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Kinase Cak1 functionally interacts with the PAF1 complex and phosphatase Ssu72 via kinases Ctk1 and Bur1

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Abstract Protein kinases orthologous with Cak1 of *Saccharomyces cerevisiae* (ScCak1) appear specific to ascomycetes. ScCak1 phosphorylates Cdc28, the cyclin-dependent kinase (CDK) governing the cell cycle, as well as Kin28, Bur1 and Ctk1, CDKs required for the transcription process performed by RNA polymerase II (RNA Pol II). Using genetic methods, we found that Cak1 genetically interacts with Paf1 and Ctr9, two components belonging to the PAF1 elongation complex needed for histone modifications, and with Ssu72, a protein phosphatase that dephosphorylates serine-5 phosphate in the RNA Pol II C-terminal domain. We present evidence suggesting that the interactions linking Cak1 with the PAF1 complex and with Ssu72 are not direct but mediated via Ctk1 and Bur1. We discuss the possibility that Ssu72 intervenes at the capping checkpoint step of the transcription cycle.

Keywords Cak1 · Paf1 complex · Phosphatase Ssu72 · Ctk1 · Bur1

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Introduction

The transcription of protein-coding genes in eukaryotes is mediated by RNA polymerase II (RNA Pol II) and the general transcription factors acting during the different phases of the transcription cycle (pre-initiation, initiation, promoter clearance, elongation and termination) and those participating in pre-mRNA maturation (pre-mRNA capping, splicing, surveillance and export) (Sims et al. 2004). Gene expression is regulated by transcriptional regulatory proteins interacting with binding motifs in DNA (upstream regulatory sequences). These regulatory proteins recruit and regulate chromatin-modifying complexes and components of the transcription machinery (Lee et al. 2002).

In *Saccharomyces cerevisiae*, six cyclin-dependent kinases (CDKs) are involved in transcription: Cdc28 and Pho85 phosphorylate regulatory proteins (Wittenberg and Reed 2005; Carroll and O'Shea 2002) whereas Kin28, Bur1, Ctk1 and Srb10, interacting with components of general transcription complexes, phosphorylate the C-terminal domain (CTD) of the RNA Pol II large subunit Rpo21 (Prelich 2002) and some other substrates (Liu et al. 2004). The protein kinase ScCak1 is the CAK enzyme (CDK activating kinase) (Kaldis et al. 1996; Thuret et al. 1996; Espinoza et al. 1996; for review see Kaldis 1999). It activates CDKs Cdc28, Kin28, Bur1 and Ctk1, but not Pho85 and Srb10 (Espinoza et al. 2000; Yao and Prelich 2002; Ostapenko and Solomon 2005). Cak1 phosphorylates a

conserved threonyl residue within the so-called T loop, at positions 169, 162, 240 and 338 of Cdc28, Kin28, Bur1 and Ctk1, respectively. Cdc28, the major regulator of cell cycle, is inactive when it is not phosphorylated. Therefore Cak1 activity appears essential for cell cycle progression. Unlike Cdc28-T169A, the T162A mutant of Kin28 exhibits no particular phenotype (Kimmelman et al. 1999; Keogh et al. 2002). The biological significance of Kin28 phosphorylation remains elusive. The phosphorylation of Bur1 is not essential for the cells to survive (Yao and Prelich 2002; Keogh et al. 2003). Also, the phosphorylation of Ctk1 is not necessary for cell proliferation on complete medium (Ostapenko and Solomon 2005). Cak1 is dispersed throughout the cell with the bulk found in the cytoplasm (Kaldis et al. 1998). In metazoans, the Kin28 ortholog Cdk7 was described as a CAK (Keriel and Egly 2002). However, in *S. cerevisiae*, Kin28 possesses no CAK activity (Feaver et al. 1994; Cismowski et al. 1995).

Cross and Levine (1998) have isolated *CDC28* mutants, notably *cdc28-43244*, which allow viability in the absence of Cak1 at temperatures below 35°C. This demonstrates that Cdc28 phosphorylation is the only essential function of Cak1 in rich glucose medium at permissive temperatures. However, a *cak1Δ cdc28Δ* strain harboring allele *cdc28-43244* in a plasmid does not grow as well as a *CAK1 cdc28Δ* strain harboring the same plasmid. Cross and Levine (1998) suggested that Cak1 fulfills additional nonessential roles required for optimal cell growth in rich medium.

ScCak1 belongs to the extended CDK family (Liu and Kipreos 2000). Gene *CAK1* is a high-copy-number suppressor of *kin28-ts* (Valay et al. 1995) and *bur1-ts* mutants (Yao and Prelich 2002). Given that ScCak1 resembles the CDKs and that Cak1 of metazoans (Cdk7) has a double function (the activation of CDKs and the phosphorylation of the CTD of the largest subunit of RNA Pol II), we asked whether ScCak1 might be, by itself, a direct regulator of transcription in vegetative growth. We present data indicating that proteins Paf1 and Ctr9, belonging to the same transcriptional elongation complex required for histone modifications (Krogan et al. 2003a; Wood et al. 2003; Ng et al. 2003), interact functionally with Cak1. Furthermore, we show that the thermosensitivity of a *cak1Δ cdc28-43244* strain is rescued by mutant alleles of gene *SSU72*. Ssu72 is a protein phosphatase that interacts with components required for transcription initiation of mRNA and transcription termination of snoRNAs and specific mRNA (Pappas and Hampsey 2000; Sanders et al. 2002; Dichtl et al. 2002; Ganem et al. 2003). Ssu72 catalyzes CTD serine-5 phosphate dephosphorylation (Krishnamurthy et al. 2004). We present evidence that the interactions linking Cak1 with the Paf1 complex on the one hand, and Cak1 with Ssu72 on the other hand, are not direct but are mediated via Ctk1 and Bur1.

Materials and methods

Strains and media

Strains used in this work are described in Table 1. Complete medium (YPD) is 1% (w/v) yeast extract, 1% (w/v) bactopeptone, 2% (w/v) glucose. G8 is complete medium with 8% (w/v) glucose. Synthetic complete medium (SC) with 2% glucose is as described by Sherman (1991). SPO2 sporulation medium is 0.5% yeast extract, 0.5% bactopeptone, 2% potassium acetate. Yeast cells were transformed by the lithium acetate procedure (Gietz et al. 1992).

Plasmids

Plasmids used in this study are listed in Table 2. Plasmids p1053 and pRS306-P3 were constructed by cloning the 314 kb *Pst*I–*Kpn*I DNA fragment encompassing gene *SSU72* at the *Pst*I–*Kpn*I sites of pBluescript (Stratagene) and at the *Nsi*I–*Kpn*I sites of vector pRS306, respectively. Plasmid p1054 was constructed by replacing the *Eco*RI–*SSU72*–*Hin*dIII DNA fragment (1,052 bp) of p1053 by a 2,362 bp DNA fragment (prepared from plasmid p443; Reynaud et al. 2001) harboring genes *TRP1* and *CYH2*.

Plasmid p1055, a 824 bp *Eco*RI–*Pst*I DNA fragment isolated from p443 and containing gene *TRP1*, was inserted at the *Sna*BI site of Litmus 28. Plasmid p1057, a 1,150 bp *Hin*cII–*Bsi*WI DNA fragment containing the C-terminal part of gene *KIN28* with mutation *kin28-T162A*, was inserted between the *Stu*I–*Bsi*WI sites of p1055.

