The EMBO Journal vol.15 no.8 pp.1877–1884, 1996

MAT1, cdk7 and cyclin H form a kinase complex which is UV light-sensitive upon association with TFIIH

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MAT1, cyclin H and cdk7 are part of TFIIH, a class II transcription factor which possesses numerous subunits of which several have been shown to be involved in processes other than transcription. Two of them, XPD (ERCC2) and XPB (ERCC3), are helicases involved in nucleotide excision repair (NER), whereas cdk7, cyclin H and MAT1 are thought to participate in cell cycle regulation. MAT1, cyclin H and cdk7 exist as a ternary complex either free or associated with TFIIH from which the latter can be dissociated at high salt concentration. MAT1 is strongly associated with cdk7 and cyclin H. Although not strictly required for the formation and activity of the complex, it stimulates its kinase activity. The kinase activity of TFIIH, which is constant during the cell cycle, is reduced after UV light irradiation.

Keywords: cdk7/cyclin H/UV sensitivity/MAT1/TFIIH

Introduction

The various phases of the cell cycle are devoted to some cellular specializations such as DNA replication, chromosome segregation, etc. At several transitions, the cyclin-dependent kinases (Morgan, 1995; Nigg, 1995) ensure that cell cycle events occur in the proper order; checkpoint mechanisms will, in response to intra- or extracellular signals, lead to a pause in the progression of the cell cycle for example in order to allow DNA repair (Carr and Hoelstra, 1995). It is not surprising that transcription activity may be modulated as a function of cell cycle events. Indeed, the mitotic state is accompanied by a cessation of nuclear gene expression; in the case of class III genes, this inhibition is associated with specific inactivation of transcription factor TFIIIB (Gottesfeld *et al.*, 1994; White *et al.*, 1995).

Transcription initiation of protein coding genes is a multistep process, requiring the assembly of several basal transcription factors as well as RNA polymerase II (Chalut *et al.*, 1994). A key step is the phosphorylation of the C-terminal domain (CTD) of the largest subunit of RNA polymerase II, a modification that occurs before elongation but whose precise role has never been determined (Dahmus, 1994). The non-phosphorylated form interacts preferentially with TBP, suggesting that phosphorylation might serve to free the elongation complex from the promoter-bound TBP (Usheva *et al.*, 1992). However, *in vitro* transcription is possible in the presence of protein kinase inhibitors (Serizawa *et al.*, 1993), or kinase-deficient TFIIH (Mäkelä *et al.*, 1995) as well as in the presence of a RNA polymerase without the CTD (Zehring *et al.*, 1988). The requirement for the CTD and for its phosphorylation may depend on the promoter, on the extracts used (Li and Kornberg, 1994) and on the difference between basal and activated transcription (Gerber *et al.*, 1995). Of the highly purified transcription factors, only TFIIH possesses CTD kinase activity (Lu *et al.*, 1992). TFIIH consists of at least nine subunits, two of which are ATP-dependent DNA helicases, implicated in DNA repair (Schaeffer *et al.*, 1993; Aboussekhra *et al.*, 1995). Mutations in both helicases can lead to various disorders such as xeroderma pigmentosum, Cockayne's syndrome and trichothiodystrophy (Vermeulen *et al.*, 1994; Cleaver and Hultner, 1995). We and others have recently identified the catalytic subunit of the TFIIH-associated CTD kinase as cdk7, also called MO15 (Feaver *et al.*, 1994; Roy *et al.*, 1994b; Serizawa *et al.*, 1995; Shiekhatar *et al.*, 1995). Cdk7, a member of the cdc2 superfamily of protein kinases, is the catalytic subunit of the cdk-activating kinase (CAK) (Fesquet *et al.*, 1993; Poon *et al.*, 1993; Solomon *et al.*, 1993; Darbon *et al.*, 1994). The phosphorylation of Thr161/160 of $p34^{cdc2}$ and $p33^{cdk2}$ by CAK is necessary for the activity of these key cell cycle regulatory kinases. However, the precise function of CAK remains unclear. Cdk7 levels and CAK activity are constant during the cell cycle (Tassan *et al.*, 1994) and it has not yet been possible to integrate CAK into a signal transduction pathway.

The role of TFIIH in transcription and DNA repair and the fact that one of its subunits is cdk7, a kinase thought to be involved in cell cycle regulation, prompted us to further characterize this multisubunit complex. Here we show that two of the nine polypeptides that form TFIIH are cyclin H and MAT1. These polypeptides, in addition to cdk7, can be resolved as a ternary complex from TFIIH. Since the complex of cdk7, cyclin H and MAT1 is believed to play a role in cell cycle regulation, we studied the CTD kinase activity of TFIIH during the various phases of the cell cycle and after induction of DNA damage.

Results

MAT1 as well as cyclin H and cdk7 is part of TFIIH

A careful analysis of the fractions from glycerol gradients onto which TFIIH previously treated with high salt concen-

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tration or detergent had been loaded (see below), on silver-stained protein gels, showed that three polypeptides of low molecular weight co-migrated with the CTD kinase activity (data not shown; see also Roy *et al.*, 1994b). Preparations of these polypeptides were subjected to tryptic digestion and microsequencing. While the tryptic oligopeptides of two of them were part of previously described proteins, cyclin H and cdk7, having calculated molecular weights of 38 kDa (324 amino acids) and 39 kDa (347 amino acids) respectively, none of the third (previously called p32) derived oligopeptide sequences (ALQDAFSGLFWQP, LEEALYEYQPLQIETYG and EEDFPSLR) corresponded to a known protein in the databases. However, we found that all three micro-sequenced oligopeptides were contained in the protein which had been recently cloned and termed MAT1 (36 kDa; 309 amino acids) (Devault *et al.*, 1995; Fisher *et al.*, 1995; Tassan *et al.*, 1995).

