Cdk-activating kinase complex is a component of human transcription factor TFIIH

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TRANSCRIPTION factor IIH (TFIIH) contains a kinase capable of phosphorylating the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II (RNAPII)[1–3]. Here we report the identification of the Cdk-activating kinase (Cak) complex (Cdk7 and cyclin H) as a component of TFIIH after extensive purification of TFIIH by chromatography. We find that affinity-purified antibodies directed against cyclin H inhibit TFIIH-dependent transcription and that both cyclin H and Cdk7 antibodies inhibit phosphorylation of the CTD of the largest subunit of the RNAPII in the preinitiation complex. Cak is present in at least two distinct complexes, TFIIH and a smaller complex that is unable to phosphorylate RNAPII in the preinitiation complex. Both Cak complexes, as well as recombinant Cak, phosphorylate a CTD peptide. Finally, TFIIH was shown to phosphorylate both Cdc2 and Cdk2, suggesting that there could be a link between transcription and the cell cycle machinery.

Immunoaffinity purification using antibodies directed against the 62K subunit of TFIIH was used to identify any CTD kinase

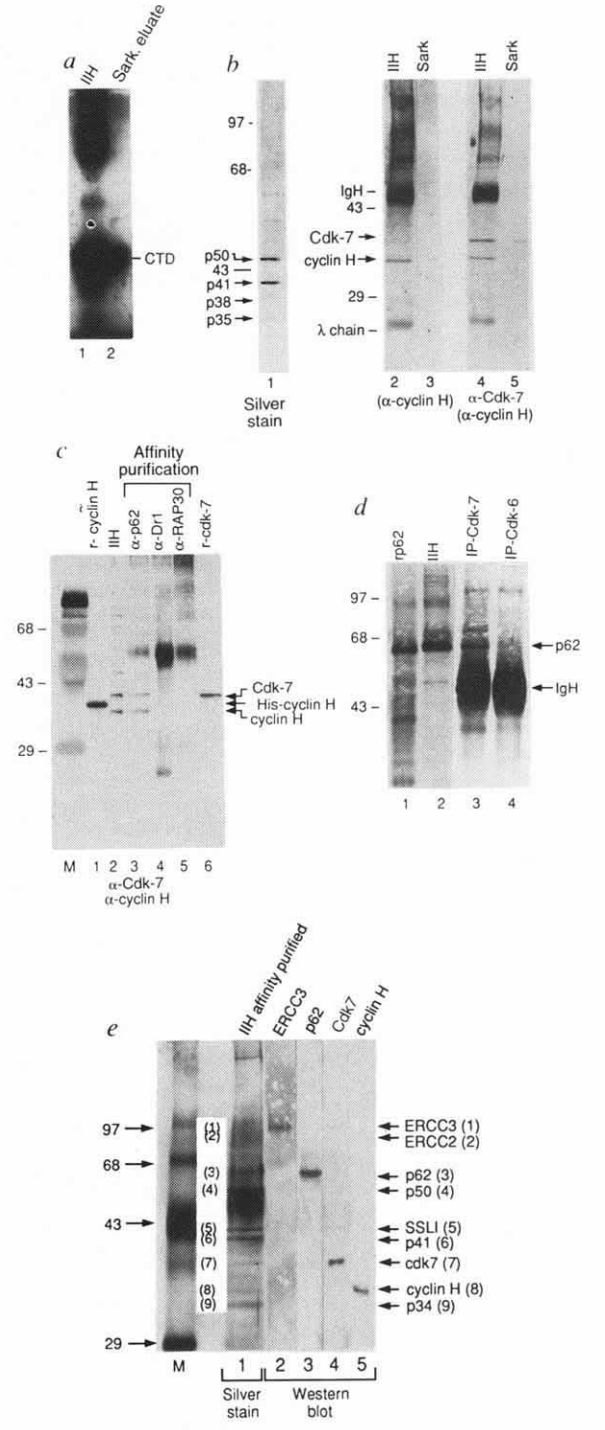

FIG. 1 *a*, The Sarkosyl eluate contains CTD kinase activity. lane 1, TFIIH (phenyl-Superose, 230 ng); lane 2, Sarkosyl eluate (5 μl). Labelled products near the origin of the gel in lane 1 represent phosphorylation of other proteins in the phenyl-Superose preparation. The phosphorylation assay has been described[1]. *b*, Silver-stained and western blot analysis of polypeptides dissociated from immunoaffinity-purified TFIIH by the Sarkosyl wash. lane 1, silver stain of the peak Sarkosyl fraction. Arrows indicate enriched polypeptides in this fraction. Lanes 2–5, western blot of Cdk7 and cyclin H in an immunopurified TFIIH (lanes 2 and 4) and Sarkosyl eluate of the p62 immunoaffinity purification (lanes 3 and 5). Lanes 2 and 3 of the blot were incubated with cyclin H antibodies only. Lanes 4 and 5, western blot incubated with both cyclin H and Cdk7 antibodies. Arrows to the left indicate cyclin H and Cdk7. IgH represents the immunoglobulin heavy chain; λ chain is the immunoglobulin light chain. Molecular weight markers (in thousands) are indicated at the left. *c*, Identification of Cdk7 and cyclin H as components of TFIIH using immunoaffinity chromatography with p62 antibodies coupled to protein A–agarose. Lanes: 1, recombinant hexahistidine-tagged cyclin H (50 ng); 2, TFIIH (phenyl–Superose 2.3 μg); 3, anti-p62 eluate; 4, anti-Dr_1 