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A role for Ctr9p and Paf1p in the regulation of G_1 cyclin expression in yeast

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ABSTRACT

Entry into the cell cycle in budding yeast involves transcriptional activation of G_1 cyclin genes and DNA synthesis genes when cells reach a critical size in late G_1. Expression of G_1 cyclins *CLN1* and *CLN2* is regulated by the transcription factor SBF (composed of Swi4p and Swi6p) and depends on the cyclin-dependent Cdc28 protein kinase and cyclin Cln3p. To identify novel regulators of SBF-dependent gene expression we screened for mutants that fail to activate transcription of G_1 cyclins. We found mutations in a gene called *CTR9*. *ctr9* mutants are inviable at 37°C and accumulate large cells. *CTR9* is identical to *CDP1*. *CTR9* encodes a conserved nuclear protein of 125 kDa containing several TPR repeats implicated in protein–protein interactions. We show that Ctr9p is a component of a high molecular weight protein complex. Using immunoaffinity chromatography we found that Ctr9p associates with polypeptides of 50 and 65 kDa. By mass spectrometry these were identified as Cdc73p and Paf1p. We show that Paf1p, like Ctr9p, is required for efficient *CLN2* transcription, whereas Cdc73p is not. Paf1p and Cdc73p were previously reported to be RNA polymerase II-associated proteins, suggesting that the Ctr9p complex may interact with the general transcription apparatus.

INTRODUCTION

Transcriptional controls play an important role in the regulation of the eukaryotic cell cycle, both in yeast and higher eukaryotes (1). Transcriptional activation of regulatory genes at the G_1 to S phase transition is of particular importance for the decision to enter S phase. Understanding the molecular basis of this regulation is therefore of considerable interest. In budding yeast, the timing of S phase entry is largely determined by the transcriptional activation of G_1 cyclin genes (2–4). G_1 cyclins *CLN1* and *CLN2* are coordinately expressed with a number of genes involved in regulating DNA synthesis (5). Their expression is dependent on two transcription factor complexes termed SBF (Swi4/Swi6) and MBF (Mbp1/Swi6) that bind to specific sequences in the respective promoters (6,7).

SBF is largely responsible for regulating transcription of *CLN1*, *CLN2* and the *HO* endonuclease gene (8,9), whereas MBF regulates the transcription of DNA replication enzymes and S phase cyclins *CLB5* and *CLB6*. Different cyclin–Cdc28p complexes regulate late G_1-specific transcription. Activation of transcription in late G_1 when cells reach the critical cell size requires the Cln3p–Cdc28p kinase (2–4). Repression of SBF-dependent transcription is due to Clb1–4-associated Cdc28 kinases and Clb2 was shown to bind Swi4p (10,11). How Cln3p–Cdc28p kinase acts to activate transcription is not known. It has recently become clear that general transcription factors can also have rather specific effects on the expression of cell cycle-regulated genes. It was demonstrated that mutants in $TAF_{II}145$, an essential component of TFIID, arrest the cell cycle in G_1 and fail to express the SBF and MBF target genes (12). Thus, general transcription factors could also be targets for growth and cell cycle control (13).

We do not understand how gene expression of *CLN1* and *CLN2* is activated when cells reach a critical size. We therefore performed a genetic screen for novel regulators of Start-specific gene expression. We report the identification of two novel genes involved in transcription in late G_1, *CTR9* and *PAF1*. We show that Ctr9p and Paf1p are components of a high molecular weight protein complex that includes Cdc73p. Cdc73p and Paf1p were previously reported to be associated with RNA polymerase II (14–16), suggesting that the Ctr9p complex may be a novel transcription factor complex interacting with the general transcription machinery.

MATERIALS AND METHODS

Yeast strains

Standard techniques were used for culturing yeast and for genetic crosses (17). Strains were congenic derivatives of K699 (W303) whose genotype is: MAT**a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3* Gal^+ psi^+ *ssd1-d*. The ubiquitin *Y–LacZ* fusion under the control of a 90 bp fragment from the URS2 region (four copies of SCB elements) of the *HO* promoter (*URA3*::*sRS2-ubiY-LacZ*) and the *ADH–CLN2* gene fusion have been described (18,19). Strain CY918 (MATα *HMLα*hmr*:*TRP1 HIS3 URA3*::*sRS2-ubiY-LacZ HO-ADE2 HO-CAN TRP1*::*Adh-CLN2*) is a derivative of strain K4535 (20). Strain CY1154 (*mat*Δ::*LEU2 HMLα*hmr:TRP1 his3 URA3*::*sRS2-ubiY-LacZ HO-ADE2 HO-CAN TRP1*::*Adh-CLN2*) and isogenic swi4 (CY929, *swi4*::*LEU2*) and swi6 mutants (CY928, *swi6*::*TRP1*) were used for complementation analysis.

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Gene disruptions

The *CTR9* disruption allele was constructed by replacing most of the coding region (residues 1–1045) of *CTR9* with the *LEU2* gene. For disrupting the *PAF1* gene, the complete coding region was replaced with the *URA3* gene. The complete reading frame of *CDC73* was replaced with the *HIS3* gene to generate a disruption construct. Gene disruption cassettes were used to transform diploid strains. Diploids were sporulated and meiotic segregants carrying the disruption alleles were isolated by tetrad analysis (CY1332, *ctr9*Δ::*LEU2*; CY1994, *paf1*Δ::*URA3*; CY2003, *cdc73*Δ::*HIS3*).

Construction of epitope-tagged genes

The tagged *CTR9* allele was constructed in a *LEU2*-based integrative plasmid carrying 700 nt from the C-terminal part of *CTR9* and 500 nt from the 3′-untranslated region. A *Not*I site was introduced at codon 1044 of *CTR9*. Subsequently, a *Not*I cassette encoding six copies of the myc epitope was inserted. The plasmid was linearised within the *CTR9* gene and integrated at the *CTR9* locus of strain K699 by transformation to generate strain CY1546 (MAT**a**, *CTR9-myc6*). The insertion puts the tagged gene under the control of the *CTR9* promoter and inactivates the endogenous gene. The insertion was confirmed by Southern blotting and the tagged allele shown to be functional. The same strategy was used to generate a tagged *PAF1* gene. A *Not*I fragment encoding three copies of the influenza HA epitope was inserted into a *Not*I site created at the C-terminus of the *PAF1* coding region. A *TRP1*-based integrative plasmid carrying the modified gene fragment was integrated into the *PAF1* locus (CY2038, MAT**a** *PAF1-HA3*). The HA tag was replaced by a *Not*I fragment encoding six copies of the myc tag to create an myc-tagged *PAF1* allele (CY2117, MAT**a** *PAF1-myc6*). The HA-tagged *CDC73* allele was generated by inserting three copies of the HA epitope at the N-terminus of the coding region. The modified gene, including its promoter, was cloned into a *URA3* plasmid and integrated at the *URA3* locus (strain CY1999, MAT**a** *URA3*::*CDC73-HA3*). Other yeast strains were constructed using standard genetic crosses. Their relevant genotypes are indicated in the figure legends.

