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The Yeast Carboxyl-Terminal Repeat Domain Kinase CTDK-I Is a Divergent Cyclin–Cyclin-Dependent Kinase Complex

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Saccharomyces cerevisiae **CTDK-I is a protein kinase complex that specifically and efficiently hyperphosphorylates the carboxyl-terminal repeat domain (CTD) of RNA polymerase II and is composed of three subunits of 58, 38, and 32 kDa. The kinase is essential in vivo for normal phosphorylation of the CTD and for normal growth and differentiation. We have now cloned the genes for the two smaller kinase subunits, CTK2 and CTK3, and found that they form a unique, divergent cyclin–cyclin-dependent kinase complex with the previously characterized largest subunit protein CTK1, a cyclin-dependent kinase homolog. The *CTK2* gene encodes a cyclin-related protein with limited homology to cyclin C, while CTK3 shows no similarity to other known proteins. Copurification of the three gene products with each other and CTDK-I activity by means of conventional chromatography and antibody affinity columns has verified their participation in the complex in vitro. In addition, null mutations of each of the genes and all combinations thereof conferred very similar growth-impaired, cold-sensitive phenotypes, consistent with their involvement in the same function in vivo. These characterizations and the availability of all of the genes encoding CTDK-I and reagents derivable from them will facilitate investigations into CTD phosphorylation and its functional consequences both in vivo and in vitro.**

The largest subunit of eukaryotic RNA polymerase II (Pol II) contains a unique carboxyl-terminal domain (CTD) which in most eukaryotes consists of multiple heptapeptide repeats with the consensus sequence YSPTSPS (for reviews, see references 10 and 71). The CTD is essential for RNA polymerase function in vivo (5, 50, 72) and in unfractionated in vitro systems (44), but it is not required for accurate initiation in fractionated systems using purified general transcription factors. Earlier suggestions that the CTD interacts with factors involved in transcription initiation have received experimental support, especially in the recent descriptions of Pol II holoenzyme (33, 34). Several other roles for the CTD, including postinitiation functions, have been suggested, but most await experimental tests (e.g., reference 19).

The CTD has been shown to exist in both unphosphorylated and highly phosphorylated forms in vivo, and CTD kinase activities have been identified in various organisms (reviewed in reference 11). The first of these activities purified, *Saccharomyces cerevisiae* CTDK-I, efficiently hyperphosphorylates the CTD on both native Pol II and bacterially produced fusion proteins, and it consists of three subunits of 58, 38, and 32 kDa (α, β, and γ subunits, respectively) (39). The gene for the α subunit, *CTK1*, was cloned and found to encode a novel cdc2/CDC28-related kinase (also called cyclin-dependent kinase [CDK]), apparently the catalytic subunit of the CTDK-I complex. Null mutant *ctk1* strains lacked CTDK-I activity and displayed slow growth, abnormal morphology, defective sporulation, and other defects at normal temperatures, and they failed to grow at low temperatures (cold-sensitive phenotype); experiments with phospho-CTD-specific antibodies revealed that these mutant strains were grossly deficient in the normally phosphorylated form of the Pol II CTD in vivo (40). These properties, combined with its high in vitro efficiency and discrete substrate specificity, strongly implicate CTDK-I as a transcriptionally important CTD kinase.

Several other complexes with CTD kinase activity in vitro have subsequently been purified and characterized in terms of subunit composition; like CTDK-I, each contains a CDK-related component. Two complexes purified from mouse cells on the basis of the ability to phosphorylate a synthetic peptide substrate contained cdc2 (8, 9). More recently, CTD kinase catalytic activities associated with transcription factor TFIIH were found to reside in CDK homologs, namely, MO15 (also called cdk7) in human factor and KIN28 in yeast factor (16, 59). However, these subunits are known to be involved in other in vitro and in vivo activities as well: cdc2 is integral to cell cycle control and phosphorylates a number of different substrates in vitro, and MO15 acts as a CDK-activating kinase which regulates cdc2 and cdk2 by phosphorylation. It is also notable that TFIIH (including kinase) is required for transcription initiation in a purified system dependent on a form of Pol II entirely lacking a CTD (44, 47). Thus, the relationship between CTD kinase function and each of these activities is still unclear.

With the catalytic subunit of CTDK-I known to be a CDK homolog, it was desirable to clone the genes for the two remaining subunits in order to better characterize the complex. These genes could be used for further genetic studies and could engender reagents for biochemical experiments; furthermore, their sequences could provide additional insights into the function of CTDK-I. Through antibody screening of an expression library and partial protein sequencing of purified CTDK-I subunits, we have cloned the genes for the β and γ subunits, *CTK2* and *CTK3*. Here we report the sequences of these genes, one of which appears to encode a divergent cyclin homolog. In addition, we present new biochemical evidence that the subunits are indeed part of the same complex, and we report null mutant studies which indicate that these subunits are important for CTDK-I function in vivo.

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MATERIALS AND METHODS

Strains and media. The primary *S. cerevisiae* strains used in this study were YPH274 (**a**/α *ura3-52*/*ura3-52 lys2-801*/*lys2-801 ade2-101*/*ade2-101 trp1-Δ1*/*trp1-Δ1 his3-Δ200*/*his3-Δ200 leu2-Δ1*/*leu2-Δ1*) and its parent strains YPH250 (**a** *ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1*) and YPH252 (α *ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1*), all described by Sikorski and Hieter (65). Cells were grown in YPD liquid medium (1% yeast extract, 2% peptone, 2% glucose) or on plates (with 2% agar) of YPD or minimal medium (2% glucose, 0.67% yeast nitrogen base without amino acids) supplemented with appropriate nutrients as described by Sherman et al. (63). Yeast extract, peptone, and agar were from Difco, and supplements were from Sigma.

Escherichia coli XL-1 Blue (7) from Stratagene was used for the maintenance and production of plasmids, and BMH 71-18 (49) was used for the production of fusion proteins. These strains were typically cultured in LB medium (61).

CTDK-I, antibodies, and protein sequencing. CTDK-I was purified from dry commercial yeast by the method of Lee and Greenleaf (39). For antibody affinity purification, β- and γ-subunit protein was bound to nitrocellulose membrane (0.45-μm pore size; Bio-Rad Trans-Blot) by the following procedure. Samples of Mono S column peak fractions were run on a preparative sodium dodecyl sulfate (SDS)–12% polyacrylamide gel (36), stained briefly with 0.25% Coomassie blue in 10% acetic acid–10% methanol, destained with 10% acetic acid–10% methanol, and soaked in water. The 38- and 32-kDa bands (approximately 600 pmol of protein each) were cut out, chopped, and agitated overnight in a solution of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, and 0.1 mg of bovine serum albumin (BSA) per ml in order to elute the protein by diffusion. Methanol was added to 20% to the resulting supernatant, which was agitated with a nitrocellulose strip overnight to effect subunit binding. The nitrocellulose strips were then blocked with 1% BSA in rinse buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100) overnight.

Affinity purification of the antibodies was accomplished by gentle agitation of each strip with 0.6 ml of rabbit anti-CTDK-I antiserum (40) in 5.4 ml of rinse buffer–1 mM phenylmethylsulfonyl fluoride at 4°C overnight. Washes and the elution of antibodies with low-pH buffer were as described by Kelly et al. (32). Antibody specificity was verified and titer was estimated by spotting small amounts of purified subunits (reserved from the above-described procedure) on nitrocellulose, reacting with antibodies (68), and visualizing with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G antibody (Boehringer Mannheim) and 100 μg of nitroblue tetrazolium per ml–50 μg of 5-bromo-4-chloro-3-indolyl phosphate per ml–100 mM Tris-HCl (pH 9.6)–50 mM $MgCl_2$ essentially as recommended by the manufacturer.