eluate; 5, anti-RAP30 eluate; 6, recombinant Cdk7 (50 ng). Arrows to the right indicate the position of Cdk7, cyclin H, and His-tagged cyclin H. Molecular weight markers are indicated at the left of the gel. Purification was as already described, except that the column was eluted with 100 mM glycine, pH 2.6, after the low-salt wash. *d*, Cdk7 antibodies, but not Cdk6 antibodies, immunoprecipitate the 62K subunit of TFIIH. Lanes: 1, recombinant p62 (100 ng); 2, TFIIH (phenyl-Superose, 2.6 μg); 3 and 4, as indicated. *e*, Silver-stained and western blot analysis of TFIIH was resolved on the same gel and directly compared with known TFIIH subunits. Lanes: 1, silver staining of immunopurified TFIIH; 2–5, western blots of TFIIH (phenyl-Superose, 2.3 μg) using the antibodies indicated (top). Arrows on the right depict known TFIIH polypeptides. Numbers to the right of the figure correspond to the polypeptides indicated by arrows on the left of the gel. Molecular weight markers are shown by arrows on the left. The same polypeptides are also shown in the silver stain of Fig. 4.

METHODS. In *b*, polyclonal rabbit antibodies (2 mg) directed against the p62 subunit of TFIIH were covalently crosslinked to 1 ml protein A–agarose using 20 mM dimethylpimelimidate as described[13]. The resin was incubated for 90 min at 4 °C with nuclear extract (4 ml, 9 mg ml^{-1}) prepared as described[14] and dialysed against buffer containing 20 mM Tris–HCl, pH 7.9, 0.2 mM EDTA, 50 mM NaF, 100 μM $NaVO_3$, 100 mM KCl, 1% N-P40 and 1 mM PMSF. Immunoadsorbed TFIIH was then washed with 10 ml of this buffer containing 1 M KCl and 1% N-P40. This was followed by a low-salt wash (100 mM KCl; 10 ml) and elution in low-salt buffer containing 0.05% Sarkosyl; remaining bound material was washed again in low-salt buffer and eluted with 0.1 M glycine-HCl, pH 2.6.

activity associated with this factor. In an attempt to dissociate the polypeptide(s) containing kinase activity, the immunoadsorbed TFIIH complex was treated with the ionic detergent Sarkosyl. The remaining immunoadsorbed TFIIH polypeptides were eluted at low pH (a glycine wash). Analysis of the Sarkosyl eluate revealed the presence of a kinase activity capable of phosphorylating a tetra-heptapeptide repeat (YSPTSPS) of the CTD complex (Fig. 1*a*), but not the CTD of RNAPII in the preinitiation complex (data not shown). Silver-stain analysis of this fraction indicated the presence of polypeptides ranging in size from 35K to 50K (Fig. 1*b*, lane 1). Cdk-related kinases phosphorylate the CTD[4–6], and we observed that the molecular mass of the