Isolation of *rst* mutants and cloning of *CTR9*

The mutant screen performed to isolate mutants defective in expression of Swi4p-dependent genes followed the method described by Jansen *et al*. (20). The rationale of the screen was to directly select for mutants defective in *HO* gene expression by using a *HO–CAN1* gene fusion. Mutants that fail to express this gene fusion are canavanine resistant and can be directly selected. To identify mutants defective in Swi4-dependent gene expression we constructed a strain (CY918) that also contained a *LacZ* reporter gene under the control of multiple SCB elements. Complete loss of late G_1-specific transcription is lethal. We therefore integrated an extra copy of the *CLN2* gene expressed from the *Schizosaccharomyces pombe Adh* promoter to suppress potential lethal mutations.

Strain CY918 was mutagenised with EMS to 50% survival (21) and canavanine-resistant mutants were isolated and tested for their ability to express the *LacZ* reporter gene. Mutants that failed to express the Swi4p-dependent *LacZ* reporter were tested for their ability to complement *swi4* and *swi6* mutants. Among 200 mutants we recovered several *rst* mutants (‘regulation of start’) that complemented both *swi4* and *swi6* mutants. Two were alleles of the same gene we termed *RST1*. The *RST1* gene was isolated by complementing the temperature-sensitive phenotype of *rst1* mutants with plasmids from a centromeric yeast library (21). Four complementing plasmids were isolated that contained overlapping inserts. The only gene contained in the minimal overlap was YOL145C, known as *CDP1* (22) and *CTR9* (23). We will hereafter refer to the *RST1* gene as *CTR9*. The *CTR9* gene was shown to be tightly linked to the *rst1-2* mutation in crosses to strains carrying a tagged *CTR9* allele.

Immunoprecipitation

Cells were grown in 50 ml YEPD medium to mid-log phase (A_{600} = 1), harvested by centrifugation and washed in 1 ml stop buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 15 mM $MgCl_2$, 0.01% NaN_3, 25 mM NaF). Cells were resuspended in 0.5 ml breakage buffer [50 mM Tris–HCl pH 8.0, 150 mM NaCl, 15 mM $MgCl_2$, 1% NP-40, 1 mM DTT, 0.2 mg/ml Pefabloc, 2 µg/ml aprotinin, 2 µg/ml pepstatin, 50 µg/ml TPCK, 1 µg/ml leupeptin, 1 µg/ml E64 (Boehringer), 6 mM *p*-nitrophenyl phosphate, 0.04 mM sodium orthovanadate, 13 mM β-glycerophosphate] and lysed by vortexing with 0.4 ml glass beads (0.5 mm diameter) twice for 5 min at 4°C. Cell extracts were cleared by two successive centrifugations for 10 min at 13 000 r.p.m. (4°C). Cleared extracts were pre-adsorbed with 60 µl protein A–Sepharose beads (50% slurry equilibrated in breakage buffer containing 50 µg/ml BSA) for 30 min at 4°C on a rotating wheel. For immunoprecipitation, extracts were incubated for 1 h with antibodies and for 1 h with protein A–Sepharose beads (60 µl slurry). The beads were collected by centrifugation and washed four times with breakage buffer (0.5 ml). Bound proteins were eluted with SDS sample buffer, separated on a 10% SDS–polyacrylamide gel and analysed by immunoblotting. Polyclonal rabbit anti-HA antibodies (Y-11) were from Santa Cruz Biotechnology. Molecular size markers were from Amersham (Rainbow-Marker).

Preparative immunoaffinity chromatography and mass spectrometry

For the analysis of immunoprecipitated proteins by mass spectrometry, 1.2 l of culture were processed (~2×10^{10} cells). Aliquots of 6 ml of cell extract were precleared with 300 µl protein A–Sepharose for 30 min and incubated with 0.5 ml 9E10 beads, prepared by cross-linking the 9E10 antibody to protein A–Sepharose (24). After incubation for 2 h at 4°C, the suspension was poured over a mini column and washed with 6 ml breakage buffer. Bound proteins were eluted with 0.2 ml aliquots of 0.1 M glycine pH 3.0. Fractions containing protein were pooled, separated on a 10% SDS–polyacrylamide gel, stained briefly with Coomassie blue and destained in 10% acetic acid. Protein bands were excised and cleaved in-gel with endoproteinase LysC (Boehringer) (25). The peptides were eluted stepwise with 0.1% trifluoroacetic acid, acetonitrile and 10% formic acid. Eluted peptides were desalted on a reverse phase cartridge and subjected to MALDI mass spectrometry on a Reflex III instrument (Bruker, Bremen) using 4-hydroxy-α-cyanocinnamic acid or 2,5-dihydroxy-benzoic acid as matrix. The mass fingerprint results were analysed using the MSFIT program (UCSF Mass Spectrometry Facility).

Metabolic labelling of yeast cells

Cells were grown in SC medium lacking methionine to log phase (17). Samples of 4×10^8 cells were labelled with 750 μCi [^{35}S]methionine (TranS-Label; ICN) in 5 ml medium for 1 h. Immunoprecipitation from extracts of metabolically labelled yeast cells was performed using the 9E10 antibody cross-linked to protein A–Sepharose (24). After gel electrophoresis, immunoprecipitated proteins were analysed by fluorography.