To construct plasmid p1285, the *Xma*I–*Eco*RV DNA fragment containing gene *HIS3* isolated from pFA6a-HIS3MX6 (Longtine et al. 1998) was inserted in place of the 625 bp *Ngo*MIV–*Hpa*I fragment of pRS305 (Sikorski and Hieter 1989).

Strain constructions

Plasmid p1057 was partially digested with *Swa*I. The digestion products were used to transform strain GF1074. In this way, allele *kin28-T162A* was integrated at the *KIN28* locus and was linked (immediately downstream) with the *TRP1* gene whereas the chromosomal *KIN28* gene was disrupted. The resulting strain was named GF3945. Strains GF4011 and GF4012 are haploid segregants from GF3945.

Two PCR products (440 and 600 bp) synthesized using yeast genomic DNA and primers CTKBSI/CTKPS1 and CTKPS2/CTKBAM (CTKBSI GAATATACGTACGTTTTATACACAATAAATGG, CTKPS1 AACTGCAGCCATTATTGTAGGACATTG TTACC, CTKPS2 AACTGCAGGATATATAGCCA ATTGAAATAAGTAG, CTKBAM CGGGATCCGT

Table 1 Strains used in this study

Strain[a]	Genotype
GF1047	*MAT***a** *ura3 leu2 trp1 lys2*
GF1067	*MATα ura3 leu2 trp1 his3*
GF1074	GF1047×GF1067
GF1084	*MAT***a** *ura3 leu2 trp1 his3*
GF1288	*MAT***a** *ura3 leu2 trp1 lys2 cyh2^R*
GF2168	*MAT***a** *ura3 leu2 trp1 lys2 cyh2^R cak1::LEU2-TRP1-CYH2* pJG43 (*CAK1*)
GF2283	*MAT***a** *ura3 leu2 trp1 lys2 cyh2^R cak1-2*
GF2334	*MAT***a** *ura3 leu2 trp1 lys2 cyh2^R cak1-2334*
GF2351	*MAT***a** *ura3 leu2 trp1 lys2 cyh2^R cak1-4*
GF2414	*MAT***a** *ura3 leu2 trp1 lys2 ade2 ade3 cak1-2* (2283)
GF2415	*MATα ura3 leu2 trp1 his3 ade2 ade3 cak1-2* (2283)
GF2491	*MAT***a** *ura3 leu2 trp1 his3 ade2 ade3 cak1-4* (2351)
GF2492	*MATα ura3 leu2 trp1 lys2 ade2 ade3 cak1-4* (2351)
GF2909	*MATα ura3 leu2 trp1 his3 cak1-2*
GF2915	*MATα ura3 leu2 trp1 his3 cak1-4*
GF3195	*MAT***a** *ura3 leu2 trp1 his3 cdc28::TRP1* p789 (*cdc28-43244*)
GF3254	*MATα ura3 leu2 trp1 his3 cak1::KAN cdc28::TRP1* p789 (*cdc28-43244*)
GF3258	*MAT***a** *ura3 leu2 trp1 lys2 cak1::KAN cdc28::TRP1* p789 (*cdc28-43244*)
GF3319	*MATα ura3 leu2 trp1 his3 cak1::KAN cdc28::TRP1* p789 (*cdc28-43244*) *ssu72-3319*
GF3389	*MATα ura3 leu2 trp1 his3 cdc28::TRP1* p789 (*cdc28-43244*)
GF3390	*MAT***a** *ura3 leu2 trp1 his3 cdc28::TRP1* p789 (*cdc28-43244*)
GF3487	*MAT***a** *ura3 leu2 trp1 lys2 cak1::KAN cdc28::TRP1* p789 (*cdc28-43244*) *ssu72-3319*
GF3683	*MATα ura3 leu2 trp1 his3 cak1::KAN cdc28::TRP1* p789 (*cdc28-43244*) *SSU72 (URA3)*
GF3889	*MAT***a** *ura3 leu2 trp1 lys2 ssu72::KAN* p1037 (*ssu72-3319*)
GF4011	*MAT***a** *ura3 leu2 trp1 his3 kin28-T162A (TRP1)*
GF4012	*MATα ura3 leu2 trp1 his3 kin28-T162A (TRP1)*
GF4708	*MATα ura3 leu2 trp1 his3 paf1-78 (URA3)*
GF4709	*MAT***a** *ura3 leu2 trp1 his3 paf1-78 (URA3)*
GF4710	*MAT***a** *ura3 leu2 trp1 lys2 paf1-78* (*URA3*)
GF4711	*MATα ura3 leu2 trp1 lys2 paf1-78 (URA3)*
GF4713	*MATα ura3 leu2 trp1 his3 ctr9-39 (URA3)*
GF4714	*MAT***a** *ura3 leu2 trp1 lys2 ctr9-39* (*URA3*)
GF4715	*MATα ura3 leu2 trp1 lys2 ctr9-39 (URA3)*
GF4913	*MAT***a** *ura3 leu2 trp1 his3 bur1::TRP1* pSY40 (*bur1-T240A, LEU2*)
GF4942	*MATα ura3 leu2 trp1 his3 bur1::TRP1* pSY40 (*bur1-T240A, LEU2*)
GF4996	*MAT***a** *ura3 leu2 trp1 lys2 his3 ctk1::TRP1 ctk1-T338A (HIS3)*
GF5013	*MAT***a** *ura3 leu2 trp1 his3 bur1::TRP1 ctk1::TRP1* pSY40 (*bur1-T240A, LEU2*) *ctk1-T338A (HIS3)*

[a]All the strains are congenic

TTTGTATTGCTCATATTATATCATGCCC) were cloned end to end into vector Litmus 28, between restriction sites *Bsi*WI and *Bam*HI, yielding plasmid p1243. The 2,362 bp DNA fragment harboring genes *TRP1* and *CYH2* (see earlier) was cloned at the *Pst*I site of p1243, yielding plasmid p1257. This latter plasmid was digested with *Bsi*WI and *Acl*I and the digestion products were used to transform strain GF1074. A disruptant, GF4297, was induced to sporulate and haploid segregants (*ctk1::TRP1-CYH2*) were obtained. One of them was named GF4945. Plasmid p1555 was constructed by inserting a 2,200 bp *Sal*I–*Xma*I DNA fragment containing gene *ctk1-T338A*, between the *Sal*I and *Xma*I sites of p1285. Plasmid p1555 was digested with *Age*I and the digestion product was used to transform strain GF4945, yielding GF4949. In this way, plasmid p1555 was inserted at the *LEU2* locus of GF4949. GF4949 was crossed with GF1067. From this, segregant GF4996 was obtained.

Gene *BUR1* was amplified by PCR using genomic DNA and primers BURBAM and BURSAL (BURBAM CGCGGATCCGCGTTTGACTTGAGAAGT TACCAG, BURSAL CACTAGTGGGTCGACCGAG TGTCCTGAAGGTG) and cloned into vector pTZ19, between restriction sites *Bam*HI and *Sal*I, yielding plasmid p1475. The 2,362 bp DNA fragment harboring genes *TRP1* and *CYH2* (see earlier) was cloned in place of the *Sac*II–*Bgl*II fragment of p1475, yielding plasmid p1478. This latter plasmid was digested with *Nde*I and *Acl*I. The digestion products were used to transform strain GF1909. A transformant was isolated (GF4803) in which one copy of gene *BUR1* was disrupted with *TRP1–CYH2*. GF4803 was transformed with plasmid SY40 (*bur1-T240A*) (Yao and Prelich 2002). After meiosis, segregants GF4913 and GF4942 were obtained.

