Independent functions of yeast Pcf11p in pre-mRNA 3' end processing and in transcription termination

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Pcf11p, an essential subunit of the yeast cleavage factor IA, is required for pre-mRNA 3' end processing, binds to the C-terminal domain (CTD) of the largest subunit of RNA polymerase II (RNAP II) and is involved in transcription termination. We show that the conserved CTD interaction domain (CID) of Pcf11p is essential for cell viability. Interestingly, the CTD binding and 3' end processing activities of Pcf11p can be functionally uncoupled from each other and provided by distinct Pcf11p fragments *in trans*. Impaired CTD binding did not affect the 3' end processing activity of Pcf11p and a deficiency of Pcf11p in 3' end processing did not prevent CTD binding. Transcriptional run-on analysis with the *CYC1* gene revealed that loss of cleavage activity did not correlate with a defect in transcription termination, whereas loss of CTD binding did. We conclude that Pcf11p is a bifunctional protein and that transcript cleavage is not an obligatory step prior to RNAP II termination.

Keywords: pre-mRNA 3' end processing/RNA polymerase II C-terminal domain/transcription termination

Introduction

The 3' ends of most mRNAs are generated by endonucleolytic cleavage of the primary transcript and subsequent polyadenylation of the 5' cleavage product by poly(A) polymerase (reviewed by Zhao *et al.*, 1999). The mechanism of this two-step reaction and many of the factors involved are very similar in higher eukaryotes and *Saccharomyces cerevisiae* (Shatkin and Manley, 2000). In yeast, cleavage and polyadenylation factor (CPF), cleavage factors IA and IB (CF IA and CF IB) and poly(A) binding protein (Pab1p) reconstitute cleavage and polyadenylation activity *in vitro* (Ohnacker *et al.*, 2000). CPF is a multiprotein complex that includes homologues of the mammalian cleavage and polyadenylation specificity factor CPSF (Ohnacker *et al.*, 2000). CF IA is composed of the proteins Rna14p, Pcf11p, Clp1p and Rna15p (Minvielle-Sebastia *et al.*, 1994, 1997; Kessler *et al.*,

1996; Amrani *et al.*, 1997b). Rna14p and Rna15p are homologues of subunits of mammalian cleavage stimulation factor CstF (Takagaki and Manley, 1994; Shatkin and Manley, 2000). Homologues of yeast Pcf11p and Clp1p are contained within the mammalian cleavage factor II (de Vries *et al.*, 2000).

Primary RNA polymerase II (RNAP II) transcripts undergo several cotranscriptional processing steps (capping, splicing and 3' end processing) before maturing to functional mRNA (reviewed by Bentley, 2002; Proudfoot *et al.*, 2002). The C-terminal domain (CTD) of the largest subunit of RNAP II plays a key role in coupling these pre-mRNA processing reactions. The CTD is composed of tandem repeats (52 in vertebrates and 26 in yeast) of a heptad with the conserved consensus sequence Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7. Dynamic phosphorylation of the CTD on serine residues 2 and 5 of the heptads during the transcription cycle appears to orchestrate the association, dissociation and activity of the different pre-mRNA processing machineries as the polymerase traverses a gene (Dahmus, 1996; Ho and Shuman, 1999; Komarnitsky *et al.*, 2000; Schroeder *et al.*, 2000; Cho *et al.*, 2001; Licatalosi *et al.*, 2002).

Interactions of components of the 3' end processing machinery with the CTD have been demonstrated in mammals and yeast and are thought to help couple 3' end processing with transcription (reviewed by Bentley, 2002; Proudfoot and O'Sullivan, 2002; Proudfoot *et al.*, 2002). In mammals, CPSF is recruited to the promoter by binding to the general transcription factor TFIID and is transferred to the CTD of initiating RNAP II (Dantonel *et al.*, 1997). CPSF and the 50 kDa subunit of CstF bind equally well to the phosphorylated and the unphosphorylated CTD (McCracken *et al.*, 1997; Fong and Bentley, 2001). In yeast, the 3' end processing factors CPF, CF IA and CF IB are recruited to genes progressively, starting at the 5' end (Komarnitsky *et al.*, 2000; Licatalosi *et al.*, 2002). The CPF subunit Yhh1p/Cft1p and the CF IA subunit Pcf11p have been shown to bind preferentially to the phosphorylated CTD (Rodriguez *et al.*, 2000; Barilla *et al.*, 2001; Dichtl *et al.*, 2002a). In mammalian cells the CTD is required for splicing and 3' end processing *in vivo* (McCracken *et al.*, 1997), and RNAP II has been proposed to be an essential 3' end processing factor (Hirose and Manley, 1998; Ryan *et al.*, 2002). In yeast, transcription and 3' end processing as well as splicing also appear to be interdependent. However, deletion of the CTD did not inhibit splicing and the CTD is not essential for 3' end processing, but its absence reduced the efficiency of cleavage and polyadenylation (Licatalosi *et al.*, 2002).

A functional connection between 3' end processing and transcription is demonstrated by the fact that correct transcription termination requires a functional polyadenylation signal (reviewed by Proudfoot, 1989). Accordingly,

mutations in CPF and CF IA subunits have been found to impair correct transcription termination (Birse *et al.*, 1998; Dichtl *et al.*, 2002a,b; Hammell *et al.*, 2002). A functional 3' splice site in the last intron and sequences (e.g. pause sites) downstream of the poly(A) site also affect termination (Dye and Proudfoot, 1999; Yonaha and Proudfoot, 1999). Two models have been proposed to explain why *cis*-acting poly(A) signals are necessary for transcription termination. The 'antiterminator' model suggests that recognition of the poly(A) signal causes a change in the transcription complex (e.g. by dissociation of an antiterminator or processivity factor) prior to endonucleolytic cleavage (Logan *et al.*, 1987). The 'torpedo' model postulates that cleavage of the nascent transcript at the poly(A) site is signalled to RNAP II. It has been proposed that exonucleolytic degradation of the uncapped 3' cleavage product interferes with the paused polymerase and triggers termination (Connelly and Manley, 1988). The important difference between the two proposals is that cleavage of the nascent RNA at the poly(A) site is not required for transcription termination in the antiterminator model, whereas it is an obligatory step for the torpedo model.