Previously we showed a co-purification of cdk7 with TFIIH transcription activity (Roy *et al.*, 1994b). We tested the fractions from the different steps of the TFIIH purification scheme (Gérard *et al.*, 1991), first by immuno-blotting with monoclonal antibodies against XPB, XPD, p62, p44, p34, cdk7 and cyclin H as well as one raised against a microsequenced oligopeptide of MAT1 (see Materials and methods), and second, by measuring the phosphorylation of a synthetic peptide (ctd4) mimicking four repeats of the CTD of the largest subunit of RNA polymerase II (Roy *et al.*, 1994a). The first three steps in the purification of TFIIH are step elutions from heparin–ultrogel, DEAE and sulfopropyl columns. In these crude fractions, CTD kinase activity is difficult to analyse, but cdk7, cyclin H and MAT1 polypeptides co-purify with the other subunits of TFIIH as well as with the TFIIH transcription and NER activities when tested in the appropriate assays (data not shown). These step elutions are followed by a gradient elution from the heparin HPLC column, in which both cyclin H and MAT1 co-purify with the cdk7 polypeptide as well as with the CTD kinase activity and with the p62 polypeptide which is one of the 'core' subunits of TFIIH (Figure 1A). As previously observed, the phenyl-5PW column allowed for the partial separation of the CTD kinase activity and of the cdk7 polypeptide from the entire TFIIH (Roy *et al.*, 1994b). Indeed, we observed that part of the three polypeptides MAT1, cdk7 and cyclin H are eluted at higher salt concentration than two of the other TFIIH subunits, XPB and XPD (Figure 1B). We cannot exclude, however, that this hydrophobic column allowed a better resolution of some contaminating free kinase complex from the kinase associated with the other TFIIH components (see also below). In the last step of our TFIIH purification scheme, the pooled phenyl fractions (from 12–16), were applied onto a HAP-HPLC column; cdk7, cyclin H and MAT1 co-purify with the p62 subunit of TFIIH and the CTD kinase activity, peaking in fraction 12 (Figure 1C). When TFIIH is analysed on a glycerol gradient, the CTD kinase activity co-purifies with the XPB and p62 subunits. In this case it also co-purifies with cdk7, cyclin H and MAT1 (data not shown).

Having observed a co-purification of cdk7, cyclin H and MAT1 with TFIIH, we wanted to confirm this association by a further independent assay. Therefore, we performed immunoprecipitations from a HeLa whole cell extract using antibodies directed against either XPB, XPD, cdk7 or cyclin H (our anti-MAT1 antibodies work poorly in precipitation experiments). The immunoprecipitates were analysed by a CTD kinase assay as well as by an SDS gel electrophoresis followed by immunoblotting. Figure 1D shows that Ab-cdk7, Ab-cyclin H, Ab-XPB and Ab-XPD immunoprecipitates all contain MAT1, cdk7 and cyclin H in addition to XPD and p62, two of the TFIIH subunits, as well as the CTD kinase activity. This immunoprecipitation is highly specific, since Ab-GST is unable to retain any of TFIIH subunits (lane 1). Altogether,

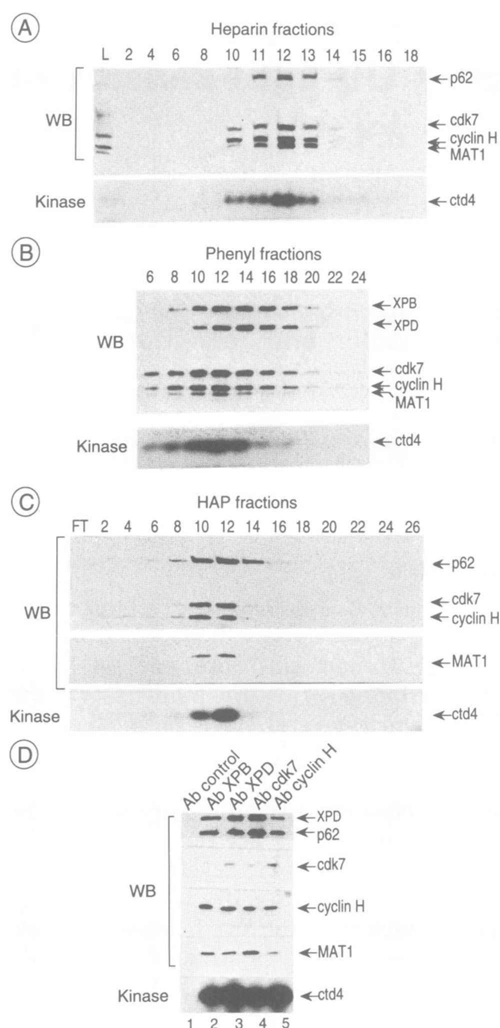

Fig. 1. Co-purification of MAT1 and cyclin H with TFIIH. A transcriptionally active sulfopropyl fraction of TFIIH was successively separated on heparin-5PW- (**A**), phenyl-5PW- (**B**) and HAP- (**C**) columns as previously described (Gérard *et al.*, 1991). The fractions were analysed on a 11% SDS–polyacrylamide gel, immunoblotted with monoclonal antibodies against p62 (Ab3c9), cdk7, cyclin H and MAT1 and in some cases XPD and XPB. L and FT represent the load and flow-through fractions respectively. HeLa whole cell extract previously diluted 10-fold in 20 mM Tris–HCl buffer, pH 7.5, containing 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT and 0.5% NP40, was immunoprecipitated (**D**) with antibodies against XPB (lane 2), XPD (lane 3), cdk7 (lane 4) and cyclin H (lane 5) as well as GST as a control (lane 1) cross-linked onto protein A–Sepharose. After extensive washing, three-quarters of the beads were eluted by boiling in SDS-sample buffer, and the proteins analysed by SDS–PAGE and immunoblotting (upper part). The remaining beads were subjected to a kinase assay using a synthetic ctd4 peptide as substrate (lower part).