Disruption of *CAK1* and isolation of thermosensitive mutants

The *CAK1* gene of strain GF1288 was disrupted by substituting a DNA fragment containing *LEU2*, *TRP1*

Table 2 Plasmids used in this study

Plasmid	Vector (relevant genotype)	Insert (gene or gene fusion)	Source/reference
pJG37	YEplac181 (*LEU2*, 2μ)	*KIN28*	Gietz and Sugino (1988)
pJG41	YEplac181 (*LEU2*, 2μ)	*CAK1*	Gietz and Sugino (1988)
pJG43	YEplac33 (*URA3*, CEN)	*CAK1*	Gietz and Sugino (1988)
pRS306-P3	pRS306 (*URA3*)	*SSU72*	Sikorski and Hieter (1989)
pSY40	(*LEU2*, CEN)	*bur1-T240A*	Yao and Prelich (2002)
p562	YEplac195 (*URA3*, 2μ)	*CAK1*, *ADE3*	Gietz and Sugino (1988)
p644	pRS416 (*URA3*, CEN)		Sikorski and Hieter (1989)
p656	YCplac111 (*LEU2*, CEN)	*CAK1*	Gietz and Sugino (1988)
p678	YEplac181 (*LEU2*, 2μ)	*CAK1*, *ADE3*	Gietz and Sugino (1988)
p681	YEp13 (*LEU2*, 2μ)	*CDC28*	Broach et al. (1979)
p785	YEp24 (*URA3*, 2μ)	*CDC28*	Biolabs
p789	pRS315 (*LEU2*, CEN)	*cdc28-43244*	Sikorski and Hieter (1989)
p1037	pRS416 (*URA3*, CEN)	*ssu72-3319*	Sikorski and Hieter (1989)
p1053	pBluescript	*SSU72*	Stratagene
p1055	Litmus 28	*TRP1*	Biolabs
p1057	p1055	*kin28-T162A*	Espinoza et al. (2000)
p1285	pRS305 (*LEU2*)	*HIS3*	Sikorski and Hieter (1989)
p1467	pRS426 (*URA3*, 2μ)	*SSU72*	Ganem et al. (2003)
p1475	pTZ19	*BUR1*	Pharmacia
p1555	p1285	*ctk1-T338A*	V. Goguel
p1571	YCplac111 (*LEU2*, CEN)	*ssu72-3319*	Gietz and Sugino (1988)

and *CYH2* in place of the 940 bp *Eco*RV–*Xcm*I segment of *CAK1*. The resulting strain was named GF2168. The disruption was complemented with plasmid pJG43. PCR mutagenesis was carried out as previously described (Thuret et al. 1996) using primer MCAMUT5 GGAAA ACGACAGAAGTCTC and MCAMUT3 ATCCTCCT GATGTCATTGC. PCR products were used to transform strain GF2168.

DNA sequencing

Genomic DNAs of mutants 2283 (*cak1-2* or *civ1-2* in Thuret et al. 1996), 2334 and 2351 (*cak1-4* or *civ1-4* in Thuret et al. 1996) were prepared. The *CAK1* sequences were amplified by PCR. PCR products were sequenced with the help of appropriate primers.

Screen for synthetic lethal mutations

We followed the method developed by Bender and Pringle (1991) to screen for mutations synthetic lethal with *cak1-2* (2283) or *cak1-4* (2351). Strains GF2414 (*cak1-2*), GF2415 (*cak1-2*), GF2491 (*cak1-4*) and GF2492 (*cak1-4*) were transformed with plasmid p562 (*CAK1*, *ADE3*). Cells of transformants were UV irradiated and then plated on G8 plates and incubated for 5–6 days at 25°C (Valay et al. 1995). Red clones were retained. To ascertain that these clones were not changed into ADE3+ by recombination, they were transformed with pJG41 (*CAK1*) or p656 (*CAK1*), or transformed with YEplac181 or YEplac111 as controls. Mutants giving rise to sectored colonies with pJG41 or p656 and only red ones with the control plasmids were kept.

For complementation tests, mutants were mated on YPD plates. Diploids were selected on SC (-HIS, -LYS). Samples of diploids were streaked on G8 plates. If both mutations are allelic every colony should be red, whereas the presence of sectored colonies indicates that both mutations belong to different complementation groups.

For identification of thermosensitive synthetic lethal mutants, 5 µl of synthetic lethal cells were spotted onto YPD plates and left for 3 days at 25 and 39°C. To assess whether synthetic lethality and thermosensitivity were due to a single mutation, thermosensitive synthetic lethal mutants harboring mutation *cak1-2* were crossed with either strain GF2414 or GF2415, and those harboring mutation *cak1-4* were crossed with either strain GF2491 or GF2492, according to their mating type. After sporulation, the thermosensitivity and the lethality of segregants were analyzed.

Identification of synthetic lethal genes

We changed plasmid p562 (*CAK1*, *ADE3, URA3*) borne by the thermosensitive synthetic lethal mutants for plasmid p678 (*CAK1*, *ADE3*, *LEU2*). After this substitution, the mutants were transformed with a wild-type genomic library built with the centromeric vector YCp50 (URA3, CEN). Transformants were selected on SC (-LEU, -URA) plates at 28°C, then replica-plated on YPD plates which were incubated for 3 days at 37°C. The YCp50-derived plasmids borne by the thermoresistant transformants were extracted and transferred into *E. coli* strain MC1066 by electroporation. They were amplified and used to transform the corresponding thermosensitive synthetic lethal mutants. Plasmids conferring thermoresistance and giving sectored colonies

were kept. Both ends of their insert DNA were sequenced.

Results

Thermosensitive *cak1* mutants

CDK-activating kinases of *S. cerevisiae* (ScCak1) and of *Schizosaccharomyces pombe* (*csk1*) (Molz and Beach 1993) have analogous functions, and phylogenetic analysis indicates that they are evolutionarily related (Liu and Kipreos 2000). ScCak1 kinase orthologs have been identified in other ascomycetes such as *Kluyveromyces lactis* (data not shown), *Zygosaccharomyces rouxii* (de Montigny et al. 2000), *Aspergillus fumigatus* (Pain et al. 2004), *Neurospora crassa* (Galagan et al. 2003) and *Candida albicans* (Bordon-Pallier et al. 2004). The amino acid sequences of ascomycetous Cak1 kinases have diverged significantly from members of the CDK family (Liu and Kipreos 2000). Nevertheless, we were able to construct a 3D model of ScCak1 using a low sequence identity approach. The model suggests that ScCak1 could adopt the conserved fold of CDKs despite large and variable insertions in external loops (data not shown).

Protein kinases often have several substrates (Liu et al. 2004; Ubersax et al. 2003). ScCak1 phosphorylates four CDKs. Three of them are directly involved in the transcription cycle mediated by RNA Pol II. To explore how Cak1 participates in transcription, we undertook a search for genes interacting with Cak1, using genetic approaches. We first prepared a collection of *cak1* conditional mutants. Mutations were generated using a PCR method under error-prone conditions. PCR products were directly inserted into the yeast genome in place of the genomic *CAK1* gene.