Some of the strongest evidence in favour of the torpedo model came from the analysis of mutants in 3' end formation factors in yeast; mutations inhibiting cleavage and polyadenylation impaired termination, whereas mutations interfering only with the polyadenylation reaction had little or no effect on termination (Birse *et al.*, 1998). In agreement with the antiterminator model, Sub1p (PC4 in mammals) was proposed to antagonize the termination activity of Rna15 (Calvo and Manley, 2001). Furthermore, termination-deficient mutants of 3' end processing factors have been described that have no defect in cleavage and polyadenylation (Aranda and Proudfoot, 2001; Dichtl *et al.*, 2002a,b). Recent reports support the notion that transcription termination downstream of the poly(A) site precedes pre-mRNA 3' end cleavage. An *in vitro* study with mammalian nuclear extract suggested that the signal for transcription termination is activated upon extrusion of the poly(A) signal from elongating RNAP II and does not require processing of the pre-mRNA (Tran *et al.*, 2001). More recently the same group presented *in vivo* evidence that termination can be driven efficiently solely by a poly(A) signal (Orozco *et al.*, 2002). Furthermore, RNAP II was proposed to pause following transcription of the poly(A) signal and to revert to a termination-competent condition prior to termination (Orozco *et al.*, 2002). Analysis of nascent transcripts of the human ϵ - and β -globin genes from transiently transfected HeLa cells detected terminated but uncleaved transcripts that extended up to 1.5 kb beyond the poly(A) site (Dye and Proudfoot, 1999). Examination of the primary transcripts of the Balbiani ring 1 gene of *Chironomus tentans* indicated that transcription termination generated uncleaved RNAs that extended up to 700 nucleotides beyond the poly(A) site (Bauren *et al.*, 1998). Electron microscopic visualization of genes in *Xenopus laevis* oocytes and in *Drosophila melanogaster* indicated that transcription termination occurs in the 3' flanking region of the genes prior to cleavage at the poly(A) site (Osheim *et al.*, 1999, 2002). Interestingly, a screen for termination defective mutants in *Schizosaccharomyces pombe* identi-

fied the Hrp1/Chd1 chromatin remodelling factors and it was suggested that the formation of specific chromatin structures at termination regions is a necessary requirement for efficient RNAP II termination (Alen *et al.*, 2002). Furthermore, we recently suggested that the yeast CPF associated Ssu72p may play a role in transcriptional elongation and termination by affecting the elongation rate of RNAP II (Dichtl *et al.*, 2002b). All these observations are consistent with the antiterminator model; however, they do not directly address the question of whether a defect in endonucleolytic cleavage of the RNA transcript interferes with termination.

We have analysed mutant *PCF11* alleles that are deficient in either pre-mRNA 3' end processing or CTD binding. We found by transcriptional run-on analysis that loss of cleavage activity did not correlate with a defect in transcription termination, whereas impaired CTD binding of Pcf11p did. Accordingly, the CTD binding and pre-mRNA 3' end processing activities of Pcf11p could be functionally uncoupled from each other *in vitro* and *in vivo*. These results indicate that the function of Pcf11p in pre-mRNA 3' end processing is independent of and not required for transcription termination.

Results

The CF IA subunits Rna14p and Pcf11p bind to the phosphorylated CTD

We searched for CTD binding proteins among the components of CF IA (Rna14p, Pcf11p, Clp1p and Rna15p), CF IB (Nab4p/Hrp1p) and Pab1p by direct two-hybrid analysis (Table I). Y190 yeast cells were cotransformed with a plasmid carrying the *GAL4* DNA-binding domain fused to the CTD (pAS2 $\Delta\Delta$ -CTD) and plasmids containing the *GAL4* activation domain fused to the test genes and assayed for activation of *HIS3* transcription. *NRD1* (pACT2-*NRD1*), which has been shown to interact with the CTD, was used as a positive control (Steinmetz and Brow, 1998).

Figure 1A shows that *RNA14* and *PCF11* as well as *NRD1*, but not *CLP1*, *RNA15*, *NAB4* or *PAB1*, resulted in a His⁺ growth phenotype under stringent conditions (35 mM 3-amino-1,2,4-triazole). These results were confirmed by an X-Gal filter lift assay which monitors β -galactosidase expression (Table I). Notably, none of the *GAL4* activation domain fusions of *RNA14*, *PCF11*, *CLP1*, *RNA15*, *NAB4*, *PAB1* or *NRD1* displayed autoactivation (results not shown). Thus, *RNA14* and *PCF11* showed a two-hybrid

Table I. Two-hybrid interactions of CF IA subunits with the CTD of RNAP II

	pACT2 fusion gene	β -galactosidase expression	<i>HIS3</i> expression
CF IA	<i>RNA14</i>	Dark blue	+
	<i>PCF11</i>	Blue	+
	<i>CLP1</i>	White	–
	<i>RNA15</i>	White	–
CF IB	<i>NAB4</i>	White	–
	<i>PAB1</i>	White	–
	<i>NRD1</i>	Blue	+

interaction with the CTD. This is in agreement with the previous observation that Pcf11p interacts with the phosphorylated CTD (Barilla *et al.*, 2001).