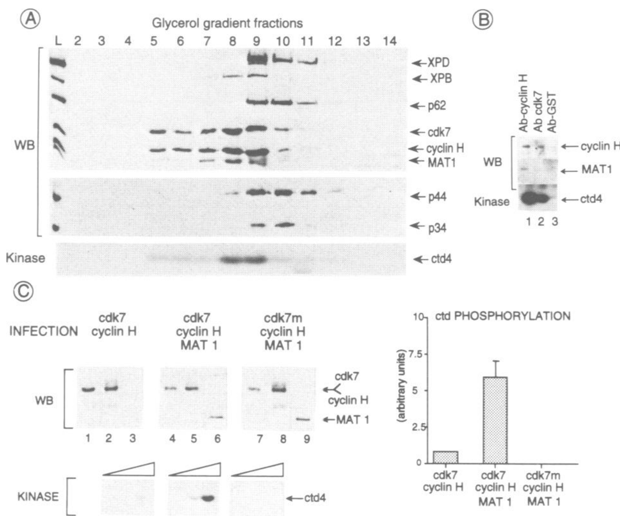

Fig. 2. Evidence for a ternary complex which contains the kinase activity. A 200 μl aliquot of TFIIH eluted from the HAP column (fractions 5–7) was dialysed for 4 h against a buffer containing 1 M KCl before loading onto a glycerol gradient (**A**) as described (Schaeffer *et al.*, 1994). The fractions were tested by Western blot (WB) for the presence of the various subunits of TFIIH, as described in the legend of Figure 1 and in a CTD kinase assay. Fractions 5–7 from the glycerol gradient were immunoprecipitated with Ab-cyclin H, Ab-cdk7 and Ab-GST (**B**, lanes 1–3 respectively) and also analysed by Western blot and in the CTD kinase assay. Insect cells (typically 1.2×10^{8} cells per assay) were infected with baculoviruses encoding cdk7, mutant cdk7 (cdk7m), cyclin H, MAT1, as indicated at the top of (**C**). The immunopurified recombinant complexes were tested by Western blot analysis (WB) using Ab-cdk7 (lanes 1, 4 and 7), Ab-cyclin H (lanes 2, 5 and 8) and Ab-MAT1 (lanes 3, 6 and 9) antibodies. The cdk7 concentration of each immunopurified fraction was evaluated by Western blot analysis and adjusted to identical values for the further kinase assays. 0.1, 0.5 and 1 μl of each recombinant complex were subjected to a kinase assay using ctd4 as a substrate (KINASE). The ctd kinase activity of these recombinant complexes of four independent parallel purifications was measured and standardized to the cyclin H/cdk7 complex. The average and standard error are shown (ctd PHOSPHORYLATION).

our results demonstrate that cdk7, cyclin H and MAT1 are part of the TFIIH transcription factor.

MAT1, cyclin H and cdk7 form an active kinase complex

When glycerol gradient analysis is performed under stringent conditions, i.e. high salt concentration (or in 0.5% sarkosyl; data not shown) TFIIH partially dissociates in at least two main fractions: the first one contains cdk7, cyclin H, as well as MAT1 and co-sediments with the CTD kinase activity at a slightly lower molecular weight than the second fraction, which contains the other subunits of TFIIH (XPB, p62, p44 and p34), and peaks in fractions 9–11 (Figure 2A). It is interesting to note that XPD is partially resolved from the other TFIIH subunits, a point that we have also mentioned previously (Roy *et al.*, 1994b; Schaeffer *et al.*, 1994).

The glycerol gradient fractions 5–7 (Figure 2A) were pooled and immunoprecipitated with either Ab-cyclin H and Ab-cdk7 antibodies. Those antibodies were able to immunoprecipitate MAT1 in addition to cyclin H, cdk7 and the CTD kinase activity (Figure 2B, lanes 1 and 2). None of these polypeptides was immunoadsorbed on the Ab-GST–Sepharose beads used as a control (lane 3). This demonstrates that cdk7, cyclin H and MAT1 are associated to form a ternary complex which possesses a kinase activity.

This result was further confirmed in our attempt to reconstitute an active kinase complex from the three components when overexpressed and reassembled. Four baculoviruses encoding cdk7, an inactive point mutation of cdk7 (cdk7m), histidine-tagged cyclin H and MAT1 were engineered (see Materials and methods and Figure 2 legend). Sf9 insect cells were co-infected with baculovirus encoding cdk7/cyclin H, cdk7/cyclin H/MAT1 or cdk7m/cyclin H/MAT1. The infected cell extracts were loaded onto a chelate resin previously saturated with nickel ions. After extensive washing, the column was eluted with the adsorption buffer containing 100 mM imidazole. The eluted fractions were then dialysed and immunoadsorbed onto protein A–Sepharose beads cross-linked with cdk7 antibodies. Finally, elution was performed with the peptide corresponding to the epitope and the eluted fractions were tested by immunoblotting and for the kinase activity. First, cdk7 interacts directly with cyclin H (Figure 2C, WB, lanes 1 and 2), a fact previously observed by Fisher and Morgan (1994). An interaction between cdk7 and cyclin H was also observed using the yeast two-hybrid system by Mäkelä *et al.* (1994) and by our laboratory (unpublished results). Second, both with affinity purification of the histidine-tagged cyclin H and with immunoabsorption using Ab-cdk7 antibodies, we obtain a complex of three polypeptides cyclin H, cdk7 and MAT1 (as shown on the immunoblot Figure 2C, WB, lanes 4–6) thus demonstrating that the three polypeptides are intimately associated to form a ternary complex. In addition, whether it is mutated in its ATP binding site or not, cdk7 is able to form a ternary complex with cyclin H and MAT1 (WB, lanes 7–9). The lower band which was detected with Ab-cyclin H antibody (Figure 2C, lanes 2, 5 and 8), is either non-specific or may result from some proteolytic degradation. Third, although already the binary complex of cdk7 and cyclin H was able to phosphorylate the ctd4 oligopeptide (Figure 2C, KINASE), this kinase activity was strongly stimulated by the presence of MAT1. In four pairs of parallel preparations of cdk7/cyclin H and cdk7/cyclin H/MAT1 we consistently observed a higher kinase activity in the preparation containing MAT1. The average stimulation was 6-fold (Figure 2C, histogram). We cannot yet tell whether this stimulation is due to a higher proportion of active cdk7 complexes in the preparation or to an increase in substrate specificity for the CTD. Preparations containing mutant cdk7 (cdk7m) or cdk7 without cyclin H (not shown) had no detectable kinase activity.