We sequenced the *CAK1* gene in three of the thermosensitive *cak1* mutants obtained: 2283 (*cak1-2*), 2334 and 2351 (*cak1-4*). The three mutants harbor multiple mutations: 2283 (M84I, V201M), 2334 (L127V, S235Y) and 2351 (D71G, V225E, S256C, V336G).

Isolation of synthetic lethal mutations

We constructed strains GF2414 and GF2415 carrying mutation *cak1-2* and strains GF2491 and GF2492 harboring mutation *cak1-4* (Table 1). The *ADE2* and *ADE3* genes of these four strains are deleted. These strains are white, but turn red when transformed with plasmid p562 (*CAK1*, *URA3*, *ADE3*) (Table 2), which is easily lost at the permissive temperature, forming sectored colonies (Valay et al. 1995). Cells of these four strains were mutagenized by UV irradiation, and then spread on G8 plates. The uniformly red colonies found among sectored or white ones were presumed to contain mutations synthetic lethal with *cak1-2* or *cak1-4*. Twenty-six red mutants were isolated from strain GF2414 (*cak1-2*), 13 from strain GF2415 (*cak1-2*), 18 from strain GF2491 (*cak1-4*) and 14 from strain GF2492 (*cak1-4*). Complementation analysis was undertaken by crossing the mutants from GF2414 (*cak1-2*) with those of GF2415 (*cak1-2*) and those from GF2491 (*cak1-4*) with those of GF2492 (*cak1-4*). Unfortunately, the crosses were not conclusive because several synthetic lethal mutations either reverted strongly or were semi-dominant. We sought synthetic lethal mutations that also conferred a thermosensitive growth phenotype, since this facilitates the cloning of the corresponding wild-type gene. Five mutants did not grow at 37°C: syn39, syn78, syn858, syn2199 and syn3118. These five mutants appeared synthetic lethal with both *cak1-2* and *cak1-4* at permissive temperatures. Since *cak1* mutations were synthetically lethal with either *kin28-ts3* (Valay et al. 1995) or *cdc28* mutation (Thuret et al. 1996), we screened the five thermosensitive mutants for those affected in either the *KIN28* or *CDC28* gene. Mutants were transformed with plasmid pJG37 (*KIN28*), plasmid p681 (*CDC28*) or plasmid p678 (*CAK1*) (Table 2) as a control. Only syn3118 transformed with pJG37 (*KIN28*) was able to grow at 37°C and form sectored colonies at the permissive temperature, indicating that it bears a mutation in the *KIN28* gene.

The wild-type allele of syn858 was cloned by complementation with a genomic library constructed in the centromeric *LEU2* plasmid p366 (P. Hieter, ATCC 77162). We selected thermoresistant transformants that were also able to form sectored colonies at the permissive temperature. One complementing plasmid, p745, was isolated. It contains an insert, belonging to chromosome VII, whose ends have the coordinates 917900–925150. Three ORFs are included in this fragment: *RTA1*, *YST1* and *GPI1*. By subcloning, we found that only *GPI1* complemented syn858. Gpi1 is involved in the first step of yeast glycosyl–phosphatidyl–inositol synthesis. At present, we do not know why *cak1* and *gpi1* mutations are synthetically lethal.

Plasmid p562 (*CAK1*, *URA3*, *ADE3*) carried by mutants syn39, syn78 and syn2199 was exchanged with plasmid p678 (*CAK1*, *LEU2*, *ADE3*). The resulting strains were transformed with a genomic library made in centromeric *URA3* plasmid Ycp50. Thermoresistant transformants forming sectored colonies at permissive temperatures were selected. Complementing plasmids were isolated and the different genes borne by the DNA inserts were subcloned. We found that syn2199, syn39 and syn78 were complemented by genes *YDJ1*, *CTR9* and *PAF1*, respectively. Ydj1 is an Hsp40 molecular chaperone interacting with Hsp70. Paf1 and Ctr9 are associated with Cdc73, Rtf1 and Leo1 to compose the Paf1 complex (Krogan et al. 2002; Mueller and Jaehning 2002; Squazzo et al. 2002) which is required for transcription elongation (Pokholok et al. 2002; Squazzo et al. 2002; Rondon et al. 2004). Inactivation of the Paf1 complex causes alterations in the transcription of a small subset of the genome that includes cell wall biosynthetic genes and many cell cycle regulated genes (Mueller and Jaehning 2002).

To check that we had indeed identified the wild-type genes corresponding to these synthetic lethal alleles that we named *ctr9-39* and *paf1-78*, we performed the following experiments. We separated mutations *ctr9-39* and *paf1-78* from mutation *cak1-2* by carrying out appropriate crosses. Using integrative plasmids, the *URA3* gene was inserted in the vicinity of *ctr9-39* and *paf1-78*. The resulting strains, GF4710 (*paf1-78*) and GF4714 (*ctr9-39*), were crossed with GF2909 (*cak1-2*) or GF2915 (*cak1-4*). After sporulation and tetrad analysis, one-quarter of the spores were unable to develop colonies at 30°C and each of these harbored the $URA3^{+}$ allele, thereby demonstrating linkage of the cloned DNA to the site of the synthetic lethal mutations.

We sequenced alleles *paf1-78* and *ctr9-39* as well as the *PAF1* and *CTR9* wild-type genes of a congenic strain. Allele *paf1-78* has its coding sequence interrupted by a stop codon at position 81 and *ctr9-39* harbors a one base deletion at codon 519. Strain GF4709 (*paf1-78*) was crossed with GF4715 (*ctr9-39*). Tetrad analysis revealed that the double-mutant *paf1-78 ctr9-79* was viable at 25°C, but unviable at 37°C.