In addition, the combinations of mutant RNAP II alleles containing a truncated CTD with only eight or nine heptad repeats with mutant alleles of *RNA14*, *PCF11* and *RNA15* impaired in 3' end processing were found to be synthetically lethal (results not shown). These genetic interactions suggested a functional connection of CF IA and the CTD. GST pull-down experiments were performed to test whether phosphorylation of the CTD is required for the binding. We used a GST fusion of the CTD expressed in *Escherichia coli* and *in vitro* translated Rna14p, Pcf11p, Clp1p, Rna15p, Nab4p and Pab1p. Nrd1p served as a positive control. Both Rna14p and Pcf11p bound to the unphosphorylated CTD (Figure 1B, lane 3). However,

Fig. 1. Interactions of CF IA subunits with the CTD. (A) Two-hybrid interactions of *RNA14* and *PCF11* with the CTD of RNAP II activate *HIS3* reporter gene expression. Y190 cells were cotransformed with pAS2ΔΔ-CTD (CTD fused to the *GAL4* DNA-binding domain) and pACT2 (*GAL4* activation domain) containing the open reading frames encoding CF IA (*RNA14*, *PCF11*, *CLP1* and *RNA15*), CF IB (*NAB4*, *PAB1* and *NRD1*). *HIS3* reporter gene expression was assayed by growth on medium lacking histidine. (B) GST (lane 2), GST-CTD (lane 3) or phosphorylated GST-CTD-P (lane 4) bound to glutathione-Sepharose 4B was incubated with *in vitro* translated ³⁵S-labelled subunits of CF IA (Rna14p, Pcf11p, Clp1p and Rna15p), CF IB (Nab4p) and Pab1p as indicated on the left of each panel. Bound proteins were separated by SDS-PAGE and visualized by autoradiography. Input (lane 1) shows 10% of *in vitro* translation reactions used in the binding reaction. The genuine CTD binding protein Nrd1p was used as a positive control.

CTD phosphorylation significantly increased the binding efficiency (lane 4). In contrast, Clp1p, Rna15p, Nab4p and Pab1p did not interact, irrespective of the phosphorylation status of the CTD. The interactions observed also occurred in the presence of DNase or RNase, indicating that binding was not mediated by nucleic acid (results not shown). Thus the CF IA subunits Rna14p and Pcf11p interacted preferentially with the phosphorylated CTD of RNAP II.

The CTD interaction domain of Pcf11p is responsible for CTD binding

Yeast Pcf11p and its putative homologues in higher and lower eukaryotes share sequence similarities with known CTD binding proteins such as the *S.cerevisiae* Nrd1p and members of the rat SR (serine-arginine-rich)-like CTD-associated factor family SCAF4 and SCAF8. Accordingly, Pcf11p, Nrd1p, SCAF4 and SCAF8 have been reported to interact with the CTD (Yuryev *et al.*, 1996; Patturajan *et al.*, 1998; Steinmetz and Brow, 1998; Barilla *et al.*, 2001). The region of highest similarity covers ~130 amino acids of their N-termini known as the CTD interaction domain (CID). The CID of SCAF8 has been shown to bind exclusively to the phosphorylated CTD *in vitro* (Patturajan *et al.*, 1998). Rna14p does not have such a CID, nor does it share any sequence similarities with other CTD binding domains of known CTD binding proteins. The domain organization of Pcf11p is shown in Figure 2A. The putative N-terminal CID is connected through a stretch of 20 consecutive glutamines to the Rna14/15p and the Clp1p interaction domains that have been mapped by two-hybrid analysis (Amrani *et al.*, 1997a,b; results not shown). The Clp1p interaction domain is flanked by two potential zinc-finger motifs (types C₂H₂ and C₂HC). As has been shown recently, Pcf11p interacts directly with Rna14p, Rna15p and Clp1p *in vitro*, presumably through the domains described above (Gross and Moore, 2001).

To analyse the CTD binding of Pcf11p and its function in mRNA biogenesis, we made a set of deletion mutants lacking different N- or C-terminal portions of the protein (Figure 2B) and we constructed the CID mutant *pcf11-13*, in which the highly conserved DSI motif of the CID was replaced by three consecutive alanines (D68A, S69A and I70A) (Figure 2B and C). We also tested the ts alleles *pcf11-2* and *pcf11-9*. Mutations in *pcf11-2* inhibit cleavage and polyadenylation (Amrani *et al.*, 1997b) and mutations in *pcf11-9* impair correct transcription termination (Birse *et al.*, 1998). The *pcf11-2* protein carries the mutations E232G, D280G, C424R, S538G, F562S and S579P, and the *pcf11-9* protein contains the amino acid changes A66D, S190P, R198G, R227G, E354V and K435V (Figure 2B). In contrast with *pcf11-9* (A66D) and *pcf11-13* (D68A, S69A and I70A), the mutations in *pcf11-2* do not change the CID sequence (Figure 2C).

First, we analysed by direct two-hybrid analysis whether the mutations in *pcf11-2*, *pcf11-9* and *pcf11-13* affect CTD binding. In addition, we tested whether an N-terminal protein fragment of Pcf11p (180 residues encoded by ΔC446) containing the putative CID is sufficient for CTD binding. Two-hybrid interactions of *PCF11*, ΔC446 and *pcf11-2* with the CTD promoted a His⁺ growth phenotype (Figure 3A) and β-galactosidase expression (Table II). In contrast, ΔN126, *pcf11-9* and *pcf11-13* failed to activate reporter gene expression. Thus the