The composition of TFIIH seems unchanged during the cell cycle

Thus, we have demonstrated that a kinase complex containing three subunits cdk7, cyclin H and MAT1 is associated with TFIIH. On the other hand, a kinase complex which contained only these three subunits, was isolated as a free form (Tassan *et al.*, 1994). Such free kinase complex was also seen in the 0.22 M KCl eluted fraction from the heparin–ultrogel column (data not shown) whereas TFIIH elutes in the 0.4 M KCl fraction (Gérard *et al.*, 1991). Given that the cdk7-containing complex activates cyclin-dependent kinases and is thus implicated

in cell cycle control, it was surprising that no change in its protein composition or activity had been found (Tassan et al., 1994). As these previous studies have concentrated on free CAK, we wondered whether the TFIIH-associated kinase might show variations during the cell cycle. JEG-3 cells (a human choriocarcinoma cell line) were arrested in G_0, at the entry of S phase and after the prophase of mitosis. Serum-starved cells arrest in G_0 outside the cell cycle; aphidicolin is an inhibitor of DNA replication, arresting cells at the entry of S phase whereas the inhibitor of microtubule polymerization, nocodazol, leads to an arrest after the prophase of mitosis (Zieve et al., 1980). The effects of the various treatments were verified by FACS analysis of ethidium bromide-stained cells (Figure 3A). As expected, serum starvation and aphidicolin treatment considerably reduced the number of cells in the S and G_2 phases, whereas nocodazol treatment arrested the cells almost quantitatively in G_2/M. The cell lysates were immunoprecipitated either with Ab-cdk7 or with Ab-XPD antibodies. These antibodies were used to discriminate between total cdk7 (free and TFIIH-associated) and TFIIH-associated cdk7 respectively. The immunoprecipitates were subsequently tested for their kinase activity against both ctd4 oligopeptide and GST-cdk2 and for their polypeptide composition by immunoblotting. As shown in Figure 3B, neither the composition nor the kinase activity of the complex undergo dramatic stoichiometric changes during arrest in different stages of the cell cycle. First, the ratios among the cdk7, cyclin H and MAT1 polypeptides as well as the core TFIIH subunits XPB, XPD and p62 immunodetected are almost identical in the three different stages of the cell cycle (see either lanes 1–4 or lanes 5–8 when the lysates are immunoprecipitated with Ab-XPD or Ab-cdk7 antibodies, respectively). Quantification of the phosphorylation of ctd4 and cdk2 (Figure 3B, lower panel) showed that the kinase activity of neither Ab-cdk7 nor Ab-XPD immunoprecipitates changes between unsynchronously growing cells and cells arrested in different cell cycle stages.

As cells arrested by drugs in a given cell cycle stage show some differences to growing cells passing through the same cell cycle phase, we decided also to analyse TFIIH in cells released from a nocodazol or aphidicolin block to detect any possible modulation of kinase activity during a normal cell cycle. Thus, we arrested JEG-3 cells by exposure to either aphidicolin or nocodazol for 24 h and subsequently released cells by changing their medium to one without either drug. In both cases we observed the cells for 16 h after release, performing immunoprecipitations against cdk7, XPD and GST every 4 h. The cells were observed from a mitotic nocodazol block up to re-entry into S phase and from the G_1/S block by aphidicolin up to their passage through mitosis, thus covering each phase of the cell cycle (see FACS analysis in the right part of Figure 4A and B). In agreement with the previous experiment we found no major changes in TFIIH composition (see the Western blots in Figure 4) or kinase activity (see the ctd4 and cdk2 phosphorylation and their quantification). Our results demonstrate that while CAK can phosphorylate and activate cell cycle kinases both in its free form and associated to TFIIH, neither complex itself undergoes any obvious cell cycle regulation.