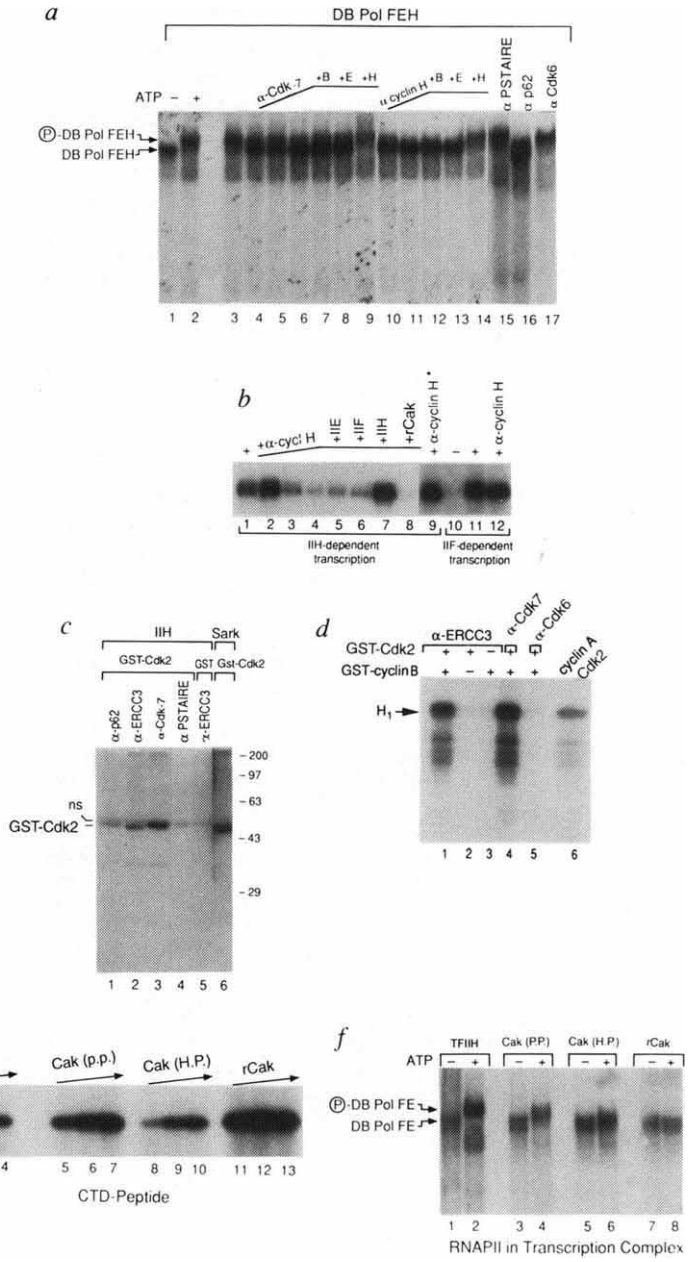

FIG. 2 *a*, Affinity-purified antibodies against Cdk7 and cyclin H inhibits the phosphorylation of RNAPII in the preinitiation complex. All antibodies were incubated with TFIIH for 1 h at 4 °C before addition to the binding reaction (lanes 4–17). Lanes: 1 and 2, complete complex in the absence and the presence of 1 mM ATP, respectively; 3–17, complete complex in the presence of 1 mM ATP (in lane 3, TFIIH was incubated in the absence of any antibody, and in lanes 4–6, TFIIH was incubated with increasing concentrations of affinity-purified Cdk7 antibodies (1, 2, 3 μg, respectively); 10 and 11, TFIIH incubated with 150 and 250 ng affinity purified cyclin H antibodies, respectively; 7–9, incubation of TFIIH with 3 μg Cdk7 antibodies, after which 2 × TFIIB (20 ng, lane 7), 2 × TFIIE (lane 8), or 2 × TFIIH (270 ng, lane 9) was added to the reaction; 12–14, products of reactions in which TFIIH was incubated with 250 ng cyclin H antibodies, after which 2 × TFIIB (20 ng, lane 12), 2 × TFIIE (lane 13), or 2 × TFIIH (270 ng, lane 14) was added; 14–17, incubation of TFIIH with 600 ng PSTAIRE, p62 or Cdk6 antibodies. DNA-binding reactions have been described[1]. The faster-migrating complex contains unphosphorylated RNAPII and is designated (DBPolFEH); the slower-migrating complex contains phosphorylated RNAPII (P-DBPolFEH). *b*, Cyclin H antibodies inhibit TFIIH-dependent transcription but not TFIIF-dependent transcription. Lane 1 shows the product of a complete TFIIH-dependent reaction; lanes 2–4 contain partially purified TFIIH incubated with increasing amounts of affinity-purified cyclin H antibodies (50, 150 and 250 ng, respectively). In the remaining lanes, TFIIH was first incubated with 250 ng cyclin H antibodies, after which the complete reaction mixture, together with saturating amounts of recombinant TFIIE (lane 5). TFIIF (lane 6), TFIIH (phenyl-Superose, lane 7) or rCAK (lane 8), was added to each tube. Lane 9, transcription reaction in which 250 ng cyclin H antibodies were added to a complete transcription mix; lanes 10 and 11, transcription reactions in the presence and the absence of TFIIF, respectively; lane 12, incubation of TFIIF with cyclin H antibodies for 1 h before addition to the transcription reaction. Transcription reactions have been described[15]. *c*, Phosphorylation of Cdk2 by TFIIH. TFIIH or CAK complexes were immunoprecipitated using p62, ERCC3, Cdk7 or PSTAIRE antibodies as described[10], except the beads were used in a kinase assay. Lanes 1–4, phosphorylation of GST–Cdk2 by immunoprecipitated complexes indicated (top); lane 5, control, using GST as the substrate; lane 6, Sarkosyl wash of the p62 immunoaffinity-purified TFIIH phosphorylates GST–Cdk2. ns, Nonspecific band. Phosphorylation was assayed as described[8]. ERCC3 monoclonal antibodies were produced in collaboration with Austral Biological-Bios, Chile. *d*, Histone H1 phosphorylation by Cdk2 in the absence or presence of cyclin B. Lanes 1–5, complexes immunoprecipitated using the antibodies shown (top); lane 6 is histone H1 phosphorylation by cyclin A–Cdk2. Activation was assayed as described[8]. *e*, Multiple Cak complexes can phosphorylate the CTD peptide. CTD peptide was incubated with different Cak-containing complexes as indicated (top). The amount of Cak complexes used in the assays in *e* and *f* were: TFIIH (2 μl of fraction 27 of gel filtration step (Fig. 4); highly purified Cak (H.P.) corresponding to a fraction from a phosphocellulose column (40 ng)[8]; partially purified Cak (P.P.) is a fraction from the S200 sizing column (1 μg)[8]. Recombinant Cak is composed of recombinant His–cyclin H and baculovirus-expressed Cdk7 (50 ng Cdk7 and 100 ng cyclin H). *f*, Gel mobility-shift assay showing the effect of different Cak complexes on the phosphorylation of RNAPII in the preinitiation complex.

