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The WD-repeat protein Pfs2p bridges two essential factors within the yeast pre-mRNA 3′-end-processing complex

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In the yeast *Saccharomyces cerevisiae*, pre-mRNA 3′-end processing requires six factors: cleavage factor IA (CF IA), cleavage factor IB (CF IB), cleavage factor II (CF II), polyadenylation factor I (PF I), poly(A) polymerase (Pap1p) and poly(A)-binding protein I (Pab1p). We report the characterization of Pfs2p, a WD-repeat protein previously identified in a multiprotein complex carrying PF I–Pap1p activity. The 3′-end-processing defects of *pfs2* mutant strains and the results of immunodepletion and immunoinactivation experiments indicate an essential function for Pfs2p in cleavage and polyadenylation. With a one-step affinity purification method that exploits protein A-tagged Pfs2p, we showed that this protein is part of a CF II–PF I complex. Pull-down experiments with GST fusion proteins revealed direct interactions of Pfs2p with subunits of CF II–PF I and CF IA. These results show that Pfs2p plays an essential role in 3′-end formation by bridging different processing factors and thereby promoting the assembly of the processing complex.
Keywords: 3′-end processing/polyadenylation/
WD repeats

Introduction

In eukaryotes, the 3′-ends of most mRNAs are generated by endonucleolytic cleavage of a primary transcript and subsequent polyadenylation of the 5′ cleavage product by poly(A) polymerase. This two-step reaction is accomplished by a surprisingly large set of protein factors (for reviews, see Colgan and Manley, 1997; Keller and Minvielle-Sebastia, 1997; Zhao *et al*., 1999). Fractionation of yeast extracts allowed the separation of activities that are required for 3′-end formation *in vitro* (Chen and Moore, 1992). Cleavage was reconstituted with fractions containing cleavage factor I (CF I) and cleavage factor II (CF II). Specific polyadenylation was obtained with combinations of fractions containing poly(A) polymerase (Pap1p), CF I and polyadenylation factor I (PF I). Further purification showed that CF I could be separated into two activities, which were named CF IA and CF IB (Kessler *et al*., 1996). CF IB consists of a single polypeptide called Nab4p/Hrp1p and is required for cleavage site selection and polyadenylation (Kessler *et al*., 1997; Minvielle-Sebastia *et al*., 1998).

CF IA is needed for both processing steps and is composed of the proteins Rna14p, Pcf11p, Rna15p and Clp1p (Minvielle-Sebastia *et al*., 1994, 1997; Kessler *et al*., 1996; Amrani *et al*., 1997b). Analyses of mutants in *RNA14*, *RNA15* and *PCF11* have confirmed that the products of these genes participate in 3′-end formation (Minvielle-Sebastia *et al*., 1994; Amrani *et al*., 1997b). Rna14p exhibits significant sequence similarity to the 77 kDa subunit of the mammalian cleavage stimulation factor CstF (Takagaki and Manley, 1994). Furthermore, the RNA-binding domain of Rna15p is homologous to that of the 64 kDa subunit of CstF. In addition to the four CF IA subunits mentioned above, the poly(A)-binding protein I (Pab1p) was detected in purified CF IA (Minvielle-Sebastia *et al*., 1997). Results from genetic and biochemical experiments indicate an involvement of Pab1p in poly(A) length control (Amrani *et al*., 1997a; Minvielle-Sebastia *et al*., 1997).