The Paf1 complex functionally interacts with Ctk1 and Bur1

At the permissive temperature, conditional mutations *cak1-2* or *cak1-4* should simultaneously, but presumably at different degrees of severity, affect the phosphorylation of Cdc28, Kin28, Bur1, Ctk1 and possibly other substrates. Porter et al. (2002) have shown that loss of Paf1 affects the expression of a subset of yeast genes among which about one-third are regulated during the cell cycle. Furthermore, mutant *paf1Δ* appears synthetic lethal with mutations affecting cell cycle regulation such as *cln3Δ* (Koch et al. 1999), *swi4Δ* or *swi6Δ* (Porter et al. 2002). On the other hand, it is accepted that Cdc28, through several actors (Wittenberg and Reed 2005) regulates the expression of many genes with cell cycle periodicity. We previously showed that mutant *cak1-4* (*civ1-4* = *cak1*-4; Thuret et al. 1996) is preferentially blocked in G1 at 37°C. One possibility to explain the unviability of double-mutant *paf1-78 cak1-4* at permissive temperatures is to suppose that the poor phosphorylation level of Cdc28 on threonine 169 plus the inactivation of the Paf1 complex impedes the expression of essential cell cycle regulated genes. However, since the Paf1 complex has general functions in the transcription cycle, we may also suspect that it is the phosphorylation deficit of at least one of the three CDKs (Kin28, Bur1 or Ctk1) that gives rise to a synthetic lethal phenotype when combined with mutants of the Paf1 complex. This second eventuality does not exclude the previous one. To test this hypothesis, we constructed congenic yeast strains in which wild-type genes *KIN28*, *BUR1* and *CTK1* were replaced by alleles *kin28-T162A*, *bur1-T240A* and *ctk1-T338A*, respectively. We postulated that the alanyl residue mimics the nonphosphorylated threonyl residues. Experiments have proven that the in vitro kinase activities of proteins Kin28-T162A, Bur1-T240A and Ctk1-T338A are significantly reduced or undetectable (Kimmelman et al. 1999; Yao and Prelich 2002; Keogh et al. 2003; Ostapenko and Solomon 2005). However, we found that strains harboring only *kin28-T162A* (e.g., GF4011), *bur1-T240A* (e.g., GF4913) or *ctk1-T338A* (e.g., GF4996) grew well at 37°C on YPD plates (cf. Figs. 1, 3). The generation time of *kin28-T162A* and *ctk1-T338A* mutants is 1 h 38 min, whereas the *bur1-T240A* mutant divides in 1 h 50 min and a congenic wild-type strain doubles in 1 h 32 min, in YPD at 28°C. We crossed strain GF4011 (*kin28-T162A*) with either strains GF4711 (*paf1-78*) or GF4715 (*ctr9-39*). We found that haploid segregants *kin28-T162A paf1-78* or *kin28-T162A ctr9-39* were not more thermosensitive than single-mutants *paf1-78* or *ctr9-39* (Fig. 1a1, a2). In contrast, haploid double-mutants obtained by crossing GF4913 (*bur1-T240A*) with either GF4711 (*paf1-78*) or GF4715 (*ctr9-39*) were not able to grow at 32°C and grew very poorly at 28°C (Fig. 1b1, b2). Finally, we crossed strain GF4996 (*ctk1-T338A*) with either strains GF4708 (*paf1-78*) or GF4713 (*ctr9-39*). Tetrad analysis revealed that double-mutants *ctk1::TRP1 paf1-78*, *ctk1-T338A paf1-78* and *ctk1::TRP1 ctr9-39* were unviable at 28°C whereas double-mutant *ctk1-T338A ctr9-39* grew very slowly (Fig. 1c1, c2). These results suggest that the functional interaction linking Cak1 with the Paf1 complex is indirect. The deficient activity of Ctk1 and Bur1 in *cak1-2* or *cak1-4* mutants, associated with a disorganized Paf1 complex, may impede transcription elongation of numerous genes.

Mutations in gene *SSU72* suppress the thermosensitivity of a *cak1Δ cdc28-43244* strain

Given that Cak1 activates four kinases which themselves have several substrates (Ubersax et al. 2003), it is understandable that the search of mutations synthetic lethal with *cak1* conditional mutations leads to the identification of numerous indirect functional interactions. Replacement of the wild-type *CDC28* gene by an allele whose product does not require Cak1 phosphorylation for its activity, should reduce the spectrum of genes genetically connected with *CAK1*. Indeed, Cross and Levine (1998) have shown that, although *CAK1* is essential in *S. cerevisiae*, *cak1Δ* mutants can proliferate when harboring the multimutated *cdc28-43244* allele. However, such strains, like GF3254 or GF3258 [(*cak1Δ cdc28Δ*, p789/ (*cdc28-43244*, *LEU2*)], are thermosensitive at 35°C (Fig. 2a) whereas a strain deleted for *CDC28* and complemented with plasmid p789 (*cdc28-43244*, *LEU2*) is thermosensitive only above 37°C (data not shown). Hoping to find pertinent interactions linking Cak1 activity and the transcription process, we have searched for extragenic suppressors of the thermosensitivity of strains GF3254 or GF3258.

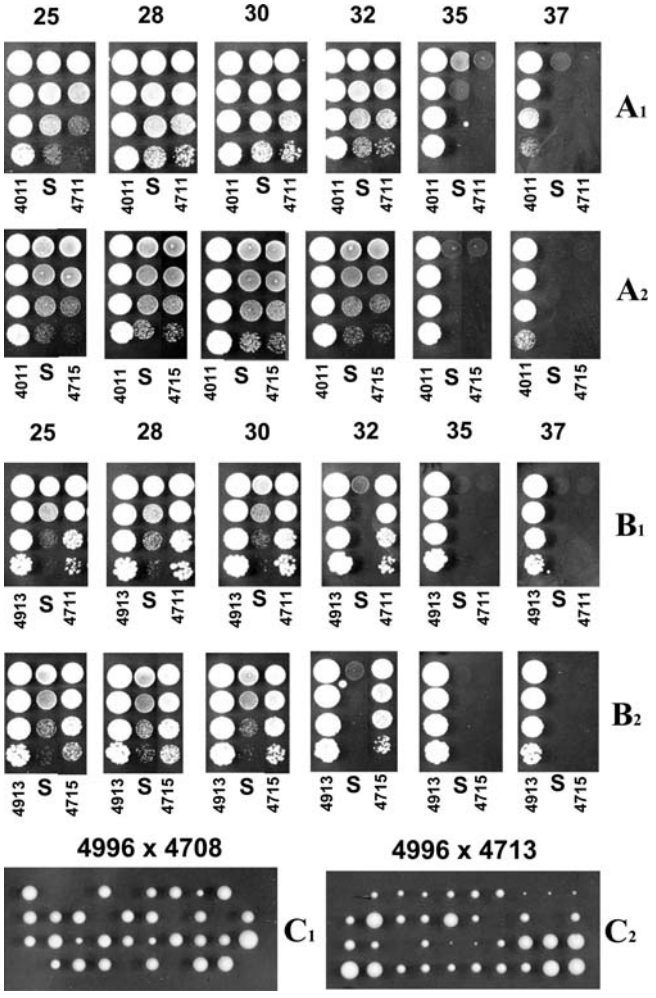

Fig. 1 Synthetic lethal interactions between *ctk1-T338A* or *bur1-T240A* and mutations in genes of the PAF1 complex. **a1** Serial dilutions (1:10) of haploid *kin28-T162A* (GF4011), haploid *paf1–78* (GF4711) and haploid double-mutant *kin28-T162A paf1–78* (S) cells, were spotted onto YPD medium and grown for 2 days at the indicated temperatures. **a2** Serial dilutions of haploid *kin28-T162A* (GF4011), haploid *ctr9–39* (GF4715) and haploid double-mutant *kin28-T162A ctr9–39* (S) cells grown for 2 days. Double-mutants are not more thermosensitive than single-mutant *paf1–78* or *ctr9-39*. **b1** Serial dilutions (1:10) of haploid *bur1-T240A* (GF4913), haploid *paf1-78* (GF4711) and haploid double-mutant *bur1-T240A paf1-78* (S) cells, were spotted onto YPD medium and grown for 3 days at the indicated temperatures. **b2** Serial dilutions of haploid *bur1-T240A* (GF4913), haploid *ctr9-39* (GF4715) and haploid double-mutant *bur1-T240A ctr9-39* (S) cells grown for 3 days. Double-mutants are clearly more thermosensitive than single-mutants *paf1-78* or *ctr9-39*. **c1** Strain GF4996 was crossed with GF4708 [*paf1-78* (*URA3*)]. The former strain has gene *ctk1* disrupted with *TRP1* and allele *ctk1-T338A* (*HIS3*) inserted at the *LEU2* locus. The three loci *CTK1*, *LEU2* and *PAF1* are unlinked. Diploids were induced to sporulate. Tetrads were dissected on YPD medium and left 6 days at 28°C. Segregants *ctk1::TRP1 ctk1-T338A* (*HIS3*) *paf1-78* (*URA3*) (1:8 spores expected) and *ctk1::TRP1 his3 paf1-78* (*URA3*) (1:8 spores expected) appeared unviable. **c2** Strain GF4996 was crossed with GF4713 [*ctr9-39* (*URA3*)]. After dissection, tetrads were left 6 days at 28°C. Segregants *ctk1::TRP1 his3 ctr9* (*URA3*) (1:8 spores expected) were unviable whereas segregants *ctk1::TRP1 ctk1-T338A* (*HIS3*) *ctr9-39* (*URA3*) (1:8 spores expected) were growing very slowly