For protein sequencing, Mono S peak samples were dialyzed against 100 mM Tris-HCl (pH 8.0)–1 mM dithiothreitol–1 mM phenylmethylsulfonyl fluoride and then reduced and alkylated by the method of Hibbs et al. (26). The resulting sample was run on a preparative SDS–12% polyacrylamide gel, and the bands were eluted and bound to nitrocellulose as described above except that (i) 0.0025% Coomassie blue was used in the staining and (ii) BSA and methanol were omitted from the elution and nitrocellulose binding steps, respectively. The following steps were performed at the Harvard Microchemistry Facility by methods previously described (38). Bound subunits (around 200 pmol of each) were subjected to in situ tryptic digestion, and the resulting peptides were separated by reverse-phase high-performance liquid chromatography. One peptide sample for each subunit was selected via information from matrix-assisted laser desorption mass spectrometry and used for automated Edman protein sequencing.

Screening of yeast genomic DNA libraries. By using the methods of Hyunh et al. (29), a λgt11 yeast genomic DNA library (provided by A. Sugino, National Institute of Environmental Health Sciences) was screened with affinity-purified anti-β-subunit (1:20 dilution) and anti-γ-subunit (1:50 dilution) antibodies and visualized with a secondary antibody as described above. After isolation of genomic DNA from purified positive phage clones, *Eco*RI fragments containing the beginning of the yeast inserts were subcloned into the pBluescript II KS+ plasmid (Stratagene) by standard recombination protocols (61).

Two adjacent restriction fragments (0.28-kb *Eco*RI-*Hin*dIII and 0.28-kb *Hin*dIII-*Nsi*I) from the subcloned plasmid containing part of the β-subunit gene (pB32) were isolated by low-melting-point agarose procedures (53) and used to produce digoxigenin-UTP-labeled probes through random-primed synthesis by way of the Genius system (Boehringer Mannheim) and its supplied protocols. A λZAP (64) yeast genomic DNA library provided by A. Sugino was screened with these probes, using a hybridization temperature of 50°C. The methods for the hybridization and visualization with alkaline phosphatase-conjugated antidigoxigenin antibody (Boehringer Mannheim) were as described in the Genius procedures recommended by the manufacturer.

For λZAP cloning of the γ-subunit gene, a 25-mer oligonucleotide (5′GAGA CAAGTTGGTACATCGAAAGG3′) derived from *ORF1* sequence information (70) was produced on a DNA synthesizer (Applied Biosystems), radiolabeled with [γ-^{32}P]ATP via bacteriophage T4 polynucleotide kinase (61), and used as a probe. The hybridization temperature was 49°C, and positive clones were visualized by autoradiography.

By way of the in vivo excision procedure described by Short et al. (64) and the manufacturer (Stratagene), purified λZAP phage clones from each of the two screenings described above were used to produce phagemids, which were recombinant pBluescript SK− plasmids containing all or part of the *CTK2* and *CTK3* genes.

Fusion proteins. Fusion proteins in which β-galactosidase was fused to portions of CTK1, CTK2, and CTK3 were produced by way of the pUR290 series of vectors (60). The CTK1 fusion protein plasmid was derived from λgt11 clone λJ7, which contained a 2.4-kb yeast insert beginning at residue 155 of CTK1 and extending beyond the stop codon. A 4.5-kb *Sac*I-*Kpn*I fragment from λJ7 was subcloned into *Sac*I-*Pst*I-digested pUR290 (the *Kpn*I and *Pst*I sites had been blunted with T4 polymerase), resulting in plasmid pUR290-78, which encodes a fusion protein containing about 71% of CTK1. The plasmid construct for fusion protein BF1, containing the middle region of CTK2 (residues 139 to 231), was formed by subcloning a 0.3-kb *Bam*HI-*Hin*dIII fragment of pB32 (including a short segment of the pBluescript polylinker) into *Bam*HI-*Hin*dIII-digested pUR292. BF2, a fusion protein containing the carboxyl-terminal region of CTK2 (residues 230 to 323), resulted from the ligation of a 1.1-kb *Hin*dIII fragment of pB32 into the *Hin*dIII site of pUR290. The 0.78-kb DNA insert for CTK3 fusion protein GF2 came from a *Sal*I-*Pst*I digest of pGZ2, a partial clone obtained from the λZAP screening. It was ligated into *Sal*I-*Pst*I-digested pUR290, and the resulting fusion protein contained an internal section of CTK3 (residues 32 to 287, about 86% of CTK3). The plasmids were transformed into *E. coli* BMH 71-18. Fusion proteins were produced by induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 1.5 h and extracted by freezing-thawing followed by boiling in SDS-polyacrylamide gel electrophoresis sample buffer. To affinity purify antibodies, samples of fusion protein extracts were run on SDS-polyacrylamide gels and electroblotted to a nitrocellulose (BF2 and GF2) or polyvinylidene difluoride (pUR290-78) membrane, which was stained briefly with India ink (22). For each, a strip containing the desired fusion protein band was cut out and used to affinity purify antibodies from the anti-CTDK-I antiserum by the methods described above. Western blotting (immunoblotting) with these antibodies was performed as previously described (32, 68), using alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G as a secondary antibody and visualizing with the color reaction described above.

For the CTDK-I assays, a modified version of the Y-FP fusion protein (39), which eliminated a nonconsensus region at the beginning of the CTD, was created. To make this construct, a *Bam*HI site was introduced into the yeast *RPO21* gene (30) on an *E. coli* plasmid by site-directed mutagenesis (35) with the oligonucleotide 5′GGAGTCTCCTCGGATCCAGGCTTT3′. A 0.85-kb *Bam*HI-*Hin*dIII fragment from this plasmid was isolated and subcloned into *Bam*HI-*Hin*dIII-digested pUR290, resulting in a plasmid construct encoding FPY1, a fusion protein containing approximately the last 24 repeats of the CTD. FPY1 was produced and purified by the methods used for Y-FP (39).

Antibody column. CTDK-I samples used for antibody column experiments were prepared by a method modified from that of Lee and Greenleaf (39). The starting material was commercial yeast blocks (Eagle brand), and cell breakage was achieved by freezing the yeast in liquid nitrogen and pulverizing it for 4.5 min in a stainless steel, 1-gal (ca. 3.8-liter)-capacity Waring blender prior to adding it to the extract buffer. The rest of the purification procedure was essentially as described previously (39) except that polyethyleneimine was added to 0.0175% and ammonium sulfate was added to 40% saturation in their respective precipitation steps. After resuspension of the ammonium sulfate precipitate, samples were dialyzed against 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.6)–150 mM KCl–0.1 mM EDTA–1 mM dithiothreitol–1 mM phenylmethylsulfonyl fluoride at 4°C for use with the antibody columns.