A multiprotein complex consisting of nine polypeptides was co-purified with PF I–Pap1p activity (Preker *et al*., 1997). Besides Pap1p, this complex includes Fip1p, Pta1p, Yhh1p/Cft1p, Ydh1p/Cft2p, Ysh1p/Brr5p, Yth1p and two uncharacterized proteins called Pfs1p and Pfs2p (for polyadenylation factor I subunit). Fip1p has been shown to interact physically with Pap1p, Yth1p and Rna14p, and is thought to tether the poly(A) polymerase to its substrate during polyadenylation (Preker *et al*., 1995). Yhh1p/Cft1p, Ydh1p/Cft2p, Ysh1p/Brr5p and Yth1p are the yeast homologues of the four subunits of mammalian cleavage and polyadenylation specificity factor CPSF: Yhh1p/Cft1p is 24% identical to the 160 kDa subunit of CPSF (Stumpf and Domdey, 1996); Ydh1p/Cft2p is 25% identical to the 100 kDa subunit of CPSF (Preker *et al*., 1997; Zhao *et al*., 1997); Ysh1p/Brr5p and the 73 kDa protein of CPSF exhibit 53% identity (Chanfreau *et al*., 1996; Jenny *et al*., 1996); and Yth1p is 40% identical to the 30 kDa polypeptide of CPSF (Barabino *et al*., 1997). Interestingly, the three large yeast CPSF homologues have also been identified in highly purified CF II fractions (Zhao *et al*., 1997). These reports are consistent with the requirement for CPSF for both steps of mammalian pre-mRNA 3′-end formation. CPSF interacts with poly(A) polymerase as well as with CstF (Murthy and Manley, 1995) and recognizes the cleavage and polyadenylation signal AAUAAA (Bienroth *et al*., 1991; Keller *et al*., 1991). While the phenotypes of conditionally lethal mutants in *PAP1*, *FIP1*, *YTH1*, *YSH1/BRR5* and *PTA1* are consistent with a role for the products of these genes in polyadenylation (Patel and Butler, 1992; Minvielle-Sebastia *et al*., 1994; Preker *et al*., 1995, 1997; Chanfreau *et al*., 1996; Jenny *et al*., 1996; Barabino *et al*., 1997), mutants in *YHH1/CFT1*, *YDH1/CFT2*, *PFS1* and *PFS2* have not been analysed to date.

Fig. 1. Sequence comparison of the WD repeats in Pfs2p. The seven repeat units were aligned manually and gaps were introduced to maximize homology within the conserved core region. Residues that are conserved among the WD-repeat units are boxed. Colours highlight conservation of identical or similar residues. The secondary structure prediction was done with the e-mail server at the EMBL (Rost and Sander, 1993, 1994). The consensus conformation was predicted to be the one that was inferred most frequently (at least five times more often than other conformations at the same position) and is only shown where a minimum of three consecutive residues matched that expression (β, β-sheet; τ, turn). A consensus (consensus A) was derived based on a frequency of at least five identical or similar residues at a given position: h, hydrophobic residues (L, I, V, C, M and A); a, aromatic residues (W, Y and F). A consensus sequence (consensus B) compiled for 269 potential WD-repeat units is shown for comparison (Neer *et al*., 1994).

Here we report the characterization of Pfs2p. Pfs2p is a 53 kDa protein that contains seven WD repeats. We found that extracts from mutants in *PFS2* have cleavage and polyadenylation defects *in vitro*. In yeast extracts, Pfs2p occurs in a multiprotein complex that exhibits CF II/PF I activity. Furthermore, Pfs2p directly interacts with Ysh1p, Fip1p and Rna14p *in vitro*. These interactions with subunits of two distinct processing factors suggest that Pfs2p functions in the assembly and stabilization of the complex that carries out 3′-end formation.

Results

PFS2 encodes an essential WD-repeat protein

Fip1p was the first PF I subunit to be identified (Preker *et al*., 1995). Subsequently, the use of a tagged version of Fip1p allowed the purification of a protein complex that included PF I–Pap1p activity and consisted of the polypeptides Fip1p, Pta1p, Yhh1p/Cft1p, Ydh1p/Cft2p, Ysh1p/Brr5p, Yth1p and two unknown proteins of 58 and 53 kDa (Preker *et al*., 1997). The peptide sequence KFTHLSSNK was obtained from the 53 kDa protein and showed a perfect match with the yeast 465 amino acid open reading frame (ORF) YNL317w, designated *PFS2*. In good agreement with its electrophoretic mobility, Pfs2p had a calculated molecular mass of 53 kDa.