Gel filtration chromatography

Whole cells extracts were prepared as described for immunoprecipitations. Aliquots of 0.2 ml of extract were applied to a 25 ml Superose 6 (10/30) column (Pharmacia) previously equilibrated in breakage buffer containing 0.1% NP-40. Fractions eluting from the column were analysed by SDS gel electrophoresis and western blotting with anti-myc (9E10) and anti-HA antibodies (12CA5). Thyroglobulin (670 kDa), γ-globin (158 kDa) and chicken ovalbumin (44 kDa) (Bio-Rad) were used as molecular weight standards.

Other techniques

Centrifugal elutriation was performed as described (26). Cell volume was determined with a Schärfe System cell counter (CASY). RNA was isolated and processed for northern blot analysis as described (27,28). Blots were hybridised with DNA fragments radiolabelled by random priming. Western blot analysis (24) was performed on Immobilon P membranes (Millipore) using horseradish peroxidase-coupled anti-mouse antibodies (Promega) and chemiluminescence detection (Amersham). *In situ* immunofluorescence was performed as described (20), using Cy3-coupled anti-mouse antibodies (Dianova).

RESULTS

Ctr9p has a role in the expression of *CLN2*

In a screen aimed at identifying novel genes involved in Swi4p-dependent gene expression (described in detail elsewhere; see Materials and Methods), we identified mutations in a gene we originally termed *RST1*. In addition to their effect on transcription, *rst1* mutants were found to have a severe growth defect and were temperature sensitive. The mutants are morphologically abnormal and accumulated large unbudded cells (Fig. 1A). This phenotype suggested a role for *RST1* in cell cycle control.

The *RST1* gene was cloned by complementing the temperature-sensitive phenotype of *rst1* cells with centromeric plasmids from a genomic library (Materials and Methods). Screening current databases showed that the *RST1* gene is identical to *CTR9* (23) and *CDP1* (22). We will hereafter refer to this gene as *CTR9*. Ctr9p is a large, conserved protein that contains several copies of so-called TPR repeats which are implicated in mediating protein–protein interactions (29). *Ctr9* mutants had been identified in a genetic screen for mutants that are inviable in the absence of Cln3p. However, no further analysis of their phenotype has been reported (23).

We disrupted the *CTR9* gene and found that *ctr9*Δ strains had the same phenotype as the original EMS alleles of *rst1* (Fig. 1A). To test whether a failure to express G_1 cyclins contributes to the temperature sensitivity of *ctr9* mutants, we analysed *CLN2* expression in *ctr9*Δ mutants synchronised by centrifugal elutriation. When a population of small unbudded cells were grown out in fresh medium at 37°C, wild-type cells synchronously started budding and activated transcription of *CLN2* upon reaching a critical cell size (Fig. 1B). In contrast, *ctr9*Δ mutants grew to a very large size without efficiently activating transcription of *CLN2*. Furthermore, only a fraction of cells started budding during the course of the experiment (Fig. 1B). From these observations we conclude that Ctr9p is important for the timely activation of *CLN2* transcription in late G_1.

Activation of *CLN* transcription when cells reach the critical cell size in late G_1 requires the Cln3p–Cdc28p kinase (2–4). We therefore analysed the phenotype of *ctr9 cln3* double mutants. We found *ctr9 cln3* double mutants to be inviable, confirming the results reported by Di Como *et al.* (23). When *CTR9* was deleted in a strain whose sole copy of *CLN3* is expressed under the control of the regulatable *GAL* promoter, cells were viable on galactose medium but inviable on glucose medium (not shown). Overexpression of *CLN3* from the *GAL* promoter ameliorated the morphological defect of *ctr9* mutants, but did not rescue their temperature-sensitive phenotype. On glucose medium *ctr9 GAL-CLN3* strains accumulated large cells, but did not exhibit a homogenous cell cycle arrest. We conclude that Ctr9p has an important role in gene expression in late G_1. Regulation of SBF- and MBF-regulated genes cannot be the sole function of Ctr9p, since *ctr9* mutants have pleiotropic phenotypes not simply explicable by a reduction in late G_1-specific RNAs. Furthermore, the growth defect of *ctr9* mutants is not substantially ameliorated by ectopic expression of *CLN2*.

Ctr9p is a nuclear protein

Does Ctr9p have a direct role in transcriptional regulation or is the effect on *CLN2* transcription an indirect consequence of other physiological defects? To investigate the function of Ctr9p, we analysed the intracellular localisation of an epitope-tagged version of Ctr9p by indirect immunofluorescence (Fig. 2). The endogenous *CTR9* gene was modified to express a protein with six copies of the myc epitope fused to the C-terminus of Ctr9p. The resulting strain showed no growth defect, indicating that the Ctr9–myc6p fusion protein is functional. Staining formaldehyde-fixed cells with the anti-myc monoclonal antibody 9E10 showed clear nuclear fluorescence in most cells. No obvious cell cycle-dependent localisation was detected. We conclude that Ctr9–myc6p is a nuclear protein, consistent with a direct role in transcription.

Ctr9p associates with Paf1p and Cdc73p

To further investigate the biochemical functions of Ctr9p, we exploited the fact that the protein contains several copies of TPR repeats. TPR repeats are implicated in mediating protein–protein interactions and several TPR repeat proteins are part of multiprotein complexes (29). This prompted us to investigate whether Ctr9p stably associates with other proteins. We metabolically labelled cells expressing epitope-tagged Ctr9p (*CTR9-myc6*) with [^{35}S]methionine and immunoprecipitated Ctr9–myc6p from whole cell extracts with anti-myc antibodies (Fig. 3). Control strains, lacking the epitope tag, were processed in parallel. Immunoprecipitates were analysed by SDS gel electrophoresis and fluorography. As shown in Figure 3, a 150 kDa protein corresponding to Ctr9–myc6p was immunoprecipitated from extracts of *CTR9-myc6* cells, but not from the *CTR9* control extracts. The apparent size of the protein is close to the predicted