proteins in our Sarkosyl eluate resembled that of Cdk-related kinases (M_r 30K to 40K). We therefore analysed purified TFIIH fractions for the presence of cell-cycle-related kinases and found that TFIIH is devoid of any immunoreactive material representing the kinases Cdk1–Cdk6, and PCTAIRE 1–3 (kinases with homology to Cdks) (data not shown). However, human Cak, composed of Cdk7/MO15 and cyclin H, contains polypeptides ranging in size from 35K to 40K[7,8]. Western blot analysis of highly purified TFIIH, affinity-purified TFIIH, or Sarkosyl eluate revealed the presence of material immunoreactive with Cdk7 and cyclin H in all three preparations (Fig. 1*b*, lanes 2–5 and Fig. 1*c*, lanes 2 and 3). The presence of Cak polypeptides in these fractions was specific, as eluates derived from Dr_1 or RAP30 affinity columns were devoid of Cdk7 and cyclin H (Fig. 1*b*, lanes 4 and 5). Furthermore, immunoprecipitation of TFIIH using Cdk7 (Fig. 1*d*, lane 3) or cyclin H (R.F. and D.M., unpublished observations) antibodies revealed P62 immunoreactive material. This p62 immunoreactivity was not evident with Cdk6 antibodies (Fig. 1*d*; compare lanes 3 and 4). Silver-stained and western blots of the p62-immunopurified TFIIH complex is shown in Fig. 1*e*. In addition to Cdk7 and cyclin H, other cloned TFIIH polypeptides and putative subunits are present in this preparation (arrows in Fig. 1*e*). Thus we have immunological evidence for the presence of Cdk7 and cyclin H in the TFIIH complex.

We previously demonstrated that when the complete transcription initiation complex (DBPolFEH) is incubated with ATP, the mobility of the complex is retarded as a result of CTD phosphorylation by TFIIH in a gel mobility shift assay (refs 1, 6, 9; Fig. 2*a*, lanes 1 and 2). Incubation of TFIIH with increasing amounts of affinity-purified antibodies against Cdk7 (lanes 4–6), cyclin H (lanes 10 and 11) or p62 (lane 16) before its addition to the reaction resulted in a change in migration of the complex: the complex now co-migrated with the complete complex formed in the absence of ATP (lane 1). Inhibition of phosphorylation was overcome by addition of excess TFIIH (lanes 9 and 14) but not by excess TFIIB or TFIIE (lanes 7, 12 and 8, 13, respectively). Furthermore, antibodies directed against the PSTAIRE motif or Cdk6 failed to inhibit the ATP- and TFIIH-dependent shift (lanes 15 and 17).