Fig. 2. Pcf11p contains a conserved CTD interaction domain. (A) Schematic representation of the domain organization of Pcf11p. The CTD interaction domain is indicated as CID, the segment of 20 consecutive glutamines as Q₂₀, the Rna14p/Rna15p interaction domain as Rna14p/Rna15p ID, the Clp1p-interaction domain as Clp1p ID, and the two zinc-finger motifs as C₂H₂ and C₂HC, respectively. The numbers correspond to the amino acid sequence of Pcf11p. (B) Schematic representation of Pcf11p mutants used in this study. The numbers and letters indicate the length of the amino acid sequence deleted either at the N- or C-terminus. (C) Sequence of the conserved CID of Pcf11p. The amino acid conservation of the CID of Pcf11p was calculated by sequence alignments of 13 known and putative CTD binding proteins containing the CID (results not shown). Invariant residues are in red boxes (100% conservation), blue boxes indicate conserved and similar amino acids that are present in 70–100% of the aligned sequences and yellow boxes indicate conserved and similar residues common in 30–70% of the aligned sequences. Point mutations in *pcf11-9* (A66D) and *pcf11-13* (D68A, S69A, I70A) changing the CID sequence are marked by arrows.

mutations in *pcf11-2*, which are exclusively outside the CID, did not affect CTD binding, whereas the point mutations of highly conserved CID residues in *pcf11-13*, as well as the mutations in *pcf11-9*, did. In addition, a 180 amino acid protein fragment containing the CID (ΔC446) was active in CTD binding, whereas deletion of nearly the complete CID in ΔN126 impaired the interaction with the CTD.

In agreement with the two-hybrid results, Pcf11-13p failed to bind to the CTD in GST pull-down experiments (Figure 3B, lanes 3 and 4). Interaction of Pcf11-9p with the phosphorylated CTD was significantly reduced compared with wild-type Pcf11p or Pcf11-2p. This suggested an interference of mutations in *pcf11-9* with its CTD binding activity as seen by two-hybrid analysis. Conversely, mutations in *pcf11-2* did not impair CTD binding even

at temperatures that restricted Pcf11-2p function (results not shown). These results demonstrated that the CID is responsible for CTD binding of Pcf11p. Furthermore, mutations in *pcf11-9* and *pcf11-13*, which change the CID sequence, affected CTD binding; mutations in *pcf11-2*, which are located downstream of the CID, did not.

The CID of Pcf11p is essential for cell viability

Next, we analysed whether the CID is essential for cell viability. We introduced the different mutant *PCF11* alleles into the NA53 strain, which carries a complete chromosomal deletion of *PCF11* rescued by the *URA3*-marked plasmid pFL38-*PCF11* (Amrani *et al.*, 1997b). Transformants were forced to lose the *URA3*-marked plasmid pFL38-*PCF11* by counterselection with 5-FOA at 23°C. Alleles with deletions at either the N- or C-terminus

Fig. 3. The CID is responsible for CTD binding. **(A)** The N-terminal 180 amino acids comprising the CID of Pcf11p are sufficient for a two-hybrid interaction with the CTD. Y190 cells were cotransformed with pAS2ΔA-CTD and the pACT2 constructs encoding the open reading frames: *PCF11*, $\Delta N126$, $\Delta C446$, *pcf11-2*, *pcf11-9* and *pcf11-13*. *HIS3* reporter gene expression was assayed by growth on medium lacking histidine. **(B)** Analysis of the CTD binding ability of Pcf11p mutants by GST pull-down experiments. GST (lane 2), GST-CTD (lane 3) or GST-CTD-P (lane 4) bound to glutathione-Sepharose 4B was incubated with *in vitro* translated ^{35}S -labelled proteins as indicated on the left of the panel. Bound proteins were separated by SDS-PAGE and visualized by autoradiography. Input (lane 1) shows 10% of *in vitro* translation reactions included in the binding reaction.

of *PCF11* did not rescue a complete chromosomal deletion of *PCF11*, whereas *pcf11-13* did (Figure 4A). Thus the N-terminus of *PCF11* encompassing the CID is essential for cell viability. Compared with the isogenic wild-type strain, *pcf11-13* cells were temperature sensitive at 30°C and died at 37°C (Figure 4B).

As shown above, mutations in *pcf11-2* neither change the CID sequence nor affect CTD binding. However, these mutations inhibit cleavage and polyadenylation *in vitro*, and it has been suggested that this deficiency causes the ts phenotype of *pcf11-2* cells (Amrani *et al.*, 1997b). In contrast, deletions removing part or all of the CID or mutations in *pcf11-13* impair CTD binding but do not change sequences further downstream of the CID. This raised the question of whether these CID mutants were able to rescue the viability of *pcf11-2* cells at restrictive temperature. Therefore we introduced the different *PCF11* alleles into the *pcf11-2* strain and incubated transformants at restrictive temperature (37°C) (Figure 4C). Wild-type *PCF11*, $\Delta N21-71$, $\Delta N108$, $\Delta N126$ and the ts allele *pcf11-13*, but not $\Delta N266$ and $\Delta N419$ (not shown),

Table II. The CID of Pcf11p is responsible for CTD binding

pACT2 fusion gene	β -galactosidase expression	HIS3 expression
<i>PCF11</i>	Blue	+
<i>pcf11-2</i>	Blue	+
<i>pcf11-9</i>	White	–
<i>pcf11-13</i>	White	–
$\Delta N126$	White	–
$\Delta C446$	Dark blue	+

complemented the growth defect of *pcf11-2* cells at 37°C *in trans*. As expected, C-terminal deletions of *PCF11* ($\Delta C208$, $\Delta C296$, $\Delta C353$ and $\Delta C446$) did not rescue cell viability (results not shown). As tested with plasmid-encoded *pcf11-2*, rescue of cell growth was not a protein dosage effect. Thus cross-complementation of the *pcf11-2* phenotype did not require the essential CID, but depended on sequences located further downstream.