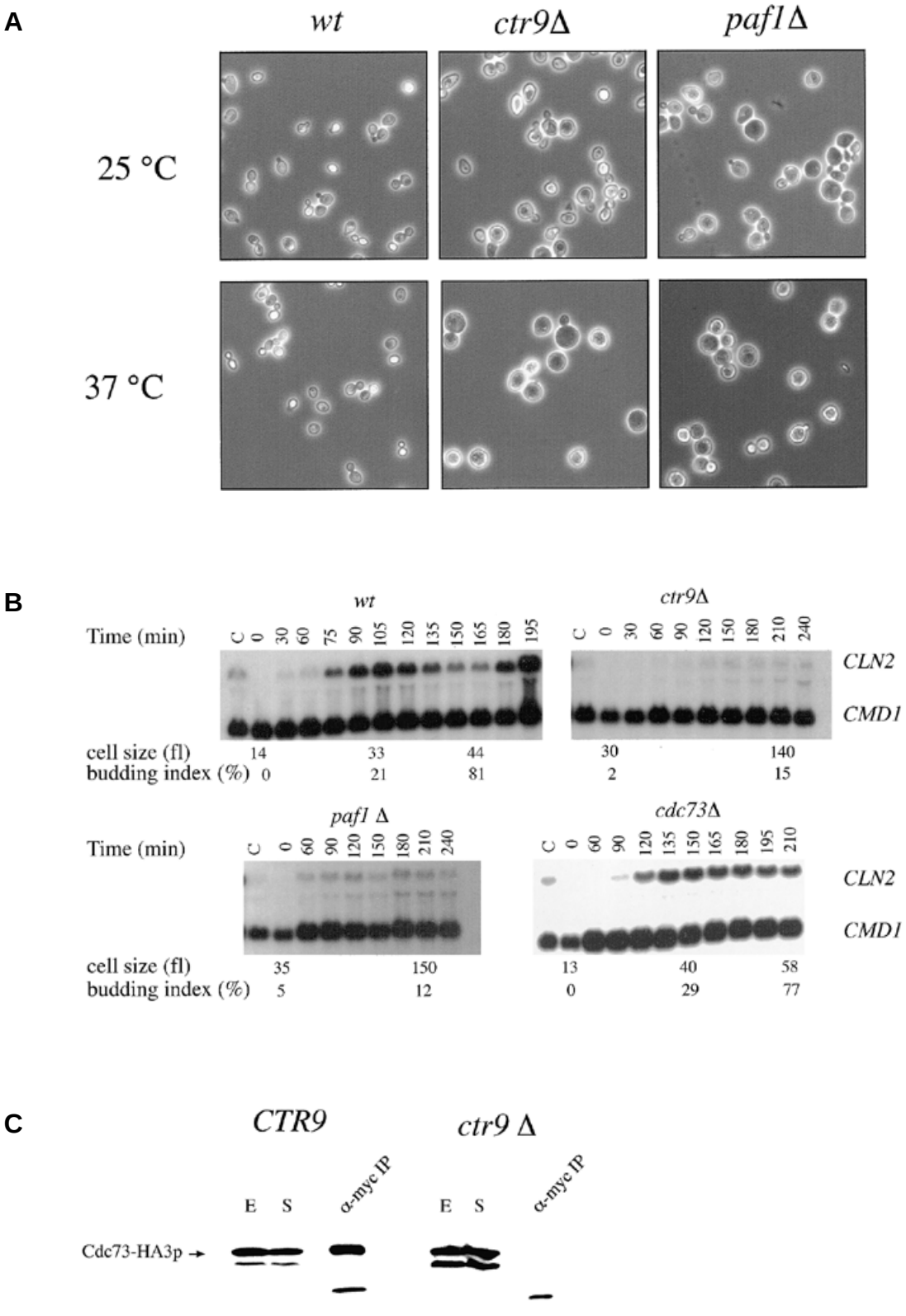

Figure 1. The phenotype of *ctr9* and *paf1* mutants. (**A**) Cells were grown in YEPD medium at 25°C to log phase and then shifted to 37°C for 5 h. Cells were analysed by phase contrast microscopy. (**B**) Expression of *CLN2* RNAs in cells synchronised by centrifugal elutriation. G_1 cells grown in YEP medium containing 2% raffinose were collected by centrifugal elutriation and released into fresh medium at 37°C. At the indicated time points, samples were taken for analysis of cell size, budding index and RNA isolation. *CLN2* RNA levels were analysed by northern blotting. The levels of *CMD1* RNA (coding for calmodulin) served as a loading control. (c), culture before elutriation. The following strains were used: K699 (wt), CY1332 (*Ctr9*Δ), CY 1994 (*paf1*Δ) and CY 2003 (*cdc73*Δ).

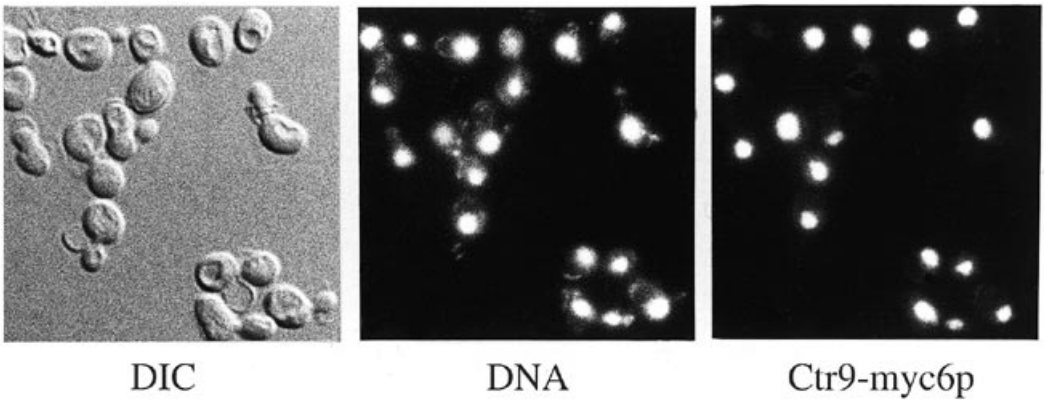

Figure 2. Ctr9p is a nuclear protein. Cells from strain CY1546 (*CTR9-myc6*), expressing an myc-tagged version of Ctr9p were grown in YEPD medium to mid-log phase. Cells were fixed with formaldehyde and analysed by fluorescence microscopy. The localisation of Ctr9–myc6p was analysed by indirect immunofluorescence using the 9E10 antibody directed against the myc epitope and Cy3-conjugated anti-mouse antibodies (Ctr9–myc6p). Nuclei were stained with 4′,6-diamino-2-phenylindole (DAPI). The same field of cells was analysed with Nomarski optics (DIC).