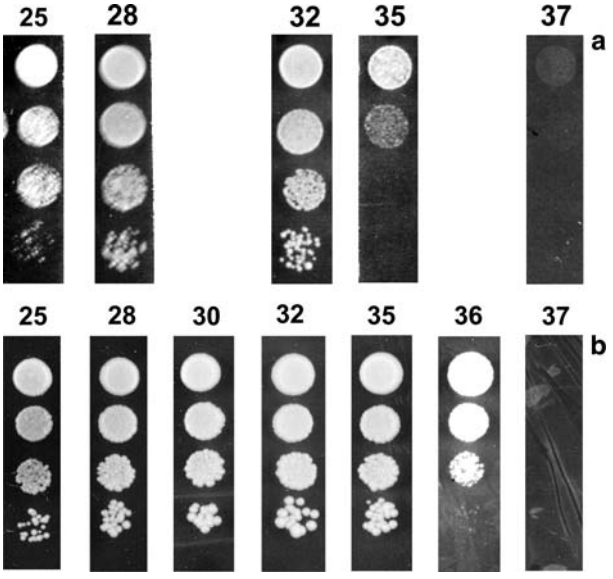

Fig. 2 Mutations in gene *SSU72* suppress the thermosensitivity of strain GF3254 (*cak1Δ cdc28Δ* p789 (*cdc28-43244*). Serial dilutions (1:10) of GF3254 (**a**) and spontaneous thermoresistant revertant GF3319 (**b**) cells were spotted onto YPD medium and grown for 3 days at the indicated temperatures

We plated strains GF3254 and GF3258 on YPD at 36°C. Seventeen spontaneous thermoresistant revertants were isolated (GF3315–GF3331), e.g., see GF3319 (Fig. 2b). Plasmid p789 (*cdc28-43244*, *LEU2*) was recovered from each of these revertants and the 17 DNA preparations were used to transform strain GF3228 [*cak1Δ cdc28Δ*, pJG43 (*CAK1*, *URA3*), p785 (*CDC28*, *URA3*)]. After elimination of plasmids pJG43 and p785, LEU+ transformants were tested for their capacity to grow on YPD at 36°C. All of them were thermosensitive, indicating that revertant mutations were not located in the *cdc28-43244* gene.

Revertants were crossed with GF3389 or GF3390 to redistribute mating type and auxotrophic markers. By doing appropriate crosses, diploids *cak1Δ*/*cak1Δ cdc28Δ*/*cdc28Δ* p789 (*cdc28-43244*) and homozygous or heterozygous for each of the 17 revertant mutations were obtained. Their thermosensitivity at 36°C was compared with that of the GF3254×GF3258 diploid. One revertant (3319) appeared clearly dominant, nine were recessive and the other seven were semidominant.

A library was prepared using the genomic DNA of strain GF3319 and vector pRS416. Strain GF3254 [(*cak1Δ cdc28Δ*, p789/ (*cdc28-43244*)] was transformed with this bank and URA+ transformants able to grow at 36°C were selected. The complementing gene is *SSU72* bearing a mutation *(ssu72-3319*) which changes the glycine of codon 42 in aspartic acid (G42D).

By doing appropriate crosses, the *SSU72-3319* mutation was placed in an otherwise wild-type context. The resulting strain, named GF3889, grows well at 37°C and does not present an altered phenotype on YPD plates.

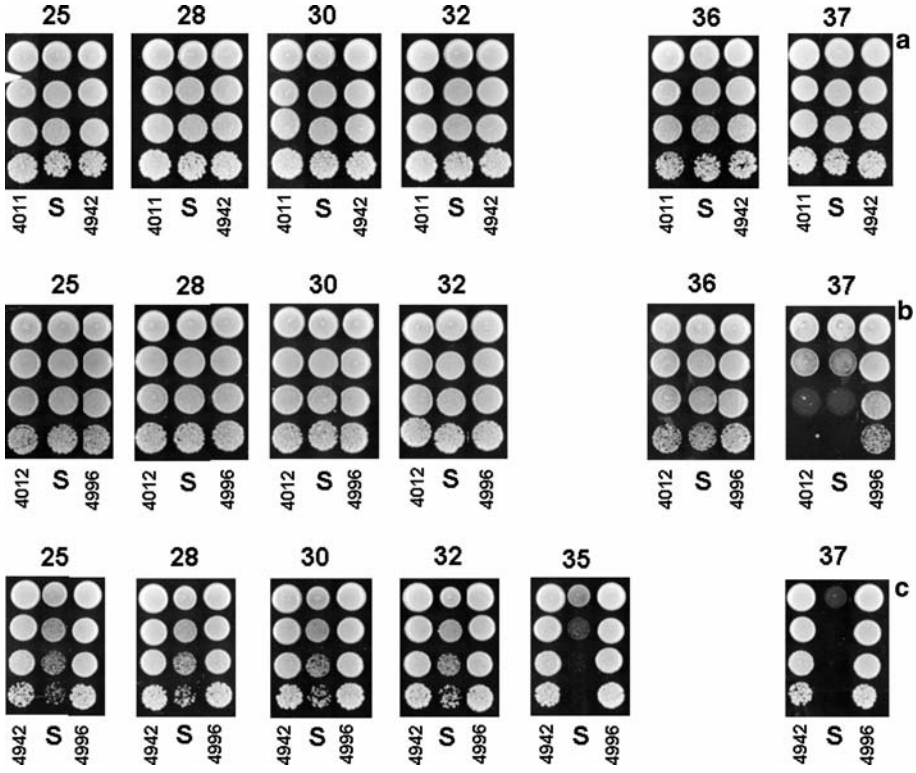

Fig. 3 Double-mutant *ctk1-T338A bur1-T240A* is thermosensitive at 35°C. **a** Serial dilutions (1:10) of haploid *kin28-T162A* (GF4011), haploid *bur1-T240A* (GF4942) and haploid double-mutant *kin28-T162A bur1-T240A* (S) cells were spotted onto YPD medium and grown for 3 days at the indicated temperatures. **b** Serial dilutions (1:10) of haploid *kin28-T162A* (GF4012), haploid *ctk1-T338A* (GF4996) and haploid double-mutant *kin28-T162A ctk1-T338A* (S) cells. **c** Serial dilutions (1:10) of haploid *bur1-T240A* (GF4942), haploid *ctk1-T338A* (GF4996) and haploid double-mutant *bur1-T240A ctk1-T338A* (S) cells

We were also curious to know whether the other revertants contained mutations in *SSU72*. To test this eventuality, these revertants were crossed with strain GF3683 [*cak1Δ cdc28Δ SSU72* (*URA3*), p789 (*cdc28-43244*)]. In this strain *URA3* is inserted upstream of *SSU72*. The diploids obtained were induced to sporulate and tetrads were analyzed. Although in the *cak1Δ cdc28Δ* p789 (*cdc28-43244*) context the sporulation yield is low (data not shown), we concluded that revertants 3320, 3324, 3325, 3329 and 3331 carry a mutation in gene *SSU72* (Table 3). By sequencing we found that revertant 3320, which is semidominant, harbors the amino acid change S43L. Interestingly, this alteration is just downstream of that of revertant 3319 (G42D). The other four revertants, which are recessive, carry the amino acid change H194P. We cannot exclude that these four revertants are different isolates of the same original mutant.