The antibodies used for the column were raised against a specific region of the CTK1 protein sequence. On the basis of a section of predicted protein sequence contained in the hydrophilic amino-terminal domain of CTK1, the 20-residue S2 peptide (LSRYNDTSFQTSSRYQGSRY) was synthesized with a Milligen/Biosearch 9600 synthesizer by the methods of Eckhardt et al. (14). S2 peptide was coupled to BSA by combining 15 mg of peptide with 2.5 mg of BSA and cross-linking for 1 h with an equal volume of 0.2% glutaraldehyde in phosphate-buffered saline (61), followed by agitation for 1 h with 1/4 volume of 1 M glycine (pH 7.2). The BSA-coupled peptide was injected into a rabbit, resulting in an antiserum. A peptide column was produced by reacting S2 peptide with 1,1′-carbonyldiimidazole-activated agarose beads (Pierce Reacti-Gel) as recommended by the manufacturer. Affinity purification of an anti-S2 antibody from the antiserum was performed as described previously (54).

The resulting anti-S2 antibody was then itself coupled to a column by binding to protein A-agarose and cross-linking with dimethylpimelimidate (23). CTDK-I binding and elution were done by a batch procedure. Dialyzed, partially pure CTDK-I was added to a small amount of anti-S2–protein A-agarose or protein A-agarose in a tube, which was gently agitated overnight at 4°C. The beads were then washed with 3 volumes of kinase dialysis buffer (see above) and eluted with 1 volume of kinase dialysis buffer containing approximately 0.03 mg of S2 peptide per ml. The resulting eluates were tested for CTDK-I activity by standard assays (39) using FPY1 and Western blotted to test for the presence of CTK1, CTK2, and CTK3.

DNA sequence analysis. The plasmid substrates for DNA sequencing of *CTK2* and *CTK3* were pB32, several phagemids from the λZAP screenings, and a series of plasmids derived from them by recombining several appropriate intragenic restriction sites with compatible sites in the pBluescript polylinker. The sequences were obtained manually by the dideoxynucleotide sequencing method (62), using the M13 universal (5′GTAAAACGACGGCCAGT3′), M13 reverse

(5′AACAGCTATGACCATG3′), and SK (5′TCTAGAACTAGTGGATC3′) primers with their respective primer sites in the polylinker.

Database searches and sequence comparisons were done via the BLAST system (1, 24) at the National Center for Biotechnology Information (NCBI). Individual protein sequence alignments were performed with the BESTFIT program from the University of Wisconsin Genetics Computer Group software (12).

Gene disruptions. To make one-step gene disruptions (58) of *CTK1*, *CTK2*, and *CTK3*, plasmid constructs containing selectable markers with flanking regions of the *CTK* genes were prepared by using pBluescript as a vector. For *CTK1* disruption, the 1.8-kb *Bam*HI fragment containing the *HIS3* gene (66) was ligated with a *Bam*HI-*Eco*RI adaptor (5′GATCCGAATTC3′, 3′GCTTAAG5′; Stratagene) and subcloned into *Eco*RI-digested pSZ17 (40) to create plasmid pSZH, which is analogous to pSZ17 but with *HIS3* substituted for *URA3*. A 2.9-kb *Sna*BI-*Vsp*I fragment was prepared from pSZH and used for yeast transformation.

The *CTK2* gene disruption involved replacing virtually the entire coding region with the *URA3* marker (56). The plasmid construct was derived from pB32, *CTK2* λZAP clone pBZ2, and the *URA3*-containing plasmid pGB310 (40) in several steps. The final construct, pBZU3, contained a 1.1-kb segment of the *CTK2* upstream region extending to a *Sna*BI site 10 nucleotides before the start codon and a 1.2-kb downstream segment extending from the *Nsi*I site overlapping the stop codon, inserted at the *Eco*RI site of pBluescript. These segments were interrupted by the 1.2-kb *Hin*dIII fragment containing *URA3*, connected via short regions from the polylinkers of the cloning vectors. A *Pvu*II-*Eco*RI fragment of 3.3 kb was isolated from pBZU3 to transform yeast strains.

For the *CTK3* gene disruption, nearly the entire gene was replaced with the *LEU2* marker (2). Plasmid construct pGZL2 was made with *CTK3* flanking sequences from λZAP phagemids pGZ2 and pGZ3; it had a 1.2-kb upstream segment extending to an *Alu*I site 13 nucleotides beyond the start codon and a 0.5-kb segment extending from the *Rsa*I site overlapping the stop codon to a downstream *Sst*II site. The insert spanned the *Eco*RI and *Sst*II sites of pBluescript, and the 2.2-kb *Xho*I-*Sal*I fragment containing the *LEU2* gene was connected between the upstream and downstream regions by way of short stretches of the pBluescript and pUC9 polylinkers. pGZL2 was digested with *Sph*I and *Sst*II to produce a 3.5-kb fragment.

To disrupt the genes in yeast cells, each of the three prepared plasmid fragments was transformed into YPH274 by the lithium acetate method (57). Prototrophic heterozygous diploid transformants were grown on appropriate selective medium plates and used for tetrad analysis. The selected diploids used were termed ADD6 (*CTK1*/*ctk1*Δ*E*::*HIS3*), BDD1 (*CTK2*/*ctk2*Δ::*URA3*), and GDD8 (*CTK3*/*ctk3*Δ::*LEU2*). Proper gene disruptions were verified by Southern blot procedures: yeast genomic DNA was prepared by the method of Holm et al. (27), digested with appropriate restriction enzymes, blotted to nitrocellulose (61), and probed with digoxigenin-labeled restriction fragments from the *CTK* flanking regions to visualize band patterns characteristic of disrupted *CTK1*, *CTK2*, and *CTK3*.

Tetrad analysis and mating. *CTK*/*ctk* strains from the transformations were sporulated for 3 to 5 days, and tetrad dissection was performed by standard methods (4). Plates were grown for 4 days at 30°C, and resulting colonies were tested for His, Ura, or Leu prototrophy. For each of the three dissection plates, four colonies of one tetrad were subjected to Southern blot analysis as described above to confirm the mutant genotypes. Mutant strains were called ADH6 (*ctk1*Δ*E*::*HIS3*), BDH1 (*ctk2*Δ::*URA3*), and GDH8 (*ctk3*Δ::*LEU2*).

Double mutants were constructed by mating the *ctk* strains described above in all three possible combinations to produce double-prototroph diploids, which then underwent sporulation and tetrad dissection. Haploid double mutants were identified on double-selective media. To make a triple *ctk* mutant, one of the double mutants (CDH1; *ctk1*Δ*E*::*HIS3 ctk2*Δ::*URA3*) was mated with a GDH8 strain to produce diploids, which were triple selected, sporulated, dissected, and triple selected again.

Nucleotide sequence accession numbers. The GenBank accession numbers for *CTK2* and *CTK3* are U30295 and U30296, respectively.

RESULTS

Cloning and sequencing of the CTDK-I β-subunit gene, *CTK2*. Antibodies against gel-isolated CTDK-I β subunit were affinity purified from an antiserum raised against all three CTDK-I subunits (40). These antibodies were used to screen a λgt11 yeast genomic DNA expression library, resulting in one phage clone containing approximately half of the β-subunit gene. Restriction fragments prepared from the DNA of that phage were used to screen a λZAP yeast genomic DNA library, and several clones containing the full-length gene were obtained. We named this gene *CTK2* (the gene product is CTK2); its restriction map is outlined in Fig. 1A. The fact that *CTK2* encodes the β subunit was verified in two ways: (i) on a Western blot, fusion proteins BF1 and BF2, containing different regions of CTK2, both reacted strongly with an anti-β-subunit antibody (data not shown), and (ii) the protein sequence of a nine-residue tryptic fragment of β subunit agreed with the predicted protein sequence of CTK2 (Fig. 1B).