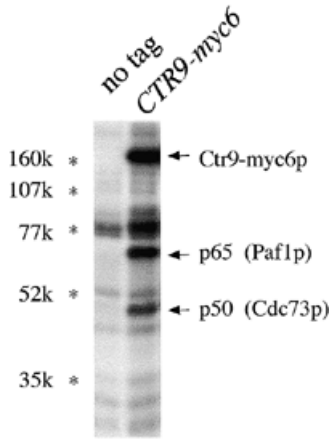

Figure 3. Detection of Ctr9p-associated proteins. Cells expressing myc-tagged Ctr9p (CY1616, *CTR9-myc6 pep4*Δ) and control cells not expressing the myc epitope (CY1615, *pep4*Δ) were metabolically labelled with [^{35}S]methionine. Whole cell extracts were subjected to immunoprecipitation with anti-myc antibodies (9E10) coupled to protein A–Sepharose. Bound proteins were analysed on a 10% SDS polyacrylamide gel and detected by fluorography. Proteins specifically precipitated in the presence of the myc-tagged Ctr9p (Ctr9–myc6p) are marked with arrows. Protein molecular weight markers are indicated to the left of the gel by asterisks.

molecular weight of Ctr9–myc6p (132 kDa). In addition to Ctr9–myc6p, two peptides of 50 and 65 kDa were reproducibly detected in immunoprecipitates from *CTR9-myc6* cells but absent from control reactions (Fig. 3). In contrast to the 150 kDa Ctr9–myc6p band, neither p50 nor p65 were detected in western blots with the anti-myc antibody 9E10 (not shown). This suggests that they are novel proteins associated with Ctr9p, rather than degradation products of Ctr9p–myc6p.

Since the complete sequence of the yeast genome is known (30), mass spectrometric analysis allows the determination of the identity of purified proteins (31). To identify p50 and p65, we purified Ctr9–myc6 and its associated proteins by immunoaffinity chromatography of whole cell extracts. From 1.2 l of cell culture we obtained enough material to visualise the proteins in Coomassie blue stained gels. As estimated from gels stained with silver or Coomassie blue, p65 and p50 co-eluted with Ctr9–myc6p in almost stoichiometric amounts (not shown). Protein bands were digested in-gel with LysC and subsequently analysed by mass spectrometry (Materials and Methods). The masses of 13 peptides unambiguously identified p65 as the product of the *PAF1* gene (YBR2016). For the band containing p50, the masses of six peptides matched the sequence of Cdc73p (YLR418c).

To confirm the mass spectrometric identification of p65 and p50, we created epitope-tagged versions of Paf1p and Cdc73p. The endogenous *PAF1* gene was replaced by a version expressing Paf1p fused at the C-terminus to three copies of the influenza HA3 epitope tag (*PAF1-HA3*). *CDC73* was N-terminally tagged with the HA3 epitope and integrated at the *URA3* locus under the control of its own promoter (*CDC73-HA3*). Using strains expressing Ctr9–myc6p and Paf1–HA3p, we immunoprecipitated Ctr9p–myc6p with anti-myc antibodies. The presence of Ctr9–myc6p and Paf1–HA3p in the immunocomplexes was analysed by western blotting. Under the conditions used, ~30% of Ctr9–myc6p was immunoprecipitated. As shown in Figure 4A, Paf1–HA3p efficiently co-immunoprecipitates with Ctr9–myc6p. The same result was obtained when Paf1–HA3p immunoprecipitates were analysed for the presence of Ctr9–myc6p. The relative amounts of Ctr9p and Paf1p in the anti-myc and anti-HA immunoprecipitates were comparable, suggesting that the two proteins are stably associated. We performed the same experiments with strains expressing Ctr9–myc6p and Cdc73–HA3p (Fig. 4B). Again, significant binding of Cdc73–HA3p to Ctr9–myc6p was detected. However, Cdc73–HA3p was less efficiently co-immunoprecipited with Ctr9–myc6p than Paf1–HA3p (Fig. 4), which could reflect weaker binding of Cdc73p to Ctr9p. These results confirm that Ctr9p is associated with Paf1p (65p) and Cdc73p (p50).

Ctr9p, Paf1p and Cdc73p are part of a large multiprotein complex

We further analysed Ctr9–myc6p complexes by gel filtration chromatography of whole cell extracts. Ctr9–myc6p and Paf1–HA3p from extracts of *CTR9-myc6 PAF1-HA3* cells