If the ts phenotype of *pcf11-13* cells is due to a loss of function of the CID and if this function can be complemented *in trans*, introduction of mutant *PCF11* alleles containing the wild-type CID sequence are expected to rescue cell viability at restrictive temperature. Indeed, wild-type *PCF11* or $\Delta C208$, $\Delta C296$, $\Delta C353$ and $\Delta C446$ compensated the temperature sensitivity of *pcf11-13* cells, although $\Delta C296$, $\Delta C353$ and $\Delta C446$ cells grew more slowly than those with wild-type *PCF11* or $\Delta C208$ (Figure 4D). In contrast, alleles containing mutations affecting the CID sequence ($\Delta N21-71$, $\Delta N108$ and $\Delta N126$) failed to rescue growth of *pcf11-13* cells at 37°C. The results of these cross-complementation experiments are summarized in Table III.

The observation that essential functions of Pcf11p can be provided by independent polypeptides *in trans* raises the possibility that more than one Pcf11p molecule is required for cell viability (see Discussion). We performed western blot analysis to verify the stable expression of N- and C-terminally truncated proteins in *pcf11-13* and *pcf11-2* mutant backgrounds. Figure 4E shows that the mutant proteins were readily detectable in total protein extracts of the respective strains. Only the $\Delta C208$ mutant protein appeared to be expressed at lower levels or to be somewhat less stable than the other protein fragments.

In conclusion, the N-terminus of *PCF11* with the CID was required for cell viability. More importantly, the functions of Pcf11p in 3' end processing and CTD binding do not seem to be tightly coupled *in vivo* because they could be carried out separately by mutant polypeptides *in trans*.

CTD binding deficiency of Pcf11p does not affect its function in cleavage and polyadenylation *in vitro*

Next, we analysed whether a deficiency in CTD binding of Pcf11p affects 3' end processing *in vitro*. Extracts of wild-type, *pcf11-2*, *pcf11-9* and *pcf11-13* strains were tested for their ability to cleave and polyadenylate a synthetic *CYC1* pre-mRNA (Figure 5A). Extracts from wild-type, *pcf11-13* and *pcf11-2* cells accurately and efficiently cleaved and polyadenylated the substrate RNA at 23°C (Figure 5A, lanes 1–3). The 3' end processing was completely

Fig. 4. The CID of Pcf11p is essential for cell viability. (A) *pcf11-13* complements a complete chromosomal deletion of *PCF11*. Strain NA53 was transformed with plasmid-encoded *PCF11*, $\Delta C208$ or the CID mutants *pcf11-13*, $\Delta N21-71$, $\Delta N108$, $\Delta N126$ and $\Delta N266$. Transformants were forced to lose the *URA3*-marked plasmid pFL38-*PCF11* on 5-FOA plates at 23°C. (B) Mutations in *pcf11-13* cause temperature sensitivity. Serial dilutions of *PCF11* and *pcf11-13* cells were incubated at 30 and 37°C. (C) Cross-complementation of the *pcf11-2* allele in trans. The *pcf11-2* strain was transformed with plasmids encoding *PCF11*, *pcf11-2*, the CID mutants *pcf11-13*, $\Delta N21-71$, $\Delta N108$, $\Delta N126$ and $\Delta N266$ or empty plasmid. Transformants were grown at restrictive conditions (37°C) for the *pcf11-2* allele. (D) Cross-complementation of the *pcf11-13* allele in trans. The *pcf11-13* strain was transformed with plasmid-encoded *PCF11*, $\Delta N21-71$, $\Delta N108$, $\Delta N126$, $\Delta C208$, $\Delta C296$, $\Delta C353$ and $\Delta C466$. Transformants were grown at restrictive growth conditions (37°C) for the *pcf11-13* allele. (E) Western blot analysis of total protein extracts obtained from *pcf11-13* (lanes 1–5) and *pcf11-2* strains (lanes 6–9) that also carry plasmid-borne N- or C-terminally truncated deletions of Pcf11p as indicated. All deletion proteins (indicated by arrows) were N-terminal in-frame fusions with two IgG binding domains. Pcf11-13p (also containing two N-terminal IgG binding domains) and Pcf11-2 proteins are indicated by asterisks. The blot was consecutively decorated with a polyclonal anti-Pcf11p serum and anti-rabbit IgG-horseradish peroxidase conjugate.

inhibited in *pcf11-2* extract at 30°C (lane 7) and 36°C (lane 11), whereas *pcf11-9* extract displayed very weak cleavage but no polyadenylation activity at all temperatures (lanes 8 and 12). In contrast, cleavage and polyadenylation activities of *pcf11-13* extract were comparable to that of wild-type extract at all temperatures tested (lanes 5, 6, 9 and 10). Addition of purified CF IA restored cleavage and polyadenylation activity of *pcf11-9* and *pcf11-2* extracts at 36°C (lanes 13 and 14). Thus 3' end processing activity of Pcf11p *in vitro* was not affected by mutations in *pcf11-13*, whereas it was inhibited by mutations in *pcf11-2* and *pcf11-9*.

To confirm these results we tested different mutant *PCF11* proteins for their ability to restore cleavage and polyadenylation of *pcf11-2* extract at 36°C (Figure 5B). Recombinant Pcf11p restored 3' end processing activity (results not shown). However, this rescue was low, possibly because the recombinant protein could not efficiently replace the mutant polypeptide of the CF IA complex in the extract. Therefore, we used a combination of recombinant Rna14p, Clp1p, Rna15p and Pcf11p or its mutant forms as GST fusion proteins to reconstitute CF IA

activity. Addition of purified CF IA (lane 2) or GST-Pcf11p (lane 3), GST- $\Delta N126$ p (lanes 4 and 5) and GST- $\Delta N266$ p (lanes 6 and 7), combined with Rna14p, Rna15p and Clp1p, effectively restored cleavage and polyadenylation activity. Addition of GST- $\Delta N419$ p combined with Rna14p/Rna15p/Clp1p (lanes 8 and 9) or Rna14p/Rna15p/Clp1p (lanes 10 and 11) without Pcf11p was not able to reconstitute 3' end processing activity of *pcf11-2* mutant extract at 36°C. Purified CF IA (not shown), GST-Pcf11p (lane 12), GST- $\Delta N126$ (lane 13), GST- $\Delta N266$ (lane 14), GST- $\Delta N419$ (lane 15) and Rna14p/Rna14p/Clp1p (lane 16) were not active on their own. Thus the N-terminal 266 amino acids encompassing the CID were dispensable for the function of Pcf11p in cleavage and polyadenylation of pre-mRNA *in vitro*.