DNA sequence was determined for 1,410 nucleotides of *CTK2* and its flanking regions; that sequence is shown in Fig. 1B. The open reading frame is 972 nucleotides long. Comparison with databases of known sequences revealed that *CTK2* is a novel gene but is located in the *S. cerevisiae* genome immediately downstream from and with opposite orientation to the *EMT3* gene (3).

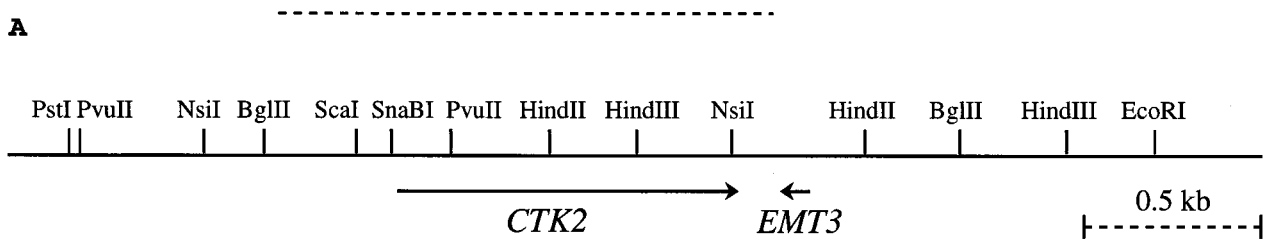
Cloning and sequencing of the CTDK-I γ-subunit gene, *CTK3*. Affinity-purified anti-γ-subunit antibodies were prepared and used to screen a λgt11 library as done for the cloning of the β-subunit gene. This procedure yielded several strong positive clones, but none encoded the γ subunit. Partial protein sequencing of gel-purified γ subunit was then performed, and the sequence of a 28-residue tryptic fragment was determined. A search for this sequence among the translations of known DNA sequences with the BLAST database program showed it to be contained within the partially sequenced but uncharacterized gene *ORF1*, which is located just downstream from the *DAT1* gene in the *S. cerevisiae* genome (70). An oligonucleotide probe was synthesized on the basis of sequence information from the known portion of the gene and was used to screen a λZAP yeast genomic DNA library, resulting in several clones containing the full-length γ-subunit gene. We termed this gene *CTK3* (the gene product is CTK3); its restriction map is shown in Fig. 2A. We sequenced 1,581 nucleotides, including the 891-nucleotide *CTK3* open reading frame; those results are shown in Fig. 2B.

Sequence analysis of the *CTK2* and *CTK3* genes. The open reading frames identified in the cloned *CTK2* and *CTK3* genes agree well with the sizes of the 38-kDa β and 32-kDa γ subunits in purified CTDK-I. *CTK2* predicts a polypeptide of 323 residues and a size of 37.9 kDa, while *CTK3* predicts a 296-residue polypeptide with a size of 34.8 kDa. The small discrepancy between the predicted and observed sizes of the γ subunit could be due to slightly aberrant mobility on an SDS-polyacrylamide gel or proteolysis of the subunit in vivo or in vitro.

Analysis of the *CTK2* and *CTK3* sequences suggests low levels of expression for these genes. The codon bias index values (6) for *CTK2* and *CTK3* are −0.03 and 0.15, respectively; these low to negative values indicate high percentages of codons nonpreferred in *S. cerevisiae*, a situation seen for genes expressed at a low level, and are in agreement with the codon bias index for *CTK1* (approximately zero) and the observation of a low abundance of CTDK-I in yeast cells (39). Within the genome, *CTK2* is close to the elongator methionine-tRNA gene *EMT3*, and *CTK3* is just downstream (272 nucleotides) from the *DAT1* gene, which encodes the dA-dT-binding protein datin. However, at this point it is not anticipated that the expressions of the neighboring genes are necessarily related, since the yeast genome is known to be very compact, with unrelated genes often close together.

Observation of the predicted amino acid compositions and structure predictions of CTK2 and CTK3 revealed few remarkable features. CTK2 contains alternating hydrophilic and hydrophobic regions, possibly indicating a globular protein. CTK3 is predicted to be somewhat acidic, and the carboxyl-terminal half of the protein is largely hydrophilic. CTK3 is also particularly glycine poor compared with the average composition of yeast proteins (20), since it contains only one glycine residue.

Database searches with the predicted amino acid sequences provided valuable information about CTK2. Using the NCBI



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-320            ATTTTTTGTC CTTATCTTTT TGCTGCACCG AAAGCACTCA TGAAGTCTAC GGCTTGATGC ACACCGTTTG GGAACCTTTG CGATCCCACG TACCTAAATG

-220 GTCCTTTTCG GTACAACTTG CTGAAGAATC ACTGTGTGGC GGGTAATGCT CTCTTTATAC TTGCATTGCG AAAATCCGCG ATGATCAAAA ATCCAAACAA GTACTGTCCT
                                                                                                          SnaBI
-110 GAAAGCCGTC TTTATACACA TAGATATATA TATATATATA TATATATCTG TATCTATAGA GCGAGAGGAG ATATAGAGCC AGCAAAACTA AGAAAATTAC GTACTTTGAT

   1 ATGCCTAGCACGTTTGAATCCCAGCTTTTTTTTTCGAGACCGTTTCTGTCTAAAAGGCAAATTCAAAGAGCCCAAAAGAATACAATATCGGACTATCGAAATTACAACCAGAAAAAACTT
   1 M P S T F E S Q L F F S R P F L S K R Q I Q R A Q K N T I S D Y R N Y N Q K K L

 121 GCCGTGTTCAAATTTCTAAGTGACTTGTGTGTTCAGCTGAAATTTCCACGGAAGACTTTAGAAACAGCAGTATACTTTTACCAAAGATACCACCTCTTTAATCGCTTCGAGACCGAGGTT
  41 A V F K F L S D L C V Q L K F P R K T L E T A V Y F Y Q R Y H L F N R F E T E V

 241 TGCTACACTGTAGCGACAAGCTGCCTGACTTTGGGCTGTAAAGAAGTTGAGACAATTAAAAAAACAAATGATATATGTACTTTGTCATTACGATTGAGGAATGTGGTTAAAATCAATACG
  81 C Y T V A T S C L T L G C K E V E T I K K T N D I C T L S L R L R N V V K I N T

 361 GACATTTTGGAAAATTTTAAAAAAAGGGTATTTCAAATAGAACTTCGGATATTAGAGTCATGCTCATTCGATTATAGAGTCAACAATTACGTTCATATTGATGAATATGTTATAAAAATT
 121 D I L E N F K K R V F Q I E L R I L E S C S F D Y R V N N Y V H I D E Y V I K I

 481 GGTAGGGAGCTATCCTTTGATTATAAGTTGTGCAATCTGGCTTGGGTAATTGCTTATGATGCTTTGAAGCTAGAAACTATTTTAGTCATACCCCAACATTCCATTGCATTGGCCATTTTG
 161 G R E L S F D Y K L C N L A W V I A Y D A L K L E T I L V I P Q H S I A L A I L

 601 AAAATAGCATATGAATTACTAGATAATAAGAATTGGTCAAGTAAAAGGTATTCTTTATTTGAAACAGACGAGAAATCCGTAAATGAAGCTTATTTTGATATAGTCAATTTTTACATCAAT
 201 K I A Y E L L D N K N W S S K R Y S L F E T D E K S V N E A Y F D I V N F Y I N