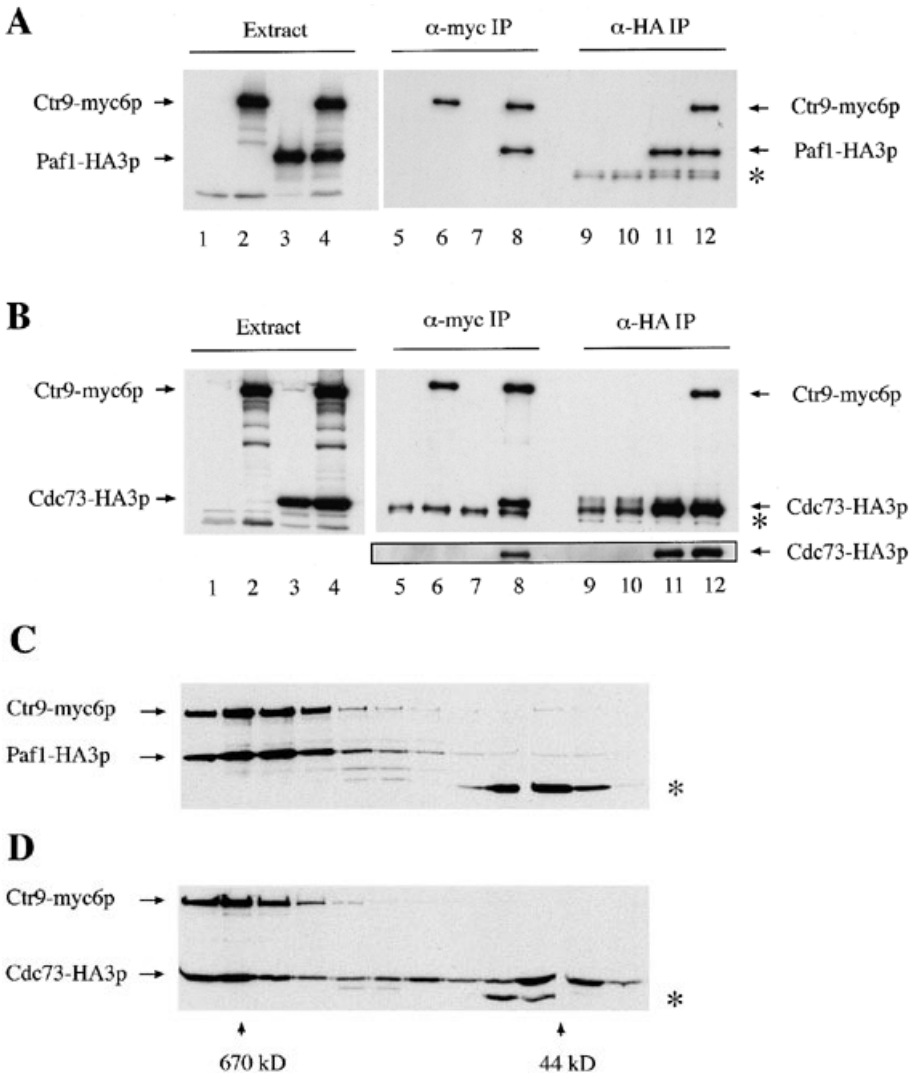

Figure 4. Ctr9p is associated with Paf1p and Cdc73p. Whole cell extracts from cells expressing epitope-tagged proteins were immunoprecipitated and analysed by western blotting. Extract, whole cell extract; α-myc IP, proteins immunoprecipitated with anti-myc antibodies; α-HA IP, proteins immunoprecipitated with anti-HA antibodies. The same fraction of total material was loaded in each lane. (**A**) Co-immunoprecipitation of Ctr9p and Paf1p. Extracts made from strains were immunoprecipitated and analysed by western blotting with anti-myc and anti-HA antibodies: lanes 1, 5 and 9, CY1615 (*pep4*Δ); lanes 2, 6 and 10, CY1616 (*CTR9-myc6 pep4*Δ); lanes 3, 7 and 11, CY2061 (*PAF1-HA3 pep4*Δ); lanes 4, 8 and 12, CY2062 (*CTR9-myc6 PAF1-HA3 pep4*Δ). In lanes 5–8, extracts were immunoprecipitated with anti-myc antibodies coupled to protein A–Sepharose. In lanes 9–12, extracts were immunoprecipitated with anti-HA antibodies (12CA5). The additional bands in lanes 2–4 are degradation products of Ctr9p and Paf1p. The fast migrating bands indicated with an asterisk correspond to the IgG used for immunoprecipitation which is detected with the secondary anti-mouse antibody. (**B**) Co-immunoprecipitation of Ctr9p and Cdc73p. Extracts from strains were analysed by immunoprecipitation and western blotting: lanes 1, 5 and 9, CY1615 (*pep4*Δ); lanes 2, 6 and 10, CY1616 (*CTR9-myc6 pep4*Δ); lanes 3, 7 and 11, CY1998 (*URA3*::*CDC73-HA3 pep4*Δ); lanes 4, 8 and 12, CY1990 (*CTR9-myc6 URA3*::*CDC73-HA3 pep4*Δ). Extracts were immunoprecipitated with anti-myc antibodies (lanes 5–8) or anti-HA antibodies (lanes 9–12). At the bottom of (B) the western blot in lanes 5–12 was reprobed with polyclonal rabbit anti-HA antibodies to distinguish the Cdc73–HA3p band from the co-migrating monoclonal anti-HA antibody used for immunoprecipitation, marked with an asterisk. (**C**) Gel filtration chromatography of Ctr9p–Paf1p complexes. Whole cell extracts (1 mg protein) from strain CY2062 (*CTR9-myc6 PAF1-HA3 pep4*Δ) were analysed by gel filtration on a Superose 6 column. The column fractions were analysed by immunoblotting. Molecular weight markers thyroglobulin (670 kDa), eluting six fractions after the void volume, and chicken ovalbumin (44 kDa) were analysed in separate runs. The low molecular weight protein eluting at ~44 kDa is an endogenous yeast protein cross-reacting with anti-HA antibodies. (**D**) Gel filtration of Ctr9p–Cdc73p complexes. Extracts from strain CY1990 (*CTR9-myc6 URA3*::*CDC73-HA3 pep4*Δ) were analysed as described for (C).

co-eluted from the column as a single peak in the high molecular weight fractions (Fig. 4C). Little or no Paf1–HA3p was found in the fractions corresponding to monomeric Paf1p (65 kDa), suggesting that Paf1p and Ctr9p are stably associated in a large protein complex. Analysis of extracts containing Ctr9–myc6p and Cdc73–HA3p showed that Cdc73p eluted in two peaks (Fig. 4D). One peak co-eluted with Ctr9–myc6p. The second peak eluted in the small molecular weight fractions presumably as a monomer. This may indicate that Cdc73p binds with lower affinity to Ctr9p than Paf1p, as also suggested by the co-immunprecipitation experiments (Fig. 4B). However, the strain expressing Cdc73–HA3p contains an additional wild-type copy of *CDC73*.

Competition of tagged and untagged Cdc73p proteins for binding to a limiting amount of Ctr9p may partly explain why a large fraction of Cdc73–HA3p elutes from the gel filtration column as a monomer. In view of these results, we conclude that Ctr9p, Paf1p and Cdc73p are associated as part of a stable multiprotein complex.

Paf1p has a role in G_1 cyclin expression

The finding that Ctr9p is found in a complex with Paf1p and Cdc73p raises the question, whether these proteins have a common function. Paf1p and Cdc73p were originally identified as proteins associated with RNA polymerase II (Pol II) *in vitro* (14–16). They are not, however, present in holoenzyme preparations. The Pol II prepar-ations containing Paf1p and Cdc73p also contained the general transcription factors TFIIB and TFIIF but lack the SRB/mediator complex (15). Whether or not Paf1p and Cdc73p bind to Pol II complexes in a stoichiometric fashion is not known. Deletion of *PAF1* is not lethal, but confers a severe temperature-sensitive phenotype. *Paf1* mutants grow slowly, are abnormally large and accumulate a large fraction of unbudded cells at the restrictive temperature, phenotypes similar to *ctr9* mutants. Deletion mutants of *CDC73* are also temperature sensitive, but have only a mild growth defect at 30°C and lack the morphological defect of *paf1* mutants (15). Paf1p and Cdc73p were found to have different effects on the expression of some genes, including *GAL1*, *MAK16*, *EGD2* and *CMK2* (14,15), but do not exhibit the global effects on transcription described for components of the holoenzyme (32).