Ssu72-3319 suppresses the transcription defects due to Ctk1-T338A and Bur1-T240A

We used genetic analyses to define the basis for the thermosensitivity of the *cak1Δ cdc28-43244* mutant (GF3254). The threonyl residues located in the T-loop of Kin28, Bur1 and Ctk1 are simultaneously nonphosphorylated in this strain.

We first tested whether it is the association of *cdc28-43244* with one of the nonphosphorylated kinases that confers the thermosensitive phenotype. We used the *kin28-T162A*, *bur1-T240A* and *ctk1-T338A* mutations to mimic the effect of the nonphosphorylated state of these kinases. We constructed strains harboring the following mutations: *cdc28-43244 kin28-T162A*, *cdc28-43244 bur1-T240A* or *cdc28-43244 ctk1-T338A*. All of these strains grew well at 36°C on YPD plates (data not shown). So the presence of allele *cdc28-43244* does not appreciably affect the thermosensitivity of yeast cells harboring mutations *kin28-T162A*, *bur1-T240A* or *ctk1-T338A*.

Next we considered whether it might be the simultaneous presence of two nonphosphorylated kinases that renders strains GF3254 (*cak1Δ cdc28-43244*) thermosensitive. Using strains GF4011 (*kin28-T162A*), GF4012 (*kin28-T162A*), GF4942 (*bur1-T240A*) and GF4996 (*ctk1-T338A*), we constructed the three possible combinations: *kin28-T162A bur1-T240A*, *kin28-T162A ctk1-T338A* and *ctk1-T338A bur1-T240A* and found that the latter combination is thermosensitive at 35°C, as are the strains GF3254 or GF3258 (*cak1Δ cdc28-43244*) (Fig. 3). Thus, the thermosensitivity of the *cak1Δ cdc28-43244* strain might be due to the simultaneous absence of phospho-T338-Ctk1 and phospho-T240-Bur1. If true, the *SSU72-3319* allele should suppress the thermosensitivity of *ctk1-T338A bur1-T240A* segregants as it did for strain GF3254. We transformed strain GF5013 (*bur1-T240A ctk1-T338A*) with plasmid p1037 (*SSU72-3319*), plasmid p1467 (*SSU72*, 2 μ) or plasmid p644 used as a control. With plasmid p1037 (Fig. 4), but not with plasmid p1467 (data not shown), strain GF5013 recovered thermoresistance. The fact that strains GF3254 and GF5013 have similar thermosensitive phenotypes and that their sensitivities are equally suppressed by mutation *ssu72-3319* supports the hypotheses that (1) proteins

Table 3 Analysis of sporulation segregants of diploids obtained by crossing GF3683 with thermoresistant revertants

Revertants	Relevant phenotypes				Gene suppressor
	URA+ t^S	Ura− t^R	Ura+ t^R	Ura− t^S	
3315	7	6	8	7	Unknown
3317	11	10	11	9	Unknown
3318	5	4	8	7	Unknown
3319	13	9	0	1	SSU72
3320	17	14	1	2	SSU72
3321	5	5	6	7	Unknown
3322	8	8	9	12	Unknown
3323	6	4	7	7	Unknown
3324	15	13	0	0	SSU72
3325	14	8	0	0	SSU72
3326	8	4	7	9	Unknown
3327	6	4	9	5	Unknown
3328	5	3	7	8	Unknown
3329	16	13	0	0	SSU72
3330	7	6	7	7	Unknown
3331	12	7	0	1	SSU72

Strain GF3683 [*cak1Δ cdc28Δ SSU72* (*URA3*$^+$) p789 (*cdc28-43244*)] was crossed with the different thermoresistant revertants obtained, the genotypes of which being either *cak1Δ cdc28Δ ssu72*^{REV} p789 (*cdc28-43244*) or *cak1Δ cdc28Δ SSU72*$^+$ *REV* p789 (*cdc28-43244*). Diploids were induced to sporulate and the meiotic products were analyzed. If a revertant is mutated in *SSU72*, it is expected that 50% of the spores will be thermo-resistant and URA- (t^R, URA-) whereas the other 50% will be thermo-sensitive and URA+ (t^S, URA+). On the other hand, if a revertant is mutated in a gene not genetically linked to *SSU72*, spores (t^R, URA-), (t^R, URA+), (t^S, URA-) and (t^S, URA+) will be observed with the same frequency of 25%

Ctk1-T338A and Bur1-T240A are not more affected in their activity than the nonphosphorylated wild-type counterparts and (2) *ssu72-3319* suppresses a transcript defect due to these nonphosphorylable kinases.

Discussion

We isolated mutations that are synthetically lethal with *cak1-ts* mutations to identify more proteins whose activities depend on Cak1 when cells are growing in rich media. Five of the synthetic lethal mutants were identified in the following genes: *KIN28*, *YDJ1*, *GPI1*, *PAF1* and *CTR9*. We previously described synthetic lethality between *kin28* and *cak1* (Valay et al. 1995). Ydj1 is a chaperone that participates in protein folding, so the observed synthetic lethality may be due to deleterious effects of the Ydj1 mutant on the residual activity of the Cak1 mutants or its downstream hypophosphorylated targets. Gpi1 catalyzes the first step in the synthesis of glycosyl–phosphatidyl–inisitol anchors of cell surface proteins. The basis for *cak1 gpi1* synthetic lethality is not clear and was not investigated further, but it may involve defects of gene expression in the *cak1* mutants.

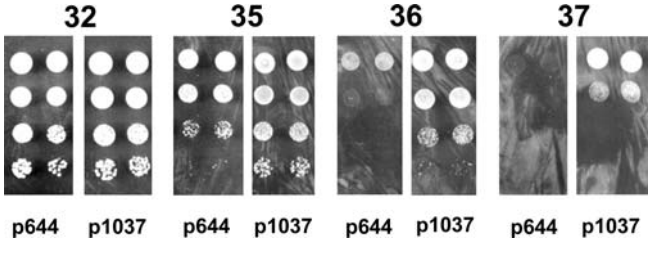

Fig. 4 Suppression of the thermosensitive phenotype of strain GF5013 (*bur1-T240A ctk1-T338A*) with gene *SSU72-3319*. Strain GF5013 was transformed with either plasmid p1037 (*SSU72-3319*) or plasmid p644, used as a control. Serial dilutions (1:10) of transformant cells were spotted onto synthetic complete medium without uracil and grown for 3 days at the indicated temperatures

Since Paf1 and Ctr9 belong to the same complex, the synthetic lethality of *cak1* with either *paf1* or *ctr9* mutants suggests a functional relationship between Cak1 and the Paf1 complex. However, we found that the combination of *ctk1-T338A* with *paf1-78* or *ctr9-39* is synthetically lethal or strongly deleterious for cell growth, while *bur1-T240A paf1-78* or *bur1-T240A ctr9-29* double-mutants survive with difficulty. From these results, we surmise that hypophosphorylation of Ctk1 and Bur1 in the *cak1-ts* mutants may explain the synthetic lethality of the *cak1-ts paf1-78* or *cak1-ts ctr9-39* double-mutants. Since the effects of *cak1-ts* mutations on the Paf1 complex appear indirect, we will turn our discussion to the relationships linking Ctk1 and Bur1 with the Paf1 complex.