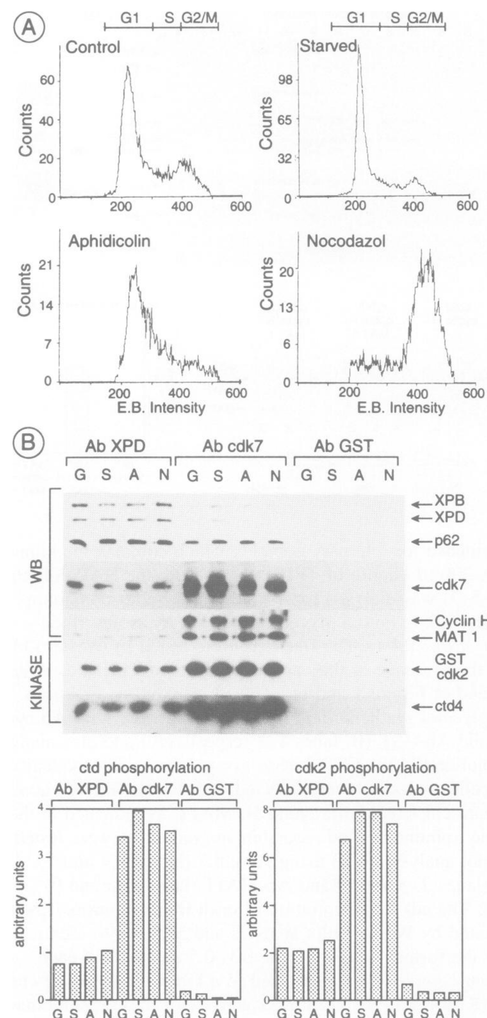

Fig. 3. The composition and kinase of TFIIH is constant over the cell cycle. Logarithmically growing JEG-3 cells were cultured either in medium containing 0.2% FCS for 48 h (S, starved) or exposed to 1 µg/ml aphidicolin (A) or 0.2 µg/ml nocodazol (N) for 24 h. Control cells (G) were grown in medium with 10% FCS. Their position in the cell cycle was determined by FACS analysis (the position of the G_1, S, G_2/M phase is indicated above of each graph of (**A**). Cells were lysed in 50 mM Tris-HCl buffer, pH 7.5 containing 0.1 mM EDTA, 1 mM DTT, 100 mM NaCl and 0.5% NP40 and adjusted for protein concentrations. Immunoprecipitations were performed with Ab-XPD (lanes 1–4), Ab-cdk7 (lanes 5–8) and Ab-GST (lanes 9–12) antibodies. The extensively washed beads were then tested by immunoblotting (WB) for the presence of XPB, XPD, p62, cdk7, cyclin H and MAT1 and for CTD and GST-cdk2 kinase activity (KINASE) (Roy et al., 1994b) The quantification of the kinase assays using a PhosphoImager are shown at the bottom of the figure.

DNA damage affects the CTD kinase activity of TFIIH

As the kinase activity of TFIIH seems invariant during the cell cycle, and given the function of TFIIH in nucleotide excision repair as well as in transcription, we wondered whether the CTD kinase activity was affected in cells subjected to DNA damage. To test this, we irradiated JEG-3 cells with ultraviolet light at a dose of 40 J/m^2 and lysed cells at different time-points after irradiation. Parallel immunoprecipitations with Ab-cdk7 and Ab-XPD were performed with extracts from the irradiated and the non-irradiated cells. We observed that UV treatment reduced

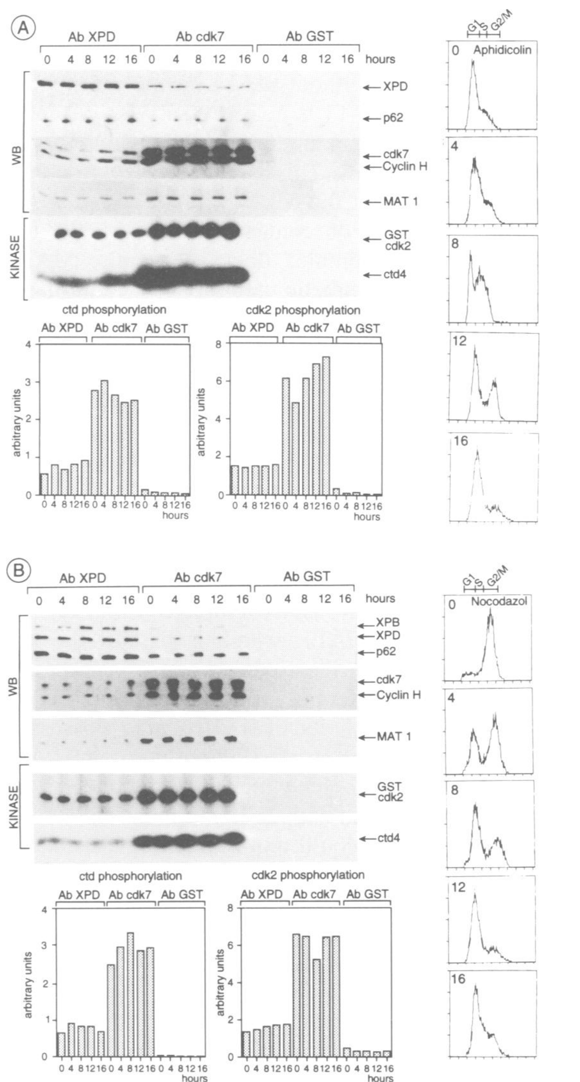

Fig. 4. Cells were exposed to aphidicolin (**A**) or nocodazol (**B**) as described earlier, and subsequently released into medium without drugs. As seen by the FACS analysis of ethidium bromide-stained cells (shown on the right of each panel), the cells begin to progress into S phase at 4 h after aphidicolin release, have their peak in S phase 8 h after release and are mainly in G_2/M after 12 h to re-enter G_1 phase 16 h after release. A minor proportion of cells (~25–30%) does not re-enter the cell cycle (A). The nocodazol arrest leads to a quantitative block in G_2/M, from which the cells report into G_1 during the 12 h following release. At 16 h post-release a significant proportion of the cells re-enters S and G_2 phases (B). The cells were analysed by Western blotting and kinase assays as described for Figure 3.

the kinase activity of the XPD immunoprecipitated fraction, but not significantly that of the cdk7 immunoprecipitates. This effect occurs when either the synthetic ctd4 or the cdk2 are used as substrates (see Figure 5A). The quantification of three irradiation experiments consistently showed a reduction of kinase activity by factor of 3–5 (see graph at the bottom of Figure 5A). This reduction in kinase activity sets in after 30–60 min and minimal kinase activity is reached after 3–4 h (Figure 5A and data not shown). Thus, we can conclude that the TFIIH-associated kinase is partially inhibited after UV irradiation (lanes 1–4) whereas the free kinase complex seems to be unaffected (lanes 5–8). This decrease in the kinase activity associated