To test whether *PAF1* and *CDC73* affect expression of *CLN2*, we created deletion alleles of *PAF1* and *CDC73* (Materials and Methods). Analysis of these strains confirmed the phenotypes reported previously for *cdc73* and *paf1* mutants (14,15). The expression of *CLN2* in *paf1* and *cdc73* strains was analysed in cells synchronised by centrifugal elutriation. As shown in Figure 1B, *paf1* mutants have the same defect in *CLN2* expression as *ctr9* cells. The smallest cells obtained after elutriation were already larger than the population of wild-type cells. They grew to a size of 150 fl without efficiently turning on *CLN2* expression. Only a fraction of these cells formed a bud during the course of the experiment. In contrast, *cdc73* mutants showed no obvious effect on cell cycle-dependent *CLN2* expression. We conclude that Paf1p is required for expression of *CLN2*, whereas Cdc73p is not.

Paf1p and Ctr9p function as a complex

Paf1 and *ctr9* mutants have very similar phenotypes (Fig. 1). They are morphologically similar, fail to grow at 37°C and do not efficiently activate *CLN2* expression. We investigated this similarity further by testing whether *paf1* mutants, like *ctr9* mutants, require Cln3p for viability. When heterozygous diploids were sporulated, no viable *paf1 cln3* spores were recovered. Using the conditional *GAL–CLN3* allele, *paf1 GAL-CLN3* strains were constructed. These strains are viable on galactose medium, but inviable on glucose medium, where they accumulate large cells. We conclude that Paf1p, like Ctr9p, becomes essential in the absence of Cln3p.

The finding that *ctr9* and *paf1* mutants show similar if not identical phenotypes suggests that their physical interaction is functionally relevant and that they might function as a complex. If this were the case, one should expect neither of them to be active in the absence of the other. Consistent with this hypothesis

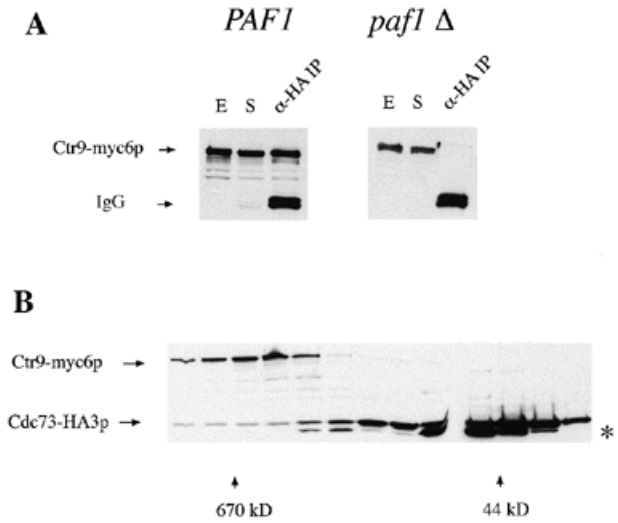

Figure 5. Binding of Cdc73p requires Ctr9p and Paf1p. (**A**) Co-immunoprecipitation of Ctr9p with Cdc73p. Whole cell extracts prepared from *wt* or *paf1*Δ mutant cells (CY1990, *CTR9-myc6 URA3*::*CDC73-HA3 pep4*Δ; CY2025, *CTR9-myc6 paf1*Δ *URA3*::*CDC73-HA3 pep4*Δ) were subjected to immunoprecipitation with anti-HA antibodies. Immunoprecipitates were analysed by western blotting using anti-myc antibodies. E, whole cell extract; S, supernatants after immunoprecipitation; α-HA IP, immunoprecipitates. (**B**) Gel filtration chromatography of Ctr9p–Cdc73p complexes. Extracts from *paf1*Δ mutant cells expressing epitope-tagged Ctr9p and Cdc73p (CY2025, *CTR9-myc6 paf1*Δ *URA3*::*CDC73-HA3 pep4*Δ) were fractionated on a Superose 6 column. Fractions eluting from the column were analysed by immunoblotting with anti-myc and anti-HA antibodies. The elution peak of molecular weight markers is indicated. The band running slightly faster than Cdc73–HA3p is a cross-reacting protein from yeast. (**C**) Co-immunoprecipitation of Paf1–myc6p with Cdc73–HA3p. Extracts from wild-type or *ctr9*Δ mutant cells (CY2162, *PAF1-myc6 URA3*::*CDC73-HA3 pep4*Δ; CY2196, *PAF1-myc6 ctr9*Δ *URA3*::*CDC73-HA3 pep4*Δ) were subjected to immunoprecipitation with anti-myc beads and analysed by western blotting using anti-HA antibodies.

we found that *paf1 ctr9* double mutants had no obvious additional growth defect compared to the individual single mutants (not shown). Furthermore, the phenotype of *ctr9* and *paf1* mutants is not exacerbated by the deletion of *CDC73*. These findings are fully consistent with the hypothesis that Ctr9p and Paf1p function as a complex.

Binding of Cdc73p requires Ctr9p and Paf1p

Our genetic analysis suggested that Ctr9p has no function in the absence of Paf1p. To investigate this possibility further, we analysed the ability of Ctr9–myc6p to interact with Cdc73–HA3p in the absence of functional Paf1p. As shown in Figure 5, Ctr9–myc6p is not detected in immunoprecipitates of Cdc73–HA3p from *paf1* mutants. Similarly, Cdc73p failed to interact with Paf1p in the absence of Ctr9p (Fig. 5C). These data suggest that Ctr9p and Paf1p are functionally interdependent; both are inactive in the absence of the other. To analyse whether the recruitment of Cdc73p into a large protein complex is due to its interaction with Ctr9p and Paf1p, we analysed the chromatographic behaviour of Cdc73p in the absence of functional Paf1p. When whole cell extracts from *paf1* mutants were analysed by gel filtration chromatography, most of Cdc73–HA3p eluted as a monomer in the low molecular weight fractions. Little or no Cdc73–HA3p co-eluted with Ctr9–myc6p. When the elution profile of Ctr9–myc6p is compared to experiments performed

with wild-type extracts (Figs 4 and 5), the protein elutes slightly later in *paf1* mutants, but still in a large complex, suggesting that Ctr9p forms multimers or also binds to other, as yet unidentified proteins. The protein levels of Ctr9–myc6p also appear to be reduced in *paf1* cells. Ctr9p may be less stable or more weakly expressed in *paf1* mutants. From these data we conclude that Cdc73p can only bind to an intact Ctr9p–Paf1p complex. Paf1p and Cdc73p could bind in a cooperative fashion to Ctr9p. These results suggest that Ctr9p and Paf1p function as a complex, consistent with the double mutant phenotype.