Consistent with this, *pcf11-13* cells displayed an *in vivo* poly(A) tail length distribution comparable to wild-type cells, whereas *pcf11-2* and *pcf11-9* cells showed a significant reduction in poly(A) tail length and accumulation of short poly(A) tails (results not shown). Furthermore, analysis of affinity purified CF IA from *pcf11-13* cells showed the same subunit composition and

Table III. Pcf11p is a bifunctional protein

Allele	Cross-complementation of		Rescue of a chromosomal <i>PCF11</i> deletion
	<i>pcf11-2</i>	<i>pcf11-13</i>	
<i>PCF11</i>	+	+	+
<i>pcf11-13</i>	+	ND	+
$\Delta N21-71$	+	—	—
$\Delta N108$	+	—	—
$\Delta N126$	+	—	—
$\Delta N266$	—	ND	—
$\Delta N419$	—	ND	—
$\Delta C446$	—	+	—
$\Delta C353$	—	+	—
$\Delta C296$	—	+	—
$\Delta C208$	—	+	—

ND, not determined.

cleavage and polyadenylation activity as wild-type CF IA (results not shown).

Correct transcription termination requires CTD binding of Pcf11p

Previous work showed that mutations in *pcf11-9* impaired correct transcription termination and it has been suggested that this defect is caused by the loss of cleavage activity (Birse *et al.*, 1998). However, we demonstrated that mutations in *pcf11-9* affect both CTD binding and cleavage and polyadenylation. These observations raised the question of whether loss of cleavage activity or loss of CTD binding caused the defect in transcription termination. With the *pcf11-2* and *pcf11-13* alleles at hand we could address this question because they affected either cleavage and polyadenylation or CTD binding.

We carried out transcriptional run-on analysis to test the requirement of the two functions of Pcf11p for transcription termination, measuring the polymerase profiles at the *CYC1* gene *in vivo*. Correct transcription termination results in run-on signals over probes 1–3, whereas faulty termination also produces signals over probes 4–6 (Birse *et al.*, 1998). The distribution of run-on transcripts over the contiguous probes 1–6 (Figure 6B) showed that transcription in wild-type (*PCF11*) and *pcf11-2* cells ceased efficiently downstream of probe 3, both at 23°C and following a shift to 37°C for 60 min. In agreement with previous results (Birse *et al.*, 1998; Barilla *et al.*, 2001), *pcf11-9* cells displayed increased run-on signals over probes 4–6 after the shift to restrictive temperature (37°C). In contrast, *pcf11-13* cells showed run-on transcripts past the poly(A) site over probes 4, 5 and 6, even at permissive temperature (23°C). Compared with the high level of read-through at 23°C, incubation of *pcf11-13* cells at 37°C changed the polymerase profile only slightly.

The temperature-sensitive phenotype of the *pcf11-13* mutant was complemented by C-terminally truncated Pcf11p fragments (Figure 4). Therefore, we tested whether the same fragments were able to complement the termination defect of *pcf11-13* cells as well. However, TRO analysis revealed that both the $\Delta C208$ fragment (Figure 6B) and the $\Delta C466$ fragment (not shown) did not significantly reduce read-through in this strain. We conclude that the truncated proteins are able to rescue

Fig. 5. CTD binding deficiency of Pcf11p does not affect its function in cleavage and polyadenylation *in vitro*. (A) Mutations in the *pcf11-13* allele do not affect cleavage and polyadenylation. ³²P-labelled *CYC1* RNA (lane 15) was assayed at 23°C (lanes 1–4), 30°C (lanes 5–8) or 36°C (lanes 9–14) under standard reaction conditions in extracts made from wild-type *PCF11* (lanes 1, 5 and 9), *pcf11-13* (lanes 2, 6 and 10), *pcf11-2* (lanes 3, 7, 11 and 14) or *pcf11-9* cells (lanes 4, 8, 12 and 13). Restoration of cleavage and polyadenylation in *pcf11-2* and *pcf11-9* extract at restrictive temperature (36°C) was examined by addition of purified CF IA (lanes 13 and 14). (B) The CID is not necessary for 3' end processing. Restoration of cleavage and polyadenylation of *pcf11-2* extract at restrictive temperature (36°C) was examined by addition of purified CF IA (lane 2) or recombinant GST fusion proteins of Pcf11p (100 ng, lane 3), $\Delta N126p$ (50 and 100 ng, lanes 4 and 5), $\Delta N266p$ (50 and 100 ng, lanes 6 and 7) and $\Delta N419p$ (50 and 100 ng, lanes 8 and 9). Before addition to the *pcf11-2* extract, proteins were incubated for 10 min with a combination of recombinant Rna14p, Clp1p and Rna15p (each 100 ng, lanes 3–11) to assemble CF IA. Recombinant proteins were also controlled for endogenous activity (lanes 12–16). Reaction products were visualized by autoradiography after electrophoresis in a gel of 6% polyacrylamide and 8.3 M urea. The positions of the full-length transcript (*CYC1*), the 5'-cleavage product (5') and the polyadenylation product (pA) are marked on the right. The length in nucleotides of marker bands (*HpaII*-digested pBR322 DNA) is indicated on the left.

viability but that they are not sufficient to provide the termination function of Pcf11p *in trans* (see Discussion). Nevertheless, our results provide evidence for the