A search for known protein motifs within Pfs2p revealed the presence of seven WD repeats between amino acids 90 and 380 (Figure 1). WD repeats, also called transducin repeats, have been detected in a large number of proteins that participate in diverse cellular processes and are frequently found in multimolecular complexes (Neer *et al*., 1994; Smith *et al*., 1999). Sequence alignments of Pfs2p with other members of the WD-repeat family yielded 16–40% identity, with the highest value for a putative *Schizosaccharomyces pombe* protein (results not shown). Among human proteins, β-transducin reached the highest score (24.5% identity).

The gene encoding Pfs2p was cloned from a genomic *Saccharomyces cerevisiae* DNA library as described in Materials and methods. To determine whether *PFS2* is essential for viability, we replaced one copy of its ORF with the *TRP1* marker in the diploid *S.cerevisiae* strain BMA41 (Baudin-Baillieu *et al*., 1997). The resulting strain MO1 was sporulated and tetrads were dissected (see Table I). Only two viable spores per tetrad were obtained and their growth depended on the presence of tryptophan in the medium. This result confirmed the previously observed lethality of a *PFS2* gene disruption (Maftahi *et al*., 1998). When MO1 was transformed with the centromeric *URA3*-marked plasmid pFL38-PFS2, tetrad dissection resulted in four viable spores per tetrad. Two out of four spores could grow without tryptophan in the medium. This haploid *PFS2* deletion strain (MO7, see Table I) was then transformed with either pFL36-PFS2 (*LEU2*, CEN) or with the empty vector pFL36. When loss of pFL38-PFS2 was forced on 5-fluoro-orotic acid (5-FOA) medium, only cells carrying pFL36-PFS2 but not those transformed with pFL36 could grow (results not shown). We conclude that *PFS2* is essential for viability, as is the case for all known genes encoding subunits of general 3′-end-processing factors.

Cleavage and polyadenylation are impaired in extracts from conditionally lethal pfs2 mutant strains

In order to characterize *PFS2* functionally, we isolated conditionally lethal mutants in this gene. Initially, mutants resulting in C-terminal deletions in Pfs2p were generated. Removal of the C-terminal extension following the last WD repeat (amino acids 388–465) allowed growth, indicating that this portion of the protein is dispensable.

Table I. Yeast strains used in this study

Strain	Relevant genotype/description
VDH2	Commercially available baker's yeast strain (Minvielle-Sebastia *et al*., 1994)
BMA41	*ura3-1/ura3-1, Δtrp1/Δtrp1, ade2-1/ade2-1, leu2-3, 112/leu2-3, 112, his3-11, 15/his3-11, 15* (Baudin-Baillieu *et al*., 1997)
MO1	*ura3-1/ura3-1, Δtrp1/Δtrp1, ade2-1/ade2-1, leu2-3, 112/leu2-3, 112, his3-11, 15/his3-11, 15, PFS2/pfs2::TRP1*
MO7	*ura3-1, Δtrp1, ade2-1, leu2-3, 112, his3-11, 15, pfs2::TRP1*, pFL38-PFS2
MO12	*ura3-1, Δtrp1, ade2-1, leu2-3, 112, his3-11, 15, pfs2::TRP1*, pFL36-PFS2
MO13	*ura3-1, Δtrp1, ade2-1, leu2-3, 112, his3-11, 15, pfs2::TRP1*, pFL36-pfs2-2
MO14	*ura3-1, Δtrp1, ade2-1, leu2-3, 112, his3-11, 15, pfs2::TRP1*, pFL36-pfs2-3
MO15	*ura3-1, Δtrp1, ade2-1, leu2-3, 112, his3-11, 15, pfs2::TRP1*, pFL36-pfs2-4
MO17	*ura3-1, Δtrp1, ade2-1, leu2-3, 112, his3-11, 15, pfs2::TRP1*, pFL36-pfs2-1
MO20	*ura3-1, Δtrp1, ade2-1, leu2-3, 112, his3-11, 15, pfs2::TRP1*, pNOP-PFS2
MO21	*ura3-1, Δtrp1, ade2-1, leu2-3, 112, his3-11, 15, pfs2::TRP1*, pNOP-pfs2-2A

None of the viable mutants had a conditional growth defect (not shown). In contrast, when one or more WD repeats were removed, the truncated forms of the gene were unable to rescue the null mutant (results not shown).