DISCUSSION

The roles of Ctr9p, Paf1p and Cdc73p in gene expression

The data presented here identify two new genes involved in cell cycle-dependent gene expression in late G_1, *CTR9* and *PAF1*.

We present several lines of evidence that Ctr9p and Paf1p form a complex that also contains Cdc73p. First, all three proteins co-immunoprecipitate. Second, the proteins co-fractionate during gel filtration chromatography. Third, neither co-immunoprecipitation nor co-fractionation of Cdc73p and Ctr9p is observed in the absence of Paf1p. Fourth, *ctr9* and *paf1* single and double mutants have the same phenotypes, consistent with the notion that Ctr9p and Paf1p function as a complex.

For Cdc73p the situation is not so clear. *Cdc73* mutants, though inviable at 37°C, have a less severe growth defect than *ctr9* and *paf1* mutants. Furthermore, *cdc73* mutants do not affect expression of *CLN2*. However, the phenotypes of *cdc73 paf1* and *cdc73 ctr9* double mutants suggest that Cdc73p has no functions that are independent of Ctr9p–Paf1p. It therefore seems reasonable to propose that Cdc73p is only required for certain functions of the Ctr9p–Paf1p complex.

Ctr9p is a conserved protein containing TPR repeats implicated in protein–protein interactions (29). Proteins with significant similarities to Ctr9p are found in human (KIAA0155; EMBL accession no. D63875), mouse ($p150^{TSP}$; EMBL accession no. L49502), *Caenorhabditis elegans* (148.5 kDa protein B0464.2; EMBL accession no. Z19152) and *S.pombe* (Tpr1p, 119 kDa protein; EMBL accession no. AF047464). The high degree of similarity suggests that some of these proteins may be orthologues sharing the same cellular function. However, only Ctr9p was identified due to its effect on gene expression. *Ctr9* mutants (*cdp1*) were independently found in a genetic screen for mutants that are inviable in the absence of Cbf1p and were shown to affect nuclear division (22). In light of our findings, the effect on nuclear division might be a consequence of defects in gene expression. $p150^{TSP}$ was identified as a nuclear protein binding to SH2 domains *in vitro* (33). The function of $p150^{TSP}$ has not been analysed, so the significance of its interaction with SH2 domains remains unclear. The hypothetical *C.elegans* protein was identified by the genome sequencing project. TPR repeats were found in a number of proteins involved in gene expression and were shown to mediate protein–protein interactions (29). We postulate that the TPR repeats in Ctr9p are involved in binding to Paf1p and Cdc73p. In current databanks we found proteins similar to Cdc73p from *Candida albicans* (Ca49C10.12c) and *C.elegans* (WORMPEP_F35F11.e). We did not find an obvious *PAF1* homologue. Nevertheless, we consider it likely that the Ctr9p-containing protein complex has a conserved function in eukaryotes.

Our data indicate a role for Ctr9p and Paf1p in controlling gene expression in late G_1. Several observations make it clear that the protein complex we describe has additional functions. First, the temperature sensitivity of *ctr9* mutants is not rescued by ectopic expression of *CLN2*. Second, *ctr9* and *paf1* mutants have pleiotropic phenotypes, including a defect in nuclear division (22). In addition, Cdc73p and Paf1p have been reported to affect the expression of several other genes (14). The most likely explanation for these findings is that the Ctr9p complex has a more general effect on gene expression.

Is the complex containing Ctr9p, Paf1p and Cdc73p part of a general transcription factor complex?

Several lines of evidence suggest a direct role for the Ctr9p–Paf1p–Cdc73p complex in transcription. The most relevant observation regarding the biochemical function of Ctr9p, Paf1p and Cdc73p is the finding that Paf1p and Cdc73p were found to be associated with Pol II *in vitro* (14–16). It was hypothesised that Cdc73p–Paf1p may be components of a novel Pol II subcomplex (15). In our preparations of Ctr9–myc6p complexes we did not detect tightly associated proteins other than Paf1p and Cdc73p. Thus, under the conditions we used, Ctr9p does not seem to be part of a stable Pol II-containing complex. Nevertheless, our finding that Ctr9p and Paf1p affect *CLN2* transcription suggests that the *in vitro* affinity of Cdc73p and Paf1p to components of the transcription machinery is functionally meaningful and that the Ctr9p–Paf1p–Cdc73p complex has a direct role in the regulation of transcription.

None of the characterised general transcription factor complexes and chromatin remodelling complexes, like the SWI/SNF complex, yeast RSC, the SRB/mediator complex or SAGA, have to date been reported to contain Cdc73p, Paf1p or Ctr9p (34). The proteins we have identified here may represent a novel transcription factor complex that interacts with the general transcription machinery.

We do not yet know the precise function of the Ctr9p complex in the regulation of Swi4p-dependent gene expression. Cln3p is required for activating Start-specific transcription as cells reach a critical cell size. In *ctr9* and *paf1* mutants, *CLN3* becomes an essential gene, consistent with the notion that they are involved in the same biological process. However, the double mutant phenotype also shows that Cln3p is at least partly functional in the absence of Ctr9p or Paf1p. Thus, Cln3p and Ctr9p–Paf1p are likely to have independent roles for activation of transcription in late G_1. Our finding that potential components of the general transcription apparatus can have specific effects on cell cycle progression is not without precedence. Yeast $TAF_{II}145$, a component of TFIID, was shown to be required for transcription of only a subset of genes and is specifically required for the G_1 to S phase transition (12,35). Analysing whether the Ctr9p complex contacts known transcription factor complexes will help to elucidate the biochemical functions of the Ctr9p complex and its components.

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