Fig. 6. Mutations in the CTD-binding domain impair correct transcription termination. (A) Schematic diagram of plasmid pUGCYC1 showing the arrangement of M13 probes relative to the *CYC1* poly(A) site (position 506). (B) Transcriptional run-on analyses performed in cells transformed with pUGCYC1 under permissive growth conditions (23°C) and after shifting to restrictive temperature (37°C) for 60 min (45 min in the case of *pcf11-13* + $\Delta C208$) as indicated. The numbers at the top of the panel correspond to the probes. Lane M marks the M13 probe used as a background hybridization control. Hybridization of transcripts to the actin probe (lane A) and RNAP III transcripts to the tRNA probe (lane t) are shown. Quantitative analyses of transcriptional run-on profiles are shown on the right of each panel. PhosphorImager quantitation was performed with IMAGEQUANT software. The M13 background was subtracted from each probe, and the results were normalized to probe 1, which was fixed at 100%. Each experiment was performed at least three times, and average values are presented.

functional importance of the CTD interaction of Pcf11p in transcription termination. Moreover, they show that loss of cleavage activity did not correlate with a defect in transcription termination.

Discussion

Pcf11p belongs to a family of CTD-binding proteins that contain a conserved N-terminal CTD interaction domain (reviewed by Proudfoot *et al.*, 2002). In agreement with previous work (Barilla *et al.*, 2001), we showed that

Pcf11p bound specifically to the phosphorylated CTD *in vitro* and that this interaction was also observed in a two-hybrid assay. Notably, binding of Pcf11p requires phosphorylation of the CTD on Ser2 of its heptad repeats, whereas phosphorylation of Ser5 has no effect (Licatalosi *et al.*, 2002). We found that the highly conserved CID of Pcf11p is essential for cell viability and is responsible for CTD binding. In agreement with this, mutations of conserved amino acids of the CID in *pcf11-13* and presumably also in *pcf11-9* impaired CTD binding. We also provided evidence that another CF IA subunit,

Rna14p, may contribute to the interaction of CF IA with the CTD. Rna14p exhibits sequence homology with the 77 kDa subunit of mammalian CstF (Takagaki and Manley, 1994), which is composed of three subunits of 77, 64 and 50 kDa. However, only CstF 50 has been found to bind to the CTD (McCracken *et al.*, 1997; Fong and Bentley, 2001).

We demonstrate that the CTD binding and 3' end processing activities of Pcf11p can be functionally uncoupled. As shown with *pcf11-13* and other CID mutants, inhibition of CTD binding did not affect the 3' end processing activity of Pcf11p *in vitro* and the mutations in *pcf11-2* inhibiting 3' end processing *in vitro* did not prevent CTD binding. Two lines of evidence support the assumption that *pcf11-13* is also functional in cleavage and polyadenylation *in vivo*: (i) *pcf11-13* cross-complemented the ts phenotype of *pcf11-2* cells; (ii) the poly(A) tail length distribution of *pcf11-13* cells was comparable to that of wild-type cells. Moreover, C-terminal fragments missing the CID could complement the ts phenotype of the 3' end processing mutant *pcf11-2*, and N-terminal fragments of Pcf11p containing the CID could complement the ts phenotype of the CTD binding mutant *pcf11-13*. These observations suggest that more than one molecule of Pcf11p may be involved per transcription and 3' end processing event. Interestingly, these N-terminal fragments lack the domains that were implied in the protein–protein contacts required for CF IA assembly. It remains to be shown whether these truncated proteins act in the absence of other associated proteins, whether they are assembled into a modified form of CF IA or whether Pcf11p is also part of a different complex. In this context it is noteworthy that the existence of alternative CF IA complexes was predicted recently (Skaar and Greenleaf, 2002). Such alternative complexes might exert a function independent of the CF IA 3' end formation factor. It is possible that the C-terminally truncated Pcf11p proteins that were able to complement the *pcf11-13* temperature-sensitive phenotype, but not the defect in transcriptional termination, provide an additional and as yet unknown function of Pcf11p that is essential and different from the function in termination. Consistent with this interpretation, it is apparent from several studies that a defect in termination does not necessarily result in a lethal phenotype (Aranda and Proudfoot, 2001; Alen *et al.*, 2002). Alternatively, these proteins might provide the essential interaction with the CTD on one hand but destabilize interactions with other factors in CF IA on the other. This may indicate that a larger deletion of the C-terminus also affects termination, but probably in a more indirect way through destabilization of the CF IA complex. Since mutations in other CF IA subunits also result in defective termination (Birse *et al.*, 1998), this explanation seems reasonable. Unfortunately, this hypothesis cannot be tested directly by TRO, since even the smallest C-terminal deletions that we produced are lethal in a *PCF11* deletion background.

The functional connection between 3' end processing and transcription termination has been known for some time. However, it remained unclear whether 3' end formation factors indirectly signal poly(A) site recognition to RNAP II by cleaving the transcript or whether they communicate in a more direct way with the polymerase. If

cleavage is an obligatory requirement for transcription termination, as proposed by the torpedo model, one would expect that the absence of cleavage activity would also impair transcription termination. Our transcriptional run-on analysis of different mutant *PCF11* alleles disproved this assumption. Although 3' end processing is impaired by mutations in both *pcf11-2* and *pcf11-9* *in vitro*, only *pcf11-9* cells displayed a defect in transcription termination. In contrast with *pcf11-2*, mutations in *pcf11-9* caused a decrease in CTD binding. In addition, mutations in *pcf11-13* affected both CTD binding and transcription termination, but not 3' end processing. Thus, loss of cleavage activity did not correlate with impaired transcription termination, whereas loss of CTD binding did. This observation underscores the functional importance of the CTD interaction of Pcf11p for transcription termination. Importantly, our experiments describe for the first time a mutant in the 3' end formation machinery that is deficient in cleavage but functional in termination at the *CYC1* terminator. Our experiments strongly argue against a strict dependence of transcriptional termination on cleavage of the RNA transcript. However, we cannot entirely exclude the possibility that cleavage contributes to termination in a wild-type cell as one of several mechanisms that act in parallel.