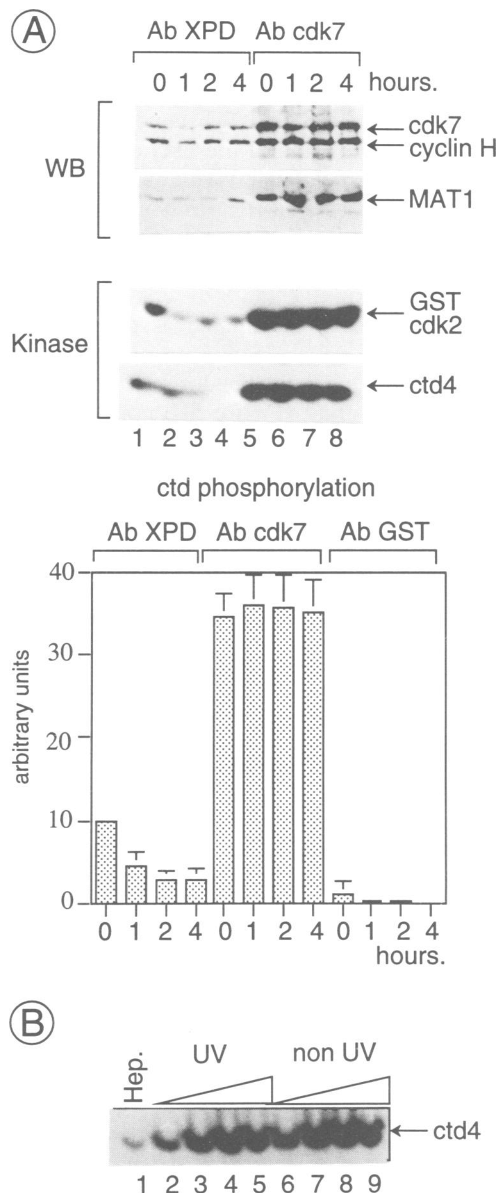

Fig. 5. The kinase activity of TFIIH drops after DNA damage. (**A**) JEG-3 cells were harvested at various times after exposure to 40 J/m^2 UV light as indicated above each part of (A) (as described in Materials and methods). Cells were then lysed and the adjusted protein concentrations immunoprecipitated with Ab-XPD (lanes 1–4) or Ab-cdk7 (lanes 5–8) antibodies previously cross-linked on protein A–Sepharose beads. After washing, the proteins immunoadsorbed onto the beads were tested by immunoblotting (WB) and for their ability to phosphorylate ctd4 as well as the cdk2 substrates (Kinase). The quantification of cdt4 phosphorylation shown represents the average of three irradiation experiments (standardized to a value of 1 for the TFIIH-associated kinase in non-irradiated cells) with standard deviations shown. (**B**) Monolayer Sf9 cells (typically, 10^8) were infected with cdk7 and cyclin H baculoviruses. At 2 days post-infection, half of the cells were UV irradiated. Irradiated (UV, lanes 2–5) and non-irradiated (non UV, lanes 6–9) cell lysates (1 ml) were incubated with 100 μl of protein A–Sepharose carrying Ab-cdk7. After extensive washing, the proteins were tested for CTD kinase activity as described earlier. Lane 1, TFIIH from the heparin HPLC fraction; lanes 2–5 and 6–9 represent 1, 2, 5 and 10 μl of each eluted fraction.

with TFIIH is not due to the absence of any of the three subunits of the kinase complex, as observed in Figure 5A (upper panel). The five other TFIIH subunits XPB, XPD,

p62, p44 and p34 are also present in each fraction (data not shown). The fact that we did not observe drastic changes in the kinase activity present in the cdk7 immunoprecipitated fraction leads to two conclusions: first, the kinase associated with TFIIH presents only a minor part of the overall cdk7 precipitated with anti-cdk7 antibodies (as could already be seen in Figures 3 and 4); and second, the free kinase complex is not (or much less) sensitive to the UV light irradiation. To further study this point, insect cells previously co-infected with cdk7 and cyclin H baculoviruses, were UV irradiated as described for the mammalian JEG-3 cells. In this case the recombinant cdk7/cyclin H complex did not lose its ability to phosphorylate ctd4 substrate (Figure 5B, compare irradiated and non-irradiated cells), thus demonstrating that UV irradiation *per se* has no direct effect on the enzymatic activity of cdk7.

Discussion

MAT1 is associated with cdk7 and cyclin H

Before we identified cdk7 as a subunit of the TFIIH transcription–DNA repair factor (Roy *et al.*, 1994b), it had been shown that this polypeptide was associated with a regulatory subunit, termed cyclin H to form the CDK-activating kinase (Fisher and Morgan, 1994, Mäkelä *et al.*, 1994). This kinase was also able to use the CTD of RNA polymerase II as a substrate (Feaver *et al.*, 1994; Roy *et al.*, 1994a,b; Mäkelä *et al.*, 1995; Serizawa *et al.*, 1995; Shiekhatar *et al.*, 1995). This prompted us to microsequence additional bands in our highly purified TFIIH preparation which contains nine subunits (Chalut *et al.*, 1994). One of these bands did indeed turn out to be cyclin H (Roy *et al.*, 1994b; also this study). In addition, we also identified another polypeptide called MAT1 (for ménage à trois) (Devault *et al.*, 1995; Fisher *et al.*, 1995; Tassan *et al.*, 1995). MAT1, which has a calculated molecular weight of 36 kDa, and cyclin H co-purifies and co-immunoprecipitates with the other TFIIH subunits. While this paper was under review, a co-immunoprecipitation of MAT1 and TFIIH was also observed by Fisher *et al.* (1995).