Incubation of TFIIH with increasing concentrations of cyclin H antibodies before its addition in the reconstituted transcription assay resulted in inhibition of transcription (Fig. 2*b*, lanes 3 and 4). At low concentrations, cyclin H antibodies had a mild stimulatory effect on transcription (Fig. 2*b*, lane 2). The dose-dependent inhibition was specific as it was overcome by addition of excess TFIIH (lane 7), but not by excess TFIIE or TFIIF (lanes 5 and 6). This inhibition was not due to a nonspecific inactivation of TFIIH by the antibodies, as incubation of these antibodies with TFIIF did not affect transcription (Fig. 2*b*, lanes 10–12). Interestingly, incubation of TFIIH with antibodies against Cdk7 was without effect in transcription (data not shown). Inhibition of transcription by antibodies against cyclin H may result from exclusion of TFIIH from the preinitiation complex, consistent with the lack of effect when cyclin H antibodies were added after the preinitiation complex was formed (Fig. 2*b*, lane 9). As TFIIH has a dual role in transcription and nucleotide-excision repair[10–12], the effect of Cdk7 antibodies was

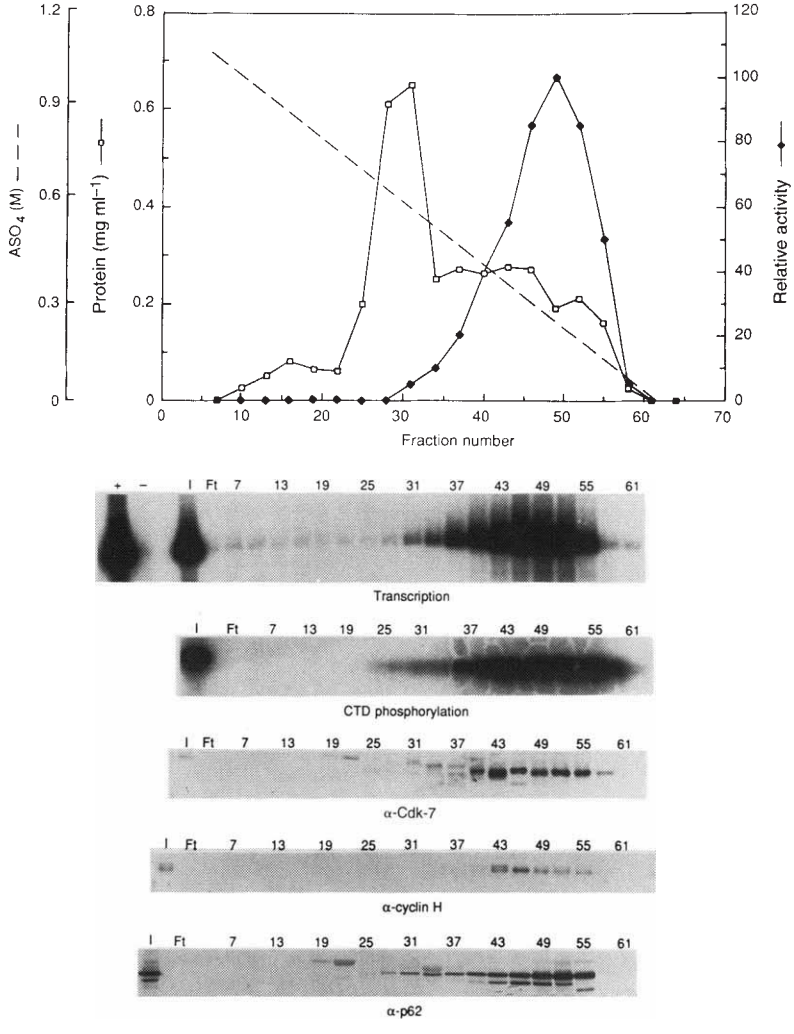

FIG. 3 TFIIH transcription activity copurifies with p62, cyclin H and Cdk7 polypeptides, and with CTD-peptide kinase activity on phenyl–Superose chromatography. METHODS. TFIIH was purified from 5.5 g HeLa nuclear extracts and fractionated as described through the fourth chromatographic step[10]. Transcription reactions were performed as described[10] except that TFIIH was omitted from the general transcription factor mix and substituted with the input (I) to the column, the flow-through (Ft), or the fractions indicated at the top of each lane.

tested in a reconstituted nucleotide-excision repair reaction where, in contrast to their effect in transcription, they were inhibitory (D. Mu, R.S., R.D., A. Sancar and D.R., unpublished observation). It is not clear why Cdk7 antibodies inhibit phosphorylation of the CTD as well as nucleotide-excision repair functions of TFIIH, while they have no effect in transcription. The epitope recognized by the antibodies could be critical for one function and not the other. Nonetheless, the inhibition of phosphorylation, transcription and nucleotide-excision repair by antibodies directed against Cak components demonstrates that Cak polypeptides are integral components of the TFIIH complex.