All these observations are consistent with the following scenario. The 3' end processing apparatus is recruited to RNAP II through interactions with the phosphorylated CTD and scans the nascent RNA for a functional poly(A) site. Poly(A) site recognition leads to the association of the 3' end processing complex with the pre-mRNA, and causes polymerase pausing and a change in polymerase processivity (Orozco *et al.*, 2002). This ultimately leads to transcription termination downstream of the poly(A) site.

Materials and methods

Yeast strains and plasmids

Manipulations and growth of *S.cerevisiae* were carried out by established procedures. The following *S.cerevisiae* strains were used in this study: NA53 [*MATa ura3-1 trp1Δ ade2-1 leu2-3, 112 his3-11, 15 pcf11-Δ::TRP1* (pFL38-*PCF11*)] (Amrani *et al.*, 1997b); NA65 [*MATa ura3-1 trp1Δ ade2-1 leu2-3, 112 his3-11, 15 pcf11-2*] (Amrani *et al.*, 1997b); NA67 [*MATa ura3-1 trp1Δ ade2-1 leu2-3, 112 his3-11, 15 pcf11-9*] (Amrani *et al.*, 1997b); Y190 [*MATa ura3-52 trp1-901 ade2-101 leu2-3, 112 his3-200 gal4Δ gal80Δ URA3::GAL1-lacZ, LYS2::GAL1-HIS3 cyh^r*; Clontech]; W303-1B [*MATa ura3-1 trp1-1 ade2-1 leu2-3, 112 his3-11, 15*]; YMS800 [*MATa ura3-1 trp1Δ ade2-1 leu2-3, 112 his3-11, 15 pcf11-Δ::TRP1* (pNOPL-*pcf11-13*)]. Construction of plasmids used in this study is described in detail in Supplementary data (available at *The EMBO Journal* Online). Fusions of the CTD with GST (pGEXAS-CTD) and the *GAL4* DNA-binding domain (pAS2ΔΔ-CTD) included the C-terminal 229 amino acids of Rpb1p (nucleotides 4514–5201).

Expression and purification of recombinant proteins

Escherichia coli BL21 cells containing a glutathione *S*-transferase (GST, pGEXAS) or a GST–CTD fusion construct (pGEXAS-CTD) were grown in 2×YT containing 100 µg/ml ampicillin at 37°C to an OD₆₀₀ of 0.8 and expression was induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) to 0.75 mM. After 3 h of expression at 37°C, cells were harvested and lysed, and proteins were purified by affinity chromatography on glutathione–Sepharose 4B (Pharmacia) according to the supplier's manual. Expression and purification of the GST–*PCF11*-H6, GST–ΔN126-H6, GST–ΔN266-H6 and GST–ΔN419-H6 constructs were carried out essentially as described above. According to the manufacturer's manual, the fusion proteins were first purified by affinity

chromatography on Ni-NTA-agarose (Qiagen) directly followed by affinity chromatography on glutathione-Sephadex 4B (Pharmacia).

Yeast two-hybrid analysis

The plasmids used for two-hybrid analysis were generated by insertion of the respective open reading frames into pAS2ΔΔ and pACT2. The *GAL4* DNA-binding domain fusion of the CTD (pAS2ΔΔ-CTD, bait) (Dichtl *et al.*, 2002a) and the *GAL4* activation domain fusions of *RNA14*, *PCF11*, *CLP1*, *RNA15*, *NAB4* and *PAB1* (pACT2 constructs, prey) are listed in Supplementary table V. Detailed information about these constructs will be given on request. The bait and prey plasmids were simultaneously introduced into the yeast strain Y190 (Clontech) and transformants were selected on YNB medium lacking leucine and tryptophan. Y190 contains two different reporter genes (*HIS3* and *LacZ*). Expression of *HIS3* was assayed by growth on YNB medium lacking tryptophan, leucine and histidine but supplemented with 35 mM 3-amino-1,2,4-triazole (3-AT). β-galactosidase expression was monitored by X-Gal colony-lift filter assay according to the Clontech manual. A two-hybrid interaction was defined as positive when cells displayed the *HIS3*⁺ and *LacZ*⁺ phenotypes.

GST pull-down experiments

In vitro translations were performed with the TNT-coupled transcription-translation system (Promega). GST-CTD fusion protein was phosphorylated with HeLa nuclear extract as described previously (Hirose and Manley, 1998). Hyperphosphorylation of the CTD and shift of its electrophoretic mobility was controlled by immunodetection with monoclonal antibodies directed against phosphoserine 2 (H5; BAbCO) and phosphoserine 5 (H14; BAbCO) of the heptapeptide repeat or the unphosphorylated CTD of Pol II (8WG16; BAbCO). GST pull-down experiments were essentially carried out as described previously (Dichtl *et al.*, 2002a).

RNA analyses

Yeast extracts were made as described previously (Ohnacker *et al.*, 2000). Internally ³²P-labelled *CYC1* RNA was produced by *in vitro* run-off transcription, and *in vitro* cleavage and polyadenylation assays were carried out in the presence of 2 mM ATP and 2 mM magnesium acetate as described previously (Minvielle-Sebastia *et al.*, 1994). For reactions at elevated temperature, the reaction mix and protein extracts were first pre-incubated separately at 36°C for 5 min, combined and assayed at 36°C for 55 min. Transcriptional run-on experiments were performed as described previously (Birse *et al.*, 1998).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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