 721 TCATTCGATATGTGCGATCTACAACGTCATTTACCGGCTGATTTGCTGCCGATTGGCGTTGAAAGGTTTATGGAACTGAAAAAAAACGCAGGACCAGAATCTGGATTGCCGCAAATACCA
 241 S F D M C D L Q R H L P A D L L P I G V E R F M E L K K N A G P E S G L P Q I P

 841 GATCACCTGTTGAATGCCGATCCTTATATCACTATTACTAGAGATAATAATGTCCAAGAAAGAAGATATGTACTTTCACTGGAGCTAATTAATGGCGAGTCCAGCATAAATAGTTCTACA
 281 D H L L N A D P Y I T I T R D N N V Q E R R Y V L S L E L I N G E S S I N S S T
       NsiI 972
 961 AGACATGCATAG AAAGTCCT ACTATAAACT TAAATAAGAT CATCATTATA AACTTTCTTT TACACAAGTA ATAAGTACCT TTTTTTATCA CAAAACATGA ATGTTGAGCT
 321 R H A (323)

1071 TCGAGGTTTA ATTCACAAAA
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FIG. 1. Restriction map and sequence of the *CTK2* gene and flanking regions. (A) Restriction map of the cloned section of the yeast genome. The relative scale is indicated at the far right, and the dotted line at the top indicates the sequenced segment shown in panel B. The arrows indicate the locations and relative positions of the *CTK2* and *EMT3* genes and their 5′-to-3′ directionality with respect to transcription. (B) Nucleotide and predicted amino acid sequences of the *CTK2* gene and its open reading frame. The 5′ region upstream of the ATG start codon is expressed by negative numbers; the total sequenced region covers from −320 to +1090. Every 30 nucleotides and every 10 amino acids are indicated by dots. Restriction enzyme sites used for gene replacement with *URA3* are indicated in the nucleotide sequence; underlined in the predicted amino acid sequence is the nine-residue sequence obtained by protein sequencing of a peptide isolated from purified β subunit.

BLAST database comparison program, we found that CTK3 showed no significant similarity to any other known proteins. CTK2, however, was revealed to have homology to *Drosophila*, human, and rat cyclin C (37, 42, 43, 67); a summary of that comparison is shown in Fig. 3. BLAST did not identify any other cyclins as being homologous to CTK2 above the standard cutoff of the program, but the two identified regions of cyclin C share homology with a variety of other cyclins. The upper sequence in Fig. 3 is in fact within the cyclin box region (41, 51), containing the most highly conserved sequences among all of the different cyclins. In addition, BLAST revealed homology in this same CTK2 region to general transcription factor TFIIB, which has been identified as evolutionarily related to cyclins (17). Overall, the relationship between CTK2 and the cyclins is clearly significant.

However, there are also indications that CTK2 is a somewhat divergent member of the cyclin family. A comparison of CTK2 and *Drosophila* cyclin C with the BESTFIT program determined a 23% identity and 49% similarity between the two, a homology that is somewhat weak for related cyclins. Other cyclins compared with CTK2 resulted in values similar to or lower than these. In addition, there are several examples of highly conserved residues within the cyclin box for which CTK2 has substitutions. The CTK2 sequence also shows no evidence of cell cycle-dependent regulation exhibited by some other yeast cyclins. It contains neither a PEST sequence (55) nor a destruction box (18), which are supposed to allow rapid degradation of cyclins, and sequencing up to approximately 700 nucleotides upstream of *CTK2* (data not shown) indicated the absence of SWI4/SWI6 or *Mlu*I cell cycle boxes, which are regulatory elements allowing cell cycle-dependent expression of certain yeast cyclins (48).

Physical association of CTK1, CTK2, and CTK3 in vitro. The fact that the *CTK2* and *CTK3* gene products are part of the active CTDK-I complex is shown by their distribution across a peak of activity in fractions from the Mono S column step in the purification (Fig. 4). When visualized with antibodies affinity purified against CTK2 and CTK3 fusion proteins, Western blots revealed 38- and 32-kDa species whose profiles across the peak each coincide with both CTD kinase activity and the