Loss of Paf1 (or Ctr9) results in severe defects affecting many cellular processes (Betz et al. 2002), but the mutants still proliferate on rich media. Similarly, strains harboring *ctk1* null alleles are viable. It was previously shown that *paf1*, *ctr9*, *cdc73*, *rtf1* and *leo1* deletion mutations show strong synthetic interactions with a *ctk1* null mutation, including lethality (Squazzo et al. 2002). Here we have shown that the slight alteration of Ctk1 brought by the T338A mutation, in combination with mutations *paf1-78* or *ctr9-39*, abolishes or strongly inhibits cell growth. These results further confirm and support the possibility that Ctk1 and the Paf1 complex cooperate in the transcription process.

The Paf1 complex as well as Ctk1 are present at promoters and throughout the coding region of the transcribed genes (Ahn et al. 2004; Cho et al. 2001). It is known that Ctk1 and the Paf1 complex have several roles that may be combined at different steps of the transcription cycle. The Paf1 complex has been shown to be required for histone H2B ubiquitination by Rad6/Bre1 and for the subsequent histone H3 methylation by Set1, Set2 and Dot1 (Ng et al. 2003; Krogan et al. 2003a, b; Wood et al. 2003; Zhang 2003; Xiao et al. 2005). The histone H3 methylation activity of Set2 is triggered by its association with serine-2 phosphorylated CTD (Krogan et al. 2003b) catalyzed by Ctk1. This phosphorylation of the CTD is also needed to resume transcription from the pre-mRNA capping checkpoint (discussed subsequently). The activities of Ctk1 and of the Paf1 complex also influence mRNA 3′-end processing and/or transcription termination. The recruitment of polyadenylation factors to 3′-regions of genes is disrupted in *ctk1Δ* strains (Ahn et al. 2004), and loss of the Paf1 complex leads to reduced RNA Pol II serine-2 phosphorylation and shortened poly(A) tails (Mueller et al. 2004). Since Ctk1 and the Paf1 complex each intervenes at multiple points in the transcription process, it is likely that the *ctk1-T338A paf1-78* double-mutant is lethal because of defects in the transcription of numerous genes.

We also discovered a functional interaction linking the Bur1 kinase and the Paf1 complex. Double-mutants *bur1-T240A paf1-78* or *bur1-T240A ctr9-39* grow slowly on YPD at 28°C. Bur1 is an essential kinase required for efficient transcription elongation by RNA Pol II and might phosphorylate a substrate other than the CTD (Murray et al. 2001; Keogh et al. 2003). As the exact role(s) of Bur1 is not clearly understood, it is difficult to envision at which step of the transcription process the interaction between Bur1 and the Paf1 complex becomes critical. A possible stage where they might both intervene is the 5′ capping checkpoint (Pei and Shuman 2003; Sims et al. 2004) as Bur1 and the Paf1 complex genetically interact with Spt4/Spt5 (Murray et al. 2001; Squazzo et al. 2002).

We found that the thermosensitivity of a *cak1Δ cdc28-43244* strain is suppressed by the *SSU72-3319* mutation. We showed that a congenic strain harboring both *ctk1-T338A* and *bur1-T240A* mutations is thermosensitive, and this phenotype is also suppressed by *SSU72-3319*. From these results, we conclude that the lack of Ctk1 and Bur1 T-loop phosphorylation in the *cak1Δ cdc28-43244* strain is sufficient to explain its thermosensitivity.

Ssu72 is an essential protein that interacts with the general transcription factor TFIIB, with RBP2 (the second largest subunit of RNA Pol II; Sun and Hampsey 1996; Wu et al. 1999; Pappas and Hampsey 2000), and with Taf150 (a component of the TFIID complex; Sanders et al. 2002). It is also associated with cleavage and polyadenylation factor CPF (Dichtl et al. 2002). We have shown that Ssu72 is a protein phosphatase that interacts genetically and physically with Kin28 and genetically with the Fcp1 phosphatase (Ganem et al. 2003), and Ssu72 was recently shown to be a CTD phosphatase with specificity for serine-5 phosphate (Krishnamurthy et al. 2004). Ssu72 appears to be required for the transcription termination of some pre-mRNAs, snRNAs and snoRNAs (Ganem et al. 2003; Nedea et al. 2003; Steinmetz and Brow 2003).

We showed previously that *ssu72-ts ctk1Δ* double-mutants are lethal (Ganem et al. 2003). The fact that the deficient activities of Ctk1-T338A and Bur1-T240A can be suppressed by Ssu72-3319, which harbors the amino change G42D but has kept its catalytic capacity (Ganem et al. 2003), strongly suggests that Ctk1/Bur1 and Ssu72 functionally interact. More precisely, since cells become thermosensitive only when they carry both the *ctk1-T338A* and *bur1-T240A* mutations, we suspect that Ssu72-3319 acts as a suppressor in a transcription step where Ctk1 and Bur1 intervene together. Such a step may be the pre-mRNA capping checkpoint (Keogh et al. 2003). It is thought that soon after transcription initiation, the Spt4/Spt5 complex interacts with RNA Pol II and promotes a pause (Sims et al. 2004). This pause allows the recruitment of the capping enzymes, stimulated both by the CTD phosphorylated on serine-5 and Spt5. After adding a 5′-cap to the nascent transcript, the capping complex is released and RNA Pol II resumes transcription through the concerted realization of three events: dephosphorylation of CTD serine-5 phosphate, phosphorylation of Spt5, and phosphorylation of CTD serine-2. It was suggested that Bur1 phosphorylates Spt5 (Keogh et al. 2003; Pei and Shuman 2003), Ctk1 phosphorylates CTD on serine-2 (Ahn et al. 2004) and a phosphatase dephosphorylates CTD serine-5 phosphate. This phosphatase might be Fcp1 (Schroeder et al. 2000). However, it is not clear whether Fcp1 dephophorylates in vivo both CTD serine-2 phosphate and serine-5 phosphate or preferentially dephosphorylates serine-2 phosphate (Cho et al. 2001; Kamenski et al. 2004). In light of these data, we would like to propose that the phosphorylations of Spt5 and CTD serine-2 are compromised in the *bur1-T240A ctk1-T338A* double-mutant and the capping checkpoint process is disturbed. The Ssu72-3319 mutant phosphatase may be particularly apt at dephosphorylating CTD serine-5 phosphate to stimulate the release of capping enzymes. As we showed previously that Ssu72 genetically and physically interacts with Kin28 (Ganem et al. 2003), an additional possibility would be that, at this stage of the transcription cycle, Ssu72 is inhibiting Kin28 and/or is facilitating the dissociation of Kin28 from the transcription complex. In this model, the Ssu72-3319 mutant would be more effective in opposing the activity of Kin28.

In summary, we may draw three main conclusions from our results. First, Cak1 may not intervene directly in the transcription process, at least when yeast cells are growing in rich media. Second, although mutations *ctk1-T338A* or *bur1-T240A* individually do not significantly slow cell growth on complete medium, their combination with mutations affecting components of the

PAF1 elongation complex is lethal or seriously deleterious. This suggests that the nonphosphorylation of proteins Ctk1 or Bur1 reduces their in vivo catalytic activities. Third, kinases Ctk1 and Bur1 interact functionally with phosphatase Ssu72 at a step of the transcription cycle that may be the pre-mRNA capping checkpoint.

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