MAT1 is the third subunit of the CAK complex, the other two being cdk7 and cyclin H. Several lines of evidence support this conclusion: first, these three subunits can be resolved together from the other subunits of TFIIH (Figure 2A); second, antibodies towards either cdk7 or cyclin H immunoprecipitate MAT1, as well as the two other subunits; third, when co-infected with three baculoviruses encoding cdk7, cyclin H or MAT1, it is possible to obtain a protein complex that contains the three gene products; and fourth, such a ternary complex was also identified as a free complex in oocytes (Devault *et al.*, 1995), in a subfraction of our TFIIH purification scheme (unpublished results) and by immunoprecipitation from crude HeLa cell extracts (Tassan *et al.*, 1995). Altogether, it seems that this complex exists in the cell at least in two forms: either incorporated into TFIIH or as a separate complex.

MAT1 and the kinase activity

At the present time MAT1 is always found associated with CAK (Devault *et al.*, 1995; Tassan *et al.*, 1995) or TFIIH. The remaining question concerns the role of MAT1 in the free kinase complex and in TFIIH. Unless salt or detergent is used, we never find TFIIH free of MAT1. In the same way, CAK without MAT1 has not been isolated up to now. In fact, MAT1 is not necessary for cdk7/cyclin H interaction; cdk7 can interact with cyclin H even at high salt concentrations. This is not surprising given that the crystal structure of the cdk2/cyclin A complex shows a large surface area of contact (Jeffrey *et al.*, 1995). The cdk7/cyclin H complex does not require MAT1 to phosphorylate the synthetic peptide ctd4 and the cdk substrates. However, we have demonstrated a significant stimulation of kinase activity by the presence of MAT1 in the complex. This stimulation could be due either to an increase in the proportion of active kinase complexes formed possibly by a stabilization of the interaction or to a change in substrate specificity. MAT1 contains a putative zinc finger referred to as a RING motif; some proteins having this RING motif have been implicated in gene regulation (Schwabe and Klug, 1994). As RNA pol II phosphorylation occurs only in the presence of the transcription factors and the DNA template (Lu *et al.*, 1992; Roy *et al.*, 1994b) MAT1, which is part of the kinase complex, would be an ideal candidate to position TFIIH on the promoter for RNA pol II phosphorylation. However, the p44 and p34 subunits of TFIIH, also possess zinc finger motifs (Humbert *et al.*, 1994).

How the ternary complex which contains cdk7, cyclin H and MAT1 is associated with TFIIH remains an interesting point. Such answers would require a complete characterization of the TFIIH subunits. Eight of the nine subunits that we have identified, are at the present time characterized and cloned. So far we have found no difference between MAT1 (or the other two subunits) associated with TFIIH or as free CAK, but we cannot exclude that they differ, for example in post-translational modification. Some subunit of the kinase complex could be involved either in the substrate selection of the phosphorylation reaction, or act as a bridging factor with TFIIH or any other factors involved in the three molecular mechanisms in which TFIIH participates (transcription, DNA repair and/or cell cycle). Both complexes (either free or TFIIH-associated) as well as the recombinant formed complex are able to phosphorylate ctd4 synthetic oligopeptide as well as GST-cdk2 (Fisher and Morgan, 1994). Whether or not they differ in substrate specificity is not yet established.

TFIIH and the cell cycle

Cyclins were originally discovered as proteins that accumulate during cell growth to be rapidly degraded at a defined cell cycle transition. Thus, the identification of cdk7 as a cyclin-dependent kinase and as the activator of other cdks posed the question of its regulation during cell cycle progression. Tassan *et al.* (1994, 1995) showed that the levels of all three subunits of CAK as well as their kinase activity stay constant over the eukaryotic cell cycle. The majority of kinase immunoprecipitated in their studies was not associated with TFIIH. This prompted us to investigate the behaviour of TFIIH-associated cdk7 and the overall composition of this transcription factor during the cell cycle. In Figures 3 and 4 we show that both overall and TFIIH-associated cdk7 stays unchanged when cells are arrested in different cell cycle stages. Thus, TFIIH kinase activity does not seem to play a role in

achieving the specificity of cell cycle-regulated transcription. This prompted us to search for other conditions which might affect TFIIH kinase activity and thus reveal its potential regulatory function.

TFIIH: a DNA damage detector?

Nucleotide excision repair is known to act preferentially on transcribed genes (Hanawalt, 1991). Additionally, it has recently been shown that DNA damage in transcribed genes is more efficient at activating p53 than damage in non-transcribed regions (Yamaizumi and Sugano, 1994). This might suggest that there is also a DNA damage detection system dependent on transcription. The simplest way to imagine such a system would be that some factor recognizes the transcription machinery stalled at a DNA damage site or that such a stalled complex itself emanates some signal. If TFIIH is associated with the elongating RNA polymerase (Yankulov *et al.*, 1995, but see also Zawel *et al.*, 1995) or can join the stalled RNA polymerase, it would be a good candidate to transmit or receive such a signal. This may be possible since TFIIH is the only basal transcription factor associated with enzymatic activities that could behave differently as a function of the cell cycle and of course change upon an external stress such as UV irradiation. One could for example imagine that a change in CAK activity after DNA damage could, at the same time, induce a change in transcriptional activity and a cell cycle arrest. Our results are partially compatible with such a model, as TFIIH-associated kinase activity does indeed diminish after DNA damage. Variation or inhibition of phosphorylation of linker histones, which is believed to effect changes in the stability of condensed chromatin, would thus, as a result, impede the access of transcription factors to DNA. The excess of free CAK which exists outside TFIIH does not seem to be affected by UV-induced DNA damage. It is not surprising since one would not expect free CAK to encounter DNA lesions as part of a transcription complex. On the other hand, this means that a cell cycle arrest due to the DNA damage cannot be due to lack of bulk CAK activity. However, we cannot exclude that the part of CAK associated with TFIIH is essential *in vivo* also in its cdk-activating function.