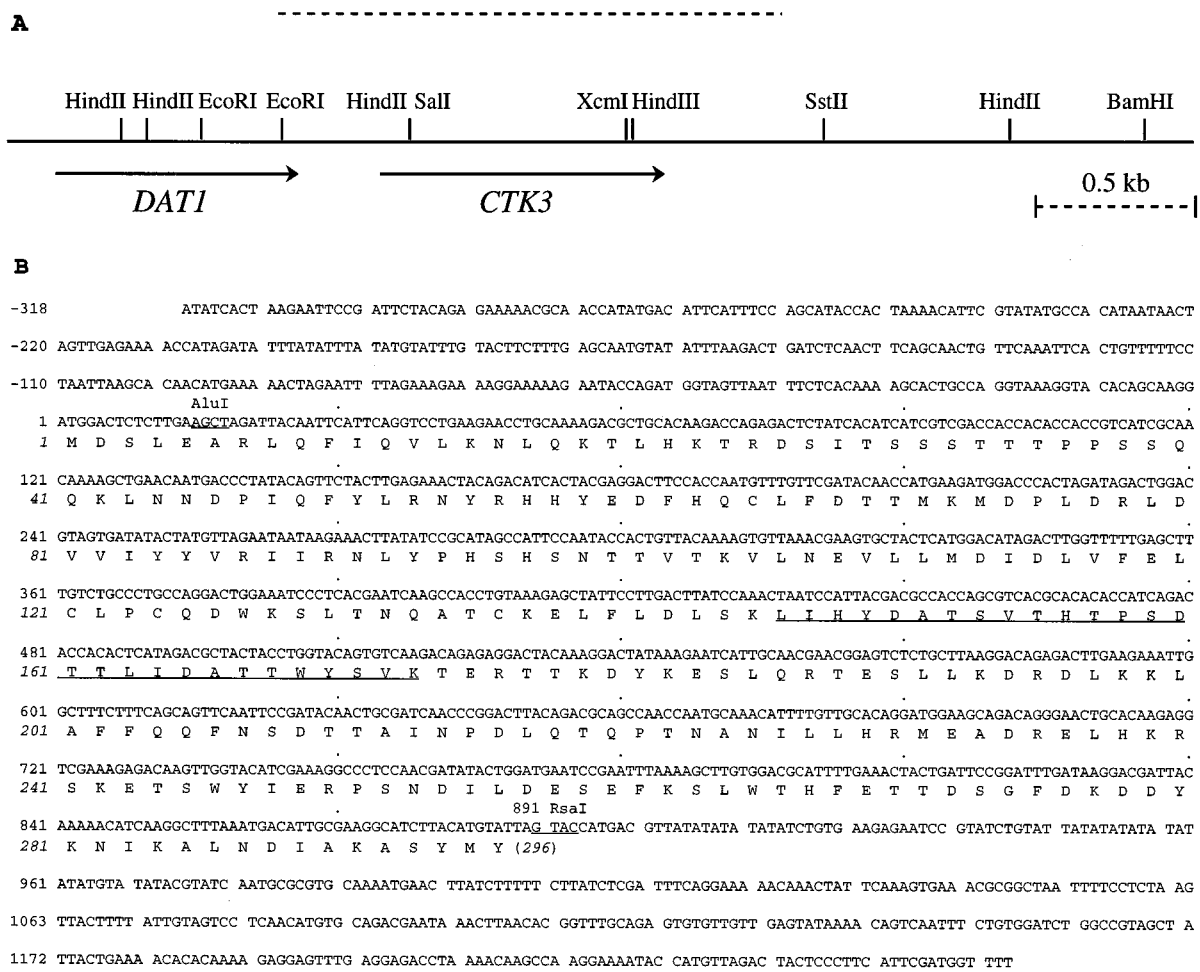

FIG. 2. Restriction map and sequence of the *CTK3* gene and flanking regions. (A) Restriction map of the cloned section of the yeast genome. The relative scale is indicated at the far right, and the dotted line at the top indicates the sequenced segment shown in panel B. The arrows indicate the locations and relative positions of the *CTK3* and *DAT1* genes and their 5′-to-3′ directionality with respect to transcription. (B) Nucleotide and predicted amino acid sequences of the *CTK3* gene and its open reading frame. The 5′ region upstream of the ATG start codon is expressed by negative numbers; the total sequenced region covers from −318 to +1263. Every 30 nucleotides and every 10 amino acids are indicated by dots. Restriction enzyme sites used for gene replacement with *LEU2* are indicated in the nucleotide sequence; underlined in the predicted amino acid sequence is the 28-residue sequence obtained by protein sequencing of a peptide isolated from purified γ subunit.

level of CTK1, visualized in a separate blot with anti-CTK1 antibodies. In addition, a Coomassie blue-stained gel of the peak fractions (data not shown) displayed similar distributions of 58-, 38-, and 32-kDa bands, the latter two of which were the source of the protein used for antibody affinity purification and protein sequencing. The Western blots therefore also confirm that the two cloned genes were correct.

The physical association of CTK1, CTK2, and CTK3 was further demonstrated by using an antibody directed against a 20-amino-acid region (S2) in the hydrophilic amino-terminal domain of CTK1. When mixed with a relatively crude fraction of partially purified CTDK-I, the agarose-linked anti-S2 antibody was able to specifically remove a majority of the 58-kDa CTK1, 38-kDa CTK2, and 32-kDa CTK3 subunits from the preparation, as visualized by anti-CTK fusion protein antibodies on Western blots (Fig. 5). The binding was antibody specific, since a control column containing no antibody bound none of the subunits. Subunit binding to the antibody column

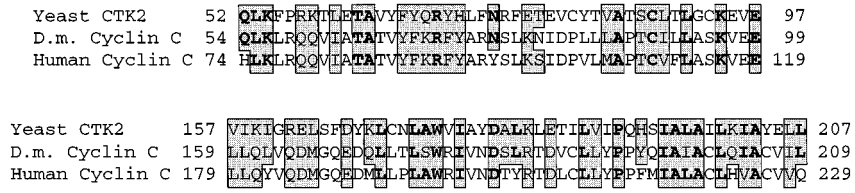

FIG. 3. Cyclin homology of CTK2. Shown are two regions of homology shared between the predicted amino acid sequences of CTK2 and *Drosophila melanogaster* and human cyclin C, as identified by a database search via the NCBI BLAST server. Positions for which CTK2 has a conservative amino acid substitution in cyclin C are boxed, and identical residues are in boldface. The upper sequence is within the conserved cyclin box region of cyclin C; the lower sequence is farther downstream and has homology with an analogous region of some, but not all, other cyclins.

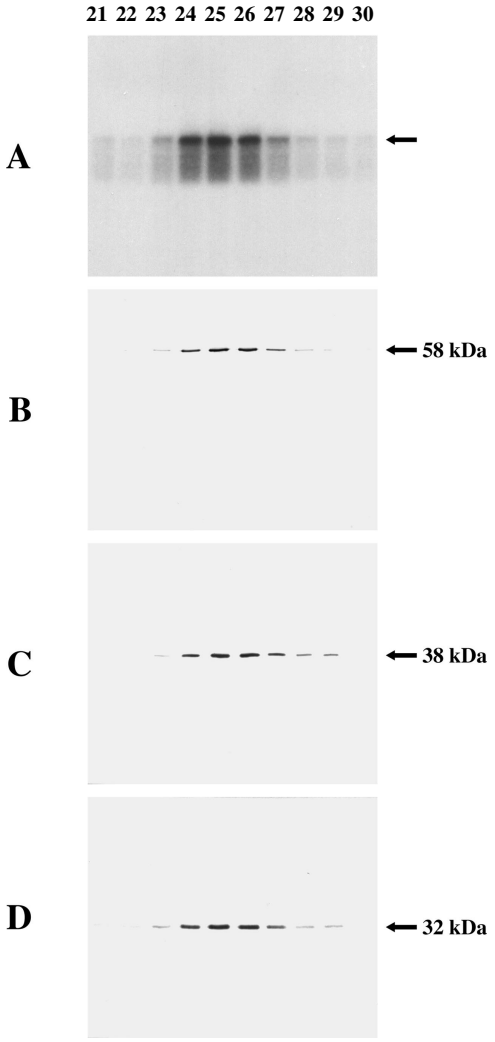

FIG. 4. Correlation of CTK1, CTK2, and CTK3 levels with CTD kinase activity in Mono S-purified CTDK-I peak fractions. (A) Autoradiogram of CTD kinase assays of Mono S fractions 21B-30B, in which CTD fusion protein FPY1 was used as a substrate in the presence of [γ-^{32}P]ATP and 0.3 μl of each fraction. Reaction products were run on an SDS–6% polyacrylamide gel. Shifted FPY1 is indicated by the arrow. (B) Samples of 0.25 μl of Mono S fractions 21B to 30B run on an SDS–12% polyacrylamide gel, transferred to nitrocellulose, and visualized with an anti-CTK1 fusion protein antibody (1:250). (C) Western blot as in panel B, but using 2 μl of each fraction and visualized with an anti-CTK2 fusion protein antibody (1:10). (D) Western blot as in panel B, but visualized with an anti-CTK3 fusion protein antibody (1:50). The amount of each fraction used for the Western blot in panel C was eightfold higher than those in panels B and D in order to compensate for the inefficient electroblotting performance of the β subunit (40).