We next examined whether the TFIIH complex has Cak activity. Analysis of immunopurified complexes using p62, ERCC3 or Cdk7 antibodies indicated that all three complexes phosphorylate the glutathione-*S*-transferase fusion protein GST–Cdk2 (Fig. 2*c*). The extent of Cdk2 phosphorylation varied among the three immunopurified complexes, with Cdk7 being the most active. This may be due to the ability of Cdk7 antibodies to precipitate other Cak-containing complexes as well as TFIIH (see below). The lower activity observed with p62 antibodies may be due to a partial inhibition of the kinase activity by these antibodies (Fig. 2*a*, lane 16). A PSTAIRE immunoprecipitated complex was devoid of any activity (Fig. 2*c*, lane 4). No kinase activity was detected when GST alone was used as a substrate (Fig. 2*c*, lane 5). The Sarkosyl eluate of a complex immunopurified using p62 antibodies could also phosphorylate Cdk2 (Fig. 2*c*, lane 6). We also tested for histone H1 activation by immunopurified TFIIH complexes using ERCC3 or Cdk7 antibodies. Figure 2*d* shows that a TFIIH complex immunopurified using ERCC3 as well as Cdk7 antibodies can activate Cdk2 in a cyclin-dependent manner (compare lane 2 with lanes 1 and 4). Cdk6 immunopurified complexes were devoid of any Cak activity (Fig. 2*d*, lane 5); results were similar when Cdc2 was used as substrate (data not shown). Together, these results demonstrate that Cak, in the context of the TFIIH complex, can activate Cdk-related kinases.

We next analysed the CTD-phosphorylating activity of recombinant Cak. Unlike TFIIH, which could phosphorylate the CTD of RNAPII in the preinitiation complex, rCak could not (Fig. 2*f*; compare lanes 1 and 2 with 7 and 8); however, it could efficiently use the CTD peptide as a substrate (Fig. 2*e*, lanes 11–13). Furthermore, we found that although either a partially or highly purified preparation of native Cak could act on the CTD peptide (Fig. 2*e*, lanes 5–10), only the less pure preparation could phosphorylate the CTD in the preinitiation complex (Fig. 2*f*, lanes 3–6). These results not only show that Cdk7 and cyclin H polypeptides (Cak) recognize the CTD heptapeptide sequence as a substrate, but they also suggest that there could be at least two different Cak-containing complexes, TFIIH and a complex that is unable to phosphorylate the CTD of RNAPII. In support of this, we found that neither highly purified native Cak nor rCak could replace TFIIH in the transcription assay (data not shown).

In an attempt to isolate different Cak-containing complexes, we assayed fractions from the different steps of the TFIIH purification for TFIIH-dependent transcription, CTD phosphorylation and Cdk2 activation (measured by phosphorylation of histone H1 activity), as well as for the presence of p62 (a core subunit of TFIIH), cyclin H and Cdk7 polypeptides by western blot. This analysis revealed that transcription, as well as CTD-peptide kinase activity copurified with p62, cyclin H and Cdk7 immunoreactivity at the fourth step of purification (phenyl-Superose chromatography; Fig. 3). But further fractionation of