In a further attempt to generate conditionally lethal mutants in *PFS2*, we used error-prone PCR (see Materials and methods). This resulted in the isolation of four temperature–formamide-sensitive (tfs) mutant strains (alleles *pfs2-1* to *pfs2-4*). These strains were viable at 23 and 37°C in the absence of formamide but failed to grow at 35°C in the presence of 3% formamide in the medium. In comparison with wild type, the four mutant strains showed reduced growth rates also under permissive conditions. The strongest effect was observed for *pfs2-1* (results not shown). DNA sequencing revealed multiple mutations in each mutant allele. The mutations were located predominantly in the WD-repeat portion of Pfs2p (results not shown). Extracts prepared from the tfs mutant strains were tested for either specific cleavage or polyadenylation activity *in vitro* (Figure 2). To enhance the effect of the mutations, the processing reactions were carried out at 32°C. However, similar results were also obtained at 30 or 23°C (not shown). Cleavage was assayed with the synthetic CYC1 pre-mRNA. Figure 2A shows that the *pfs2-1* mutant extract exhibited complete loss of cleavage activity (lane 3) and could be complemented with an extract prepared from the *rna14-1* mutant (lane 8), which is inactive on its own (lane 7) due to the lack of CF IA activity (Minvielle-Sebastia *et al*., 1994). In the *pfs2-2* and *pfs2-3* mutant extracts, cleavage activity was detectable but clearly reduced in comparison with the wild-type extract (Figure 2A, compare lanes 4 and 5 with lane 2). The extract from the *pfs2-4* mutant showed only a mild reduction of cleavage activity (compare lane 6 with lane 2).

Figure 2B shows the result of a specific *in vitro* polyadenylation assay in which a synthetic CYC1 pre-cleaved transcript that ends at the natural CYC1 cleavage site was used as substrate. The *pfs2-1* mutant extract completely lacked specific polyadenylation activity (Figure 2B, lane 3), whereas the alleles *pfs2-2* and *pfs2-3* only led to a reduction of activity (see lanes 4 and 5). Again *pfs2-4* showed the weakest effect (Figure 2B, lane 6). Polyadenylation activity of the *pfs2-1* mutant extract could be restored with *rna14-1* mutant extract (Figure 2B, lane 10) and, surprisingly, with *fip1-1* mutant extract (lane 8). Since both Fip1p and Pfs2p had been identified as subunits of the same multiprotein complex (Preker *et al*., 1997), this result indicated that subunit