An understanding of the physiological role of this reduction of TFIIH-associated kinase activity in UV-irradiated cell might finally permit us to integrate cdk7 into a signal transduction pathway and thus throw new light onto the links between transcription, DNA repair and the cell cycle.

Materials and methods

Immunological methods

Monoclonal antibody Ab-cdk7 was directed against the cdk7-derived peptide PVETLKEQSNPALAIKRK, Ab-cyclin H against the 20 C-terminal amino acids of cyclin H, and Ab-MAT1 against a microsequenced peptide: LEEALYEYQPLQIETYG.

For immunoprecipitations, the fraction to be tested was diluted 10-fold in 20 mM Tris–HCl buffer, pH 7.5, 100 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 0.1% Nonidet P-40 (NP40) and 10 μl of protein A–Sepharose beads cross-linked with antibodies were added. After 1 h of shaking at 4°C the beads were washed four times in the same buffer and once in kinase buffer (Roy *et al.*, 1994b). An aliquot was used for kinase assays and the rest taken up in SDS sample buffer.

Cell culture and UV irradiation

JEG-3 cells were grown in minimal essential medium (MEM) with 10% FCS. For irradiation, culture dishes were washed once in PBS, the PBS was almost completely aspirated and the cells exposed to UV light (40 J/m^2 at 254 nm; measured by a Bioblock VLX-3W UV meter). Subsequently, prewarmed culture medium was added, and the cells were collected at different times after UV irradiation.

For the cell cycle analysis, cells were arrested by treatment with either 1 μg/ml aphidicolin or 0.2 μg/ml nocodazol or MEM with 0.2% FCS and subsequently lysed or released by washing in MEM without serum and re-addition of MEM with 10% FCS. FACS analysis of ethanol-fixed, ethidium bromide-stained cells was performed according to Crissman and Hirons (1994).

Expression and purification of the recombinant kinase complex in insect cells

The mutation of cdk7 is a substitution of Lys41 by arginine (J.P.Tassan and E.A.Nigg, unpublished results). The *Eco*RI fragments containing the entire coding sequences of cdk7, mutant cdk7 or MAT1 were inserted into the *Eco*RI site of the pVL 1392 plasmid (PharMingen). A *Bam*HI fragment comprising the coding sequence of cyclin H was inserted into the *Bam*HI site of a modified pVL 1392 plasmid with an inserted histidine tag.

Monolayer Sf9 cells (typically, 1.2×10^8 cells) were infected with cdk7, cdk7m and cyclin H and MAT1 baculoviruses. The viruses coding for cdk7, cdk7m and cyclin H were used at a multiplicity of infection (m.o.i.) of 2 plaque-forming units each per cell, the one coding for MAT1 at a m.o.i. of 10. At 2 days post-infection, the cells were washed once with PBS containing 30% glycerol and then lysed at 4°C in 4 ml of 20 mM Tris–HCl buffer, pH 7.5, 20% glycerol, 150 mM NaCl, 5 mM β-mercaptoethanol, 0.1% NP40, 0.5 mM PMSF, 2.5 μg/ml each leupeptin, pepstatin, aprotinin, antipain and chimostatin. The lysate was frozen and thawed three times and then clarified by centrifugation (30 min at 16 000 *g* at 4°C).

Lysates were purified on a nickel ion chelated affinity column (His-Bind™ Resin, Novagen) following the manufacturer's procedure. Briefly, 4 ml of extract dialysed into the adsorption buffer (20 mM Tris–HCl buffer, pH 7.5, 250 mM NaCl) containing 5 mM imidazole were loaded onto 0.6 ml of the resin, and extensively washed with the adsorption buffer containing 40 mM imidazole. Elutions were performed sequentially with 5×0.6 ml of the adsorption buffer containing 100 mM and 500 mM imidazole. The eluted peak fractions were then pooled, dialysed against 50 mM Tris–HCl buffer, pH 7.5, 10% glycerol, 0.1 mM EDTA, 0.5 mM DTT, 50 mM KCl and incubated with protein A–Sepharose cross-linked with Ab-cdk7 antibodies for 3 h at 4°C. After extensive washing with the dialysis buffer containing 250 mM KCl and subsequently 50 mM KCl, the different recombinant kinase complexes were eluted with the peptide corresponding to the epitope (3.5 mg/ml) in dialysis buffer.

Other methods

Protein purification, glycerol gradients, immunoprecipitations and the kinase assays using ctd4 and GST-cdk were performed as previously described (Gérard *et al.*, 1991; Roy *et al.*, 1994b).

Acknowledgements

We thank Pierre Chambon and Jan H.Hoeijmakers for ideas, discussion and criticism. We are grateful to Marcel Dorée, David Morgan, John Feaver and Roger Kornberg for the communication of unpublished results and for fruitful discussions. We thank Pascal Eberling, Adrien Staub and Yves Lutz for protein microsequencing, peptide synthesis and monoclonal antibodies respectively. Richard Roy prepared the first MAT1 for microsequencing and Annabel Fery provided expert technical assistance. We are also grateful to Caroline Waltzinger for FACS analysis and Isabelle Cheynel for baculovirus expression. J.P.A. was sponsored by an EMBO long-term fellowship, and M.R. by a studentship of CNRS/Région Alsace. This work was supported by grants from the INSERM, the CNRS, the Centre Hospitalier Regional de Strasbourg and the Association pour la Recherche sur le Cancer. J.P.T. and E.A.N. acknowledge support by the Swiss National Science Fondation and the Swiss Cancer League.

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Received on September 13, 1995; revised on December 5, 1995