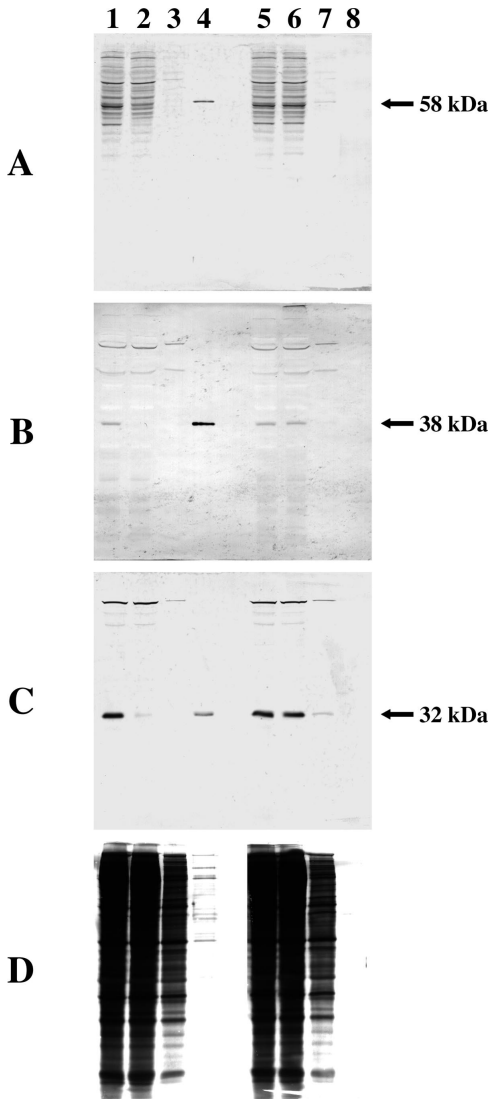

FIG. 5. Physical association of CTK1, CTK2, and CTK3 in a crude CTDK-I preparation, demonstrated by an antibody column. Anti-S2 (CTK1 domain I peptide) antibodies were cross-linked to protein A-agarose and agitated overnight with CTDK-I purified through the ammonium sulfate pelleting step. Panels A to D represent identical sets of samples run on SDS–12% polyacrylamide gels and electroblotted to nitrocellulose; all lanes are quantitatively comparable to one another, since in each case the amount of sample used is directly proportional to the volume recovered at each step in the column procedure. Lane 1, 5 μl of CTDK-I onput; lane 2, 5 μl of column supernatant after agitation with CTDK-I onput overnight at 4°C; lane 3, 15 μl of 3 combined column washes; lane 4, 5 μl of column elution with 0.03 mg of S2 peptide solution per ml. Lanes 5 to 8 are analogous to lanes 1 to 4 except that reactions were performed with a control protein A-agarose column containing no antibody. The nitrocellulose blots were visualized with antibodies affinity purified against fusion proteins of CTK1 (A), CTK2 (B), and CTK3 (C). The blot in panel D was stained with India ink to observe levels of total protein in each lane.

was apparently stable through three subsequent washes of the matrix, but the addition of an S2 peptide solution resulted in an eluate containing both subunits. This eluate also contained partial CTDK-I activity, about 10 to 30% of that in the column onput (data not shown). Thus, the three *CTK* gene products and the CTDK-I activity were all coprecipitated by anti-S2 immunoglobulin, indicating that they are part of the same active complex in vitro. The seemingly paradoxical appearance of lower levels of CTK2 in the onput than the eluate (Fig. 5B) is believed to be due to the inefficient electroblotting performance of the β subunit (40), aggravated by the high levels of other proteins in the onput samples (Fig. 5D).

Functional interaction among CTK1, CTK2, and CTK3. To investigate the in vivo roles of *CTK2* and *CTK3*, null mutations of these genes were made in strain YPH274. In addition, a *CTK1* null mutant was prepared with the same strain to provide all mutants with similar genetic backgrounds and to allow direct comparisons of phenotypes. Tetrad dissection of heterozygous diploid mutants for each of the genes (Fig. 6) shows that *ctk2* and *ctk3* mutants are viable but grow more slowly than wild-type cells at 30°C, similar to the *ctk1* mutant shown and to previously described *ctk1* mutants (40). The *ctk2* and *ctk3* mutants display phenotypes that are similar in several

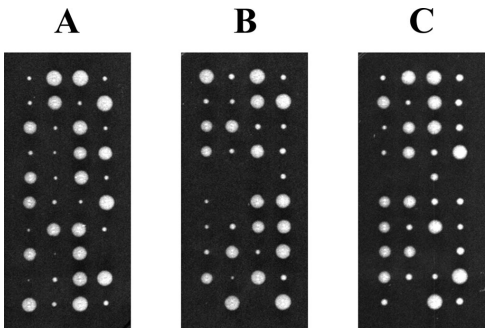

FIG. 6. Tetrad analysis of *ctk* mutants. Diploid strains heterozygous (*CTK*/*ctk*) for each gene disruption were sporulated 3 to 5 days, and resulting tetrads were dissected. Spores were grown on YPD medium for 4 days at 30°C. The results of the dissections of 10 tetrads are shown for *CTK1*/*ctk1*Δ*E*::*HIS3* (A), *CTK2*/*ctk2*Δ::*URA3* (B), and *CTK3*/*ctk3*Δ::*LEU2* (C).

other ways to those of *ctk1* mutants: they are cold sensitive, failing to grow at 12°C; they display flocculent growth in liquid media; and they show abnormal cell morphologies (for example, a significant fraction of the cells are greatly enlarged). The overall phenotypes of the three mutants are clearly similar, which could indicate related functional roles in vivo.

Further evidence for an in vivo relationship among the *CTK* gene products was provided by double and triple *ctk* null mutants. These mutants displayed phenotypes very similar to those of each of the single mutants: individual spores derived from tetrad dissection resulted in small colonies comparable in size to single mutant colonies grown in parallel (not shown), liquid cultures were flocculent, and abnormal morphology was observed. A more direct comparison of the levels of impairment was made via growth at a variety of temperatures (Fig. 7). The mutants all grew at 37, 30, and 25°C, although the growth at 25°C was somewhat spottier. When plates were incubated at 18°C, some patches showed a small amount of growth, while others showed no apparent growth during the time allowed. However, this effect seems to be a function of individual clones and not entire sets of mutants, and so it is apparently due to heterozygous background in the source strain YPH274 (heterogeneity among single mutants is also observable in the variable size of mutant colonies in Fig. 6). The nature of these background effects is not known. Upon extended incubation at 18°C (several weeks), most of the mutant clones did grow to some degree. But while there was some growth observed at 18°C, all of the mutants failed to grow at 12°C, verifying cold sensitivity. Overall, these profiles of growth at different temperatures indicate that the double and triple mutants are no more impaired than the single mutants, suggesting that the mutations are in fact redundantly affecting the same in vivo process.

