. Then, the supernatant was diluted 1:10 with 50 mM KH_2PO_4 (pH 10.7), 1 mM EDTA (pH 8.0), 50 mM NaCl and incubated at room temperature for 30 min. During this incubation, the pH of the solution was maintained at 10.7. Subsequently, the pH was adjusted to 8.0 and the incubation was continued for 30 min at room temperature. Finally, insoluble material was removed by centrifugation at 10 000 *g* for 15 min. Renatured, soluble GST–p36 protein was affinity-purified on a glutathione–Sepharose 4B column. For generation of anti-p36 antibodies, rabbits were immunized with the GST–p36 fusion protein and anti-p36 immunoglobulins were affinity-purified as described (Tassan *et al.*, 1994).

In vitro transcription–translation and in vitro association experiments

In vitro translations were performed by priming the TNT coupled reticulocyte lysate system (Promega Corp.) with appropriate plasmids in the presence of [^{35}S]methionine/cysteine (Express ^{35}S ^{35}S, Dupont NEN), as described by the manufacturer. For association experiments, lysates were mixed, incubated for 1 h at 30°C and an aliquot of each sample was analysed directly by SDS–PAGE and fluorography. For immunoprecipitation, samples were diluted 1:10 in NP40 buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP40, 0.1% deoxycholate, 0.01% SDS, 1 mM PMSF and 10 μg/ml each of leupeptin, aprotinin and pepstatin) and incubated for 2 h at 4°C with anti-CDK7 or anti-myc antibodies. Immune complexes were isolated and then subjected to SDS–PAGE and fluorography, as described previously (Krek and Nigg, 1991; Tassan *et al.*, 1994).

Cell culture, cell cycle synchronization and immunoprecipitation

HeLa cells were cultured at 37°C in a 7% CO_2 atmosphere in Dulbecco's modified Eagle's medium, supplemented with 5% fetal calf serum and penicillin–streptomycin (GIBCO BRL, Gaithersburg, MD). For cell cycle synchronization, HeLa cells were released from cell cycle blocks imposed by either thymidine–aphidicolin (G_1/S phase) or nocodazole (M phase), exactly as described previously (Fry *et al.*, 1995; Golsteyn *et al.*, 1995). For immunoprecipitation experiments, exponentially growing HeLa cells were lysed in RIPA buffer and incubated with anti-CDK7 or anti-MAT1 antibodies as described by Krek and Nigg (1991).

Miscellaneous techniques

Western blotting was performed as described (Krek and Nigg, 1991; Maridor *et al.*, 1993), using enhanced chemoluminescence (Amersham) for detection of immunoreactive proteins. The activity of CAK was assayed as described previously (Tassan *et al.*, 1994) and CTD kinase activity was assayed under the same conditions, replacing GST–CDK2 by GST–CTD (kindly provided by Dr W.Dynan; Peterson *et al.*, 1995). Indirect immunofluorescence microscopy was performed according to Tassan *et al.* (1994).

Acknowledgements

The name MAT1 (ménage à trois) appeared befitting, considering that the identification of the 36 kDa CAK subunit was first reported in France, at the Cell Division Cycle conference held in Aussois, May 12–18, 1995. We thank David Morgan, Tim Hunt and Bill Dynan for kind gifts of reagents (cyclin H cDNA and anti-cyclin H antibodies, GST–CDK2 and GST–CTD plasmids, respectively), and Rob Fisher, David Morgan and Marcel Dorée for candid and friendly discussion of unpublished data. We are also grateful to P.Zaech and C.Knabenhans for help with flow cytometry and P.Dubied and M.Allegrini for artwork. This study was supported by the Swiss National Science Foundation (31-33615.92 to E.A.N. and 5002-038919 to G.H.), and the Swiss Cancer League (For 447 to E.A.N.). A.M.F. was a recipient of a Royal Society postdoctoral fellowship.

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Received on July 7, 1995; revised on August 25, 1995