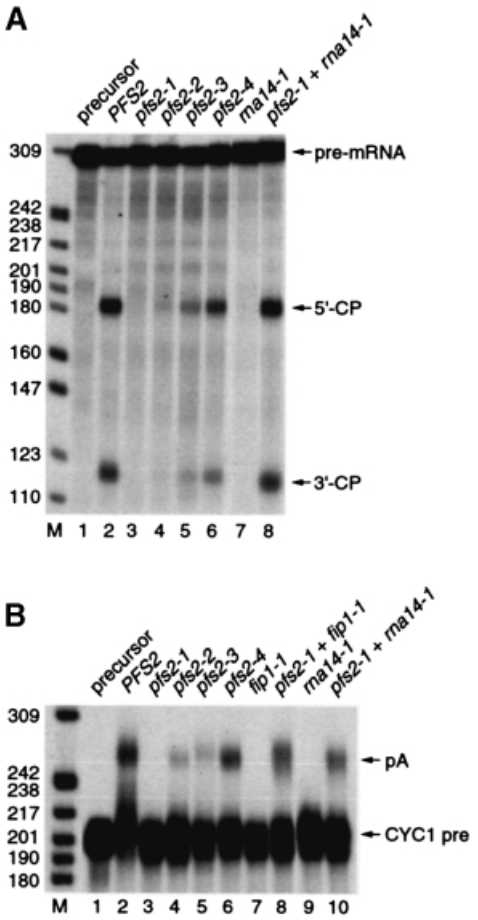

Fig. 2. *In vitro* 3′-end-processing phenotype of *pfs2* mutants. (**A**) Specific *in vitro* cleavage assay. Equal amounts of protein of extracts from strains carrying the mutant alleles *pfs2-1*, *pfs2-2*, *pfs2-3*, *pfs2-4* and *rna14-1* were added to the reaction mixtures as indicated on top of each lane. An extract from strain MO12 (*PFS2*) served as positive control. The migration positions of the cleavage substrate (pre-mRNA), the 5′ cleavage product (5′-CP) and the 3′ cleavage product (3′-CP) are indicated on the right. The position and size (in number of nucleotides) of the marker bands are indicated on the left. (**B**) Specific *in vitro* polyadenylation assay. Equal amounts of protein of extracts from strains carrying the mutant alleles *pfs2-1*, *pfs2-2*, *pfs2-3*, *pfs2-4*, *fip1-1* and *rna14-1* were added to the reaction mixtures as indicated on top of each lane. An extract from strain MO12 (*PFS2*) served as positive control. The position of the polyadenylation substrate (CYC1 pre) and the polyadenylated reaction products (pA) is indicated on the right. The position and size of the marker bands are indicated on the left.

exchange between the two mutant complexes could occur during the polyadenylation reaction. The strong *in vitro* processing defect of the *pfs2-1* mutant is consistent with the assumption that Pfs2p has an important function in 3′-end formation.

Depletion of Pfs2p from yeast extracts inhibits cleavage and polyadenylation

To test the functional requirement of Pfs2p for cleavage and polyadenylation in yeast extracts, depletion experiments were carried out with either pre-immune serum or a Pfs2p-specific antiserum (α Pfs2p).

Figure 3A shows an *in vitro* 3′-end-processing assay in which a yeast extract depleted for Pfs2p was tested for either cleavage or polyadenylation activity. In the extract depleted for Pfs2p, cleavage activity was strongly reduced and polyadenylation was not observed (Figure 3A, lanes 4 and 9). 3′-end-processing activity could be restored by addition of a fraction containing partially purified PF I, Pap1p and CF II (Figure 3A, lanes 5 and 10).

Figure 3B shows the result of an *in vitro* 3′-end-processing assay in which αPfs2p serum was tested for its ability to inhibit cleavage and polyadenylation. Both processing steps were strongly inhibited in a yeast extract when αPfs2p antibodies were added directly to the reaction (Figure 3B, lanes 4 and 8), whereas pre-immune serum did not affect activity significantly (lanes 3 and 7). These results indicated that Pfs2p was required for both cleavage and polyadenylation activity in yeast extracts.

Pfs2p is part of a CF II–PF I complex

In order to isolate proteins associated with Pfs2p, we used a tagged version of the protein for affinity purification. For this purpose, a yeast expression construct coding for a protein A–tobacco etch virus (TEV) protease cleavage site–Pfs2p fusion protein (see scheme in Figure 4A) was introduced into the *pfs2* null mutant background (see Materials and methods). An extract prepared from the resulting strain was incubated with IgG–agarose. This allowed the fusion protein to bind to the matrix via the protein A tag. After extensive washes, Pfs2p and associated proteins were eluted by cleavage with the site-specific TEV protease (Carrington and Dougherty, 1988). The advantage of this method over conventional purification procedures is that it allows a one-step purification of a tagged protein directly from a yeast extract under non-denaturing conditions (Senger *et al*., 1998).

Figure 4B shows a silver-stained gel in which the proteins eluted from IgG–agarose were separated. Strikingly, the eluate contained a limited set of bands (Figure 4B, lane 2) that strongly resembled the pattern of the previously reported PF I–Pap1p complex (Preker *et al*., 1997). The presence of Yhh1p/Cft1p, Ysh1p/Brr5p, Pap1p, Pfs2p, Fip1p and Yth1p could be confirmed by immunodetection (results not shown). Fip1p and Yth1p stain poorly with silver and are therefore difficult to detect in the protein gel (Preker *et al*., 1997). Antibodies against Ydh1p/Cft2p (105 kDa), Pta1p (85 kDa) and Pfs1p (58 kDa) were not available, but bands corresponding in size to each of these polypeptides were present in the eluate (Figure 4B, lane 2). This suggested that we had isolated a multiprotein complex that was similar and possibly identical to the complex that had been purified