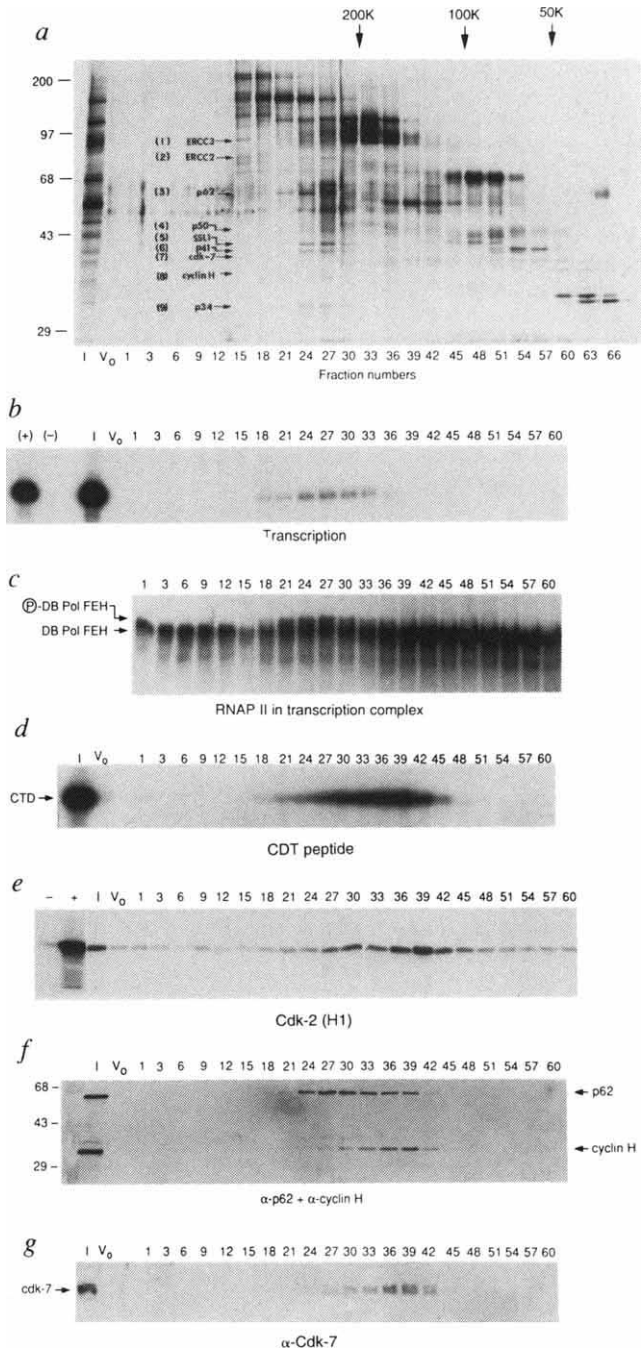

FIG. 4 Fractionation of TFIIH on gel filtration chromatography reveals the presence of two Cak-containing complexes. *a*, Silver staining of the fractions eluted from the gel-filtration column. I, input to the column; Vo, void volume. Arrows at the top of the gel represents the elution of molecular weight markers. Fraction numbers are indicated at the bottom. Migration of molecular weight protein standards is indicated on the left. The subunits of the TFIIH complex are indicated by arrows. The numbers indicated to the left of the TFIIH subunits correspond to the polypeptides observed in the affinity-purified TFIIH shown on Fig. 1*e*. *b*, TFIIH-dependent transcription analysis of gel-filtration column. *c*, Phosphorylation of RNAPII within the preinitiation complex. *d*, Phosphorylation of CTD tetra-heptapeptide. *e*, Cak activity as measured by activation of Cdk2 histone H1 activity. *f*, Western blot analysis for TFIIH (p62) and cyclin H polypeptides. *g*, Western analysis for Cdk7 protein.
METHODS. *a*, The active TFIIH fractions from the phenyl-Superose column (Fig. 3) were pooled and a portion (1.2 mg) fractionated on a Pharmacia Mono S 5/5 column. The transcriptionally active fractions were pooled (800 μg) and fractionated on a Pharmacia Superdex-200 16/60 column in the presence of 1.0 M KCl and 0.01% N-P40. Fractions were subjected to SDS–PAGE and silver staining. Activation of Cdk2 measured as phosphorylation of histone H1 was done as described[8]. 1 μg Cdk2 bound to protein A-Sepharose was incubated with 10 μl of each fraction.

TFIIH on a gel filtration column apparently separated two distinct Cak-containing complexes. The larger complex (best represented by fractions 24 and 27) contained both TFIIH-dependent transcription (Fig. 4*b*) and kinase activities (measured as phosphorylation of the CTD of RNAPII within the preinitiation complex; Fig. 4*c*). Both activities were weak, perhaps as a result of dilution by the sizing-column fractionation (fractions 18–39, peaking at fractions 24 and 27; Fig. 4*b*, *c*). These activities coeluted with polypeptides containing TFIIH (Fig. 4*a*; also western blot of p62 in Fig. 4*f*). However, the peak of CTD-peptide phosphorylation as well as Cak activity (Cdk2 phosphorylation of histone H1) was shifted to fraction 39, away from the peak of transcription. Similarly, western blot analysis of Cdk7 and cyclin H polypeptides indicated that the peak of Cdk7 and cyclin H immunoreactivity coincided with that of Cdk2 activation and CTD-peptide phosphorylation (Fig. 4*f*, *g*). Cdk7 and cyclin H immunoreactivity extended from fraction 18 to 45 (Fig. 4*f*, *g*), indicating that all fractions containing transcription activity also contained Cdk7- and cyclin-H-immunoreactive material. These results suggest the presence of at least two distinct Cak-containing complexes, a larger transcriptionally active TFIIH ($M_r > 200$K) complex and a smaller complex unable to phosphorylate RNAPII in the preinitiation complex. The smaller Cak complex(es) may lack components of TFIIH that tether the factor to the preinitiation complex. Alternatively, Cak may acquire a different substrate specificity upon interacting with the other TFIIH subunits. Also, Cak may be activating another CTD kinase within the transcription complex that is capable of phosphorylating the polymerase.