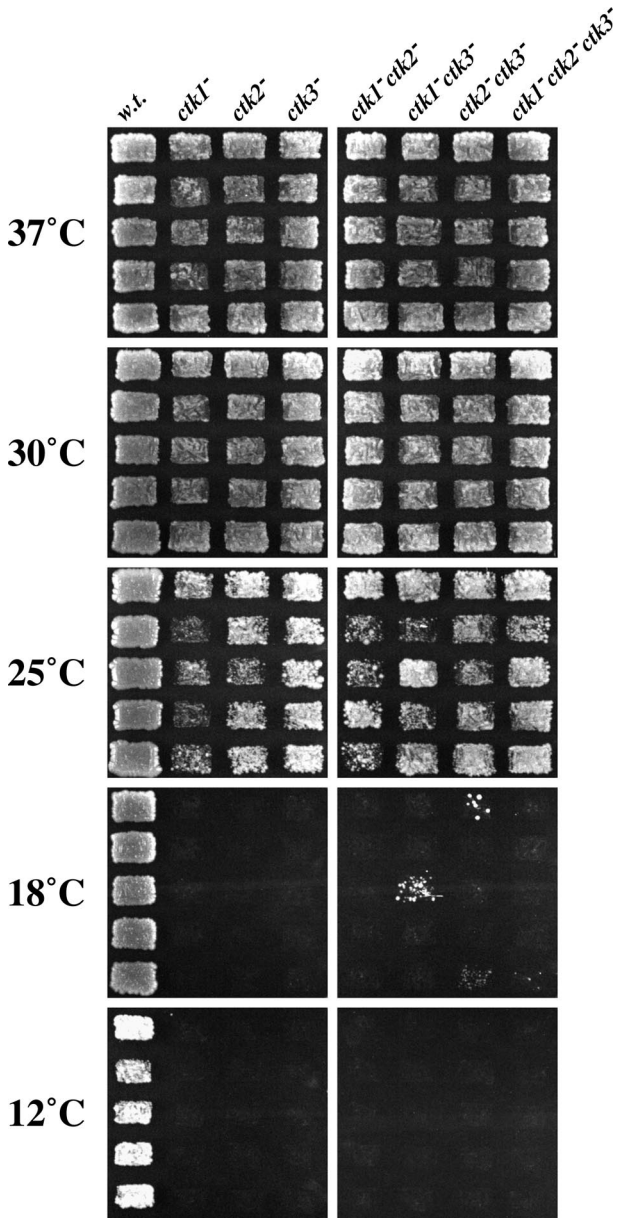

FIG. 7. Growth of *ctk* single-, double-, and triple-null mutants at various temperatures. Wild-type *CTK* control strains and five haploid strains (each arising from different spores from tetrad dissection) of each mutant were placed in patches on YPD medium and incubated at the temperatures indicated. Each set of plates was grown until mutant patches developed (37, 30, and 25°C) or until control patches approached overgrowth (18 and 12°C). Incubation times were 3 days at 37°C, 3.5 days at 30°C, 8 days at 25°C, 10 days at 18°C, and 22 days at 12°C. Haploid controls (from top to bottom) were YPH250 and YPH252 (parent strains of YPH274) and *CTK1 CTK2 CTK3* strains derived from tetrad dissection of ADD6, BDD1, and GDD8 (see Materials and Methods).

DISCUSSION

The studies presented here have identified the genes for the β and γ subunits of the yeast CTD kinase CTDK-I. Furthermore, the results have verified that the gene products of *CTK1*, *CTK2*, and *CTK3* physically associate in vitro and interact functionally in vivo.

Sequence information from *CTK1* and *CTK2* has shed light on the nature of the CTDK-I complex. As previously reported (40), CTK1 is a member of the family of cdc2/CDC28-related protein kinases. These proteins are also called CDKs because of their tendency to associate with cyclins to form active complexes capable of phosphorylating their substrates. There are numerous examples of cyclin-CDK pairs (28); the interaction between cdc2 (also known as cdk1) and cyclin B in higher organisms is perhaps the best characterized. The fact that CTDK-I composition follows this paradigm is confirmed by the revelation that CTK2 is cyclin related. The observed interaction between CTK1 and CTK2 and the apparent requirement of *CTK2* for CTDK-I activity in vivo may suggest that CTK2 binding activates CTK1, as cyclin B does cdc2. Future in vitro studies will determine if this is the case.

While CTDK-I does resemble a cyclin-dependent kinase, at this point there is no evidence to indicate a relationship between this enzyme and the cell cycle. As mentioned earlier, CTK2 is highly divergent compared with most other known cyclin-related proteins, and its sequence shows none of the described motifs for cell cycle-dependent expression or degradation. Profiles of the levels of CTK2 protein and CTDK-I activity at different points in the cell cycle may elucidate whether this protein and the associated activity actually cycle. However, some studies have shown that divergent cyclin-CDK pairs can be involved in functions other than direct cell cycle control (for example, the pair PHO80-PHO85 is involved in the regulation of a phosphate metabolism gene) (31). In addition, general transcription factor TFIIB has been identified as related to the cyclins, and a BLAST database search with CTK2 found a region with more homology to TFIIB than to any other protein besides cyclin C. Therefore, it is possible that CTDK-I is related just evolutionarily to cell cycle regulators such as cdc2 and cyclin B and does not necessarily have directly cell cycle-related physiological functions.

The role of the γ subunit in CTDK-I is more mysterious, as the sequence of the *CTK3* gene gives no significant information about its function. However, it may be notable that a third subunit has also been identified in the cdc2-cyclin complex. Known as suc1 in *Schizosaccharomyces pombe* and CKS1 in *S. cerevisiae*, this small polypeptide seems to regulate cdc2 function in some way in vivo (13, 21). CTK3 shows no apparent sequence similarity to CKS1, but this does not exclude the possibility that CTK3 fulfills an analogous regulatory function specific to CTDK-I (like CTK3, suc1/CKS1 and its homologs in higher organisms also have no significant similarity to other known proteins). Further functional characterization of CTK3 and suc1/CKS1 would be needed to investigate this possibility.

Further information about the roles of the β and γ subunits in CTDK-I was obtained by analysis of the null mutants. The similarity in the impaired phenotypes of *ctk2* and *ctk3* mutants to that of a *ctk1* mutant implies that both of these subunits are essential or important for CTDK-I function in vivo. The fact that double- and triple-mutant combinations display virtually the same phenotype strongly suggests that the various subunit deletions affect the same functional entity in vivo, namely, CTDK-I. Exactly how each subunit participates in the overall complex remains to be determined by future experimentation. In vivo roles or functional features may be elucidated with more detailed mutant studies; for example, one approach is to investigate the properties of CTDK-I with substitutions in predicted cyclin-CDK binding regions or in potential phosphorylation sites.

Another unanswered question is how CTDK-I participates in the transcription process. CTDK-I is a very efficient CTD kinase in vitro, displaying an apparent processivity in its action and a high degree of substrate specificity (reference 39 and unpublished data). In addition, CTDK-I is known to be a nuclear enzyme, since CTK1 was shown previously to contain a nuclear targeting signal and to localize as a fusion protein to the nucleus (40); the CTK2 and CTK3 sequences display no apparent nuclear targeting signals (15), but they could presumably enter the nucleus as part of a complex with CTK1, since the subunits have been shown to physically associate. Furthermore, earlier antibody experiments showed that in *ctk1* mutant cells, the CTD of RNA Pol II is aberrantly phosphorylated, virtually lacking the normally phosphorylated form found in the wild type (40); this finding demonstrates clearly that CTDK-I has an effect on the transcriptional machinery in vivo. On the other hand, recent experiments using anti-CTK antibodies have shown that the CTK1 and CTK3 subunits are not detectable in purified yeast holoenzyme (34, 45), which was demonstrated recently to harbor a different CDK-cyclin pair, SRB10-SRB11, with a potential relationship to CTD phosphorylation (46). In addition, it is now clear that CTDK-I is distinct from the CTD kinase activity of TFIIH. One possibility consistent with all of these facts is that CTDK-I is not involved in transcription initiation itself but plays a postinitiation role. This idea is consistent with data on CTD phosphorylation derived from studies in other systems. For example, it has been demonstrated that for *Drosophila* heat shock genes and certain other loci in vivo, an initiated but paused Pol II is form IIA (hypophosphorylated), whereas actively elongating enzyme is form IIO (hyperphosphorylated); these observations dissociate CTD phosphorylation from RNA chain initiation for these loci (52, 69). More recently, an intriguing set of observations indicates that human immunodeficiency virus Tat proteins, which are known to activate postinitiation phases of transcription, can bind a mammalian cellular protein kinase capable of phosphorylating the CTD in vitro (25). This Tat-associated kinase is distinct from TFIIH and may help mediate postinitiation effects of human immunodeficiency virus Tat proteins. It is interesting to speculate that the Tat-associated kinase may be a mammalian counterpart of yeast CTDK-I.

These considerations indicate that it will be fruitful to investigate further the participation of CTDK-I in initiation and postinitiation phases of transcription both in vitro, using unfractionated extracts that display CTD dependence, and in vivo, using combinations of mutations in CTDK-I and in potentially interacting components of the transcriptional machinery. The cloning and characterization of the genes for the three subunits of CTDK-I will make these studies possible.

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