TFIIH phosphorylation of Cdk-related kinases raises the question of whether TFIIH is involved in cell-cycle regulation. Our results indicate that most of the Cak is in a complex(es) distinct from TFIIH, and that the direct involvement of TFIIH in cell-cycle progression is unlikely. However, TFIIH, by phosphorylating Cdc2 or Cdk2, may play a role in the modulation of transcription and DNA repair during the cell cycle.

Note added in proof: While this Letter was under review, a report appeared demonstrating that MO15 is a component of human TFIIH (ref. 16), and another indicated that KIN28 is the MO15-homologue in yeast TFIIH (ref. 17). □

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Site-specific RNase E cleavage of oligonucleotides and inhibition by stem–loops

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THE enzyme RNase E (ref. 1) cuts RNA at specific sites within single-stranded segments[2–6]. The role of adjacent regions of secondary structure in such cleavages is controversial[7–10]. Here we report that 10–13-nucleotide oligomers lacking any stem–loop but containing the RNase E-cleaved sequence of RNA I[11–13], the antisense repressor of replication of ColE1-type plasmids, are cut at the same phosphodiester bond as, and 20 times more efficiently than, RNA I. These findings indicate that, contrary to previous proposals[8,9], stem–loops do not serve as entry sites for RNase E, but instead limit cleavage at potentially susceptible sites. Cleavage was reduced further by mutations in a non-adjacent stem–loop, suggesting that distant conformational changes can also affect enzyme access. Modulation of RNase E cleavages by stem–loop regions and to a lesser extent by higher-order structure may explain why this enzyme, which does not have stringent sequence specificity[6,12,13], cleaves complex RNAs at a limited number of sites.

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RNase E cleaves RNAs of known secondary structure (such as RNA I, 9S ribosomal RNA, S20 messenger RNA) within single-stranded regions rich in A + U nucleotides[6,8,11] (see Fig. 1*a* for pBR322 RNA I). Mutational modification of the single-stranded segment bracketing the RNase E cleavage site at the 5′ end of RNA I encoded by the pBR322 or pACYC184 plasmid indicates that there is no simple relationship between the order of nucleotides and the phosphodiester bond cleaved[11,12]. Although nearby regions of secondary structure can affect RNase E cleavages[6–10,12], the actual role of such regions has been unclear. Base pairing of nucleotides at the 5′ terminus of RNA can interfere with RNase E cleavage *in vivo*[7]. However, internally located stem–loops positioned 5′ to RNase E sites have been proposed to promote cleavage, possibly by enabling loading of the enzyme onto the substrate[8]. Others have suggested that stem–loops 3′, rather than 5′, to the site of cleavage are necessary for the entry of RNase E, which then scans the upstream segment for a sequence that can be cleaved[9]. Still another proposal is that stem–loops adjacent to RNase E cleavage sites may function as anchors to maintain the cleaved region in a single-stranded conformation[8,10].

To learn whether a stem–loop is in fact required as an entry site for RNase E, we determined whether short oligoribonucleotides that lack the potential to form stem–loops can serve as substrates. As shown in Fig. 2, RNase E purified from *Escherichia coli* by immobilized metal affinity chromatography, cleaved two decaribonucleotides, BR10 (5′-ACAGUAUUUG) and ACYC10 (5′-ACAAGUUUUG), which correspond to the 5′ ends of RNA I from pBR322 and pACYC184, respectively, at the same phosphodiester bonds found previously to be cleaved by RNase E in the corresponding native RNA I species[11,13]. Furthermore, affinity-purified RNase E also cleaved a tridecaribonucleotide, BR13 (5′-GGGACAGUAUUUG; Fig. 2) identical in sequence to the 5′ end of GGG.RNA I[14], a T7 RNA