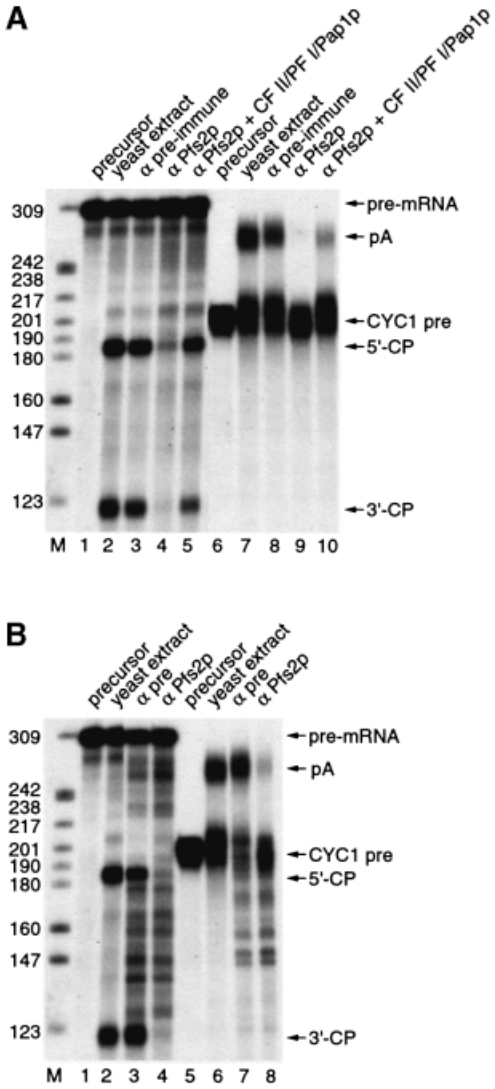

Fig. 3. Immunodepletion and immunoinhibition of 3′-end-processing activity with anti-Pfs2p antibodies. (**A**) 3′-end-processing assay with a wild-type yeast extract depleted for Pfs2p. Lanes 1–5, specific *in vitro* cleavage assay; lanes 6–10, specific *in vitro* polyadenylation assay; α pre-immune, yeast extract depleted with pre-immune serum; α Pfs2p, yeast extract depleted with serum directed against Pfs2p. Lanes 1 and 6, no protein; lanes 2 and 7, 2 µl of wild-type extract (VDH2); lanes 3 and 8, 2 µl of α pre-immune; lanes 4 and 9, 2 µl of α Pfs2p; lanes 5 and 10, 2 µl of α Pfs2p and 2 µl of partially purified CF II–PF I (Preker *et al*., 1997). The positions of the cleavage substrate (pre-mRNA), the 5′ cleavage product (5′-CP), the 3′ cleavage product (3′-CP), the polyadenylation substrate (CYC1 pre) and the polyadenylated reaction products (pA) are indicated on the right. The position and size of the marker bands are indicated on the left. (**B**) Antibodies directed against Pfs2p inhibit 3′-end-processing activity *in vitro*. Lanes 1–4, specific *in vitro* cleavage assay; lanes 5–8, specific *in vitro* polyadenylation assay. Lanes 1 and 5, no protein; lanes 2 and 6, 2 µl of wild-type extract (VDH2); lanes 3 and 7, 2 µl of wild-type extract and 1 µl of pre-immune serum; lanes 4 and 8, 2 µl of wild-type extract and 1 µl of anti-Pfs2p antiserum. The positions of the marker, the reaction substrates and products are indicated as in (A).

as PF I–Pap1p previously (Preker *et al*., 1997). More stringent washing conditions (250 mM NaCl instead of 150 mM) resulted in a reduction in the amount of Pap1p recovered in the eluate, suggesting that the polymerase is associated less strongly with an otherwise stable complex (results not shown). This is consistent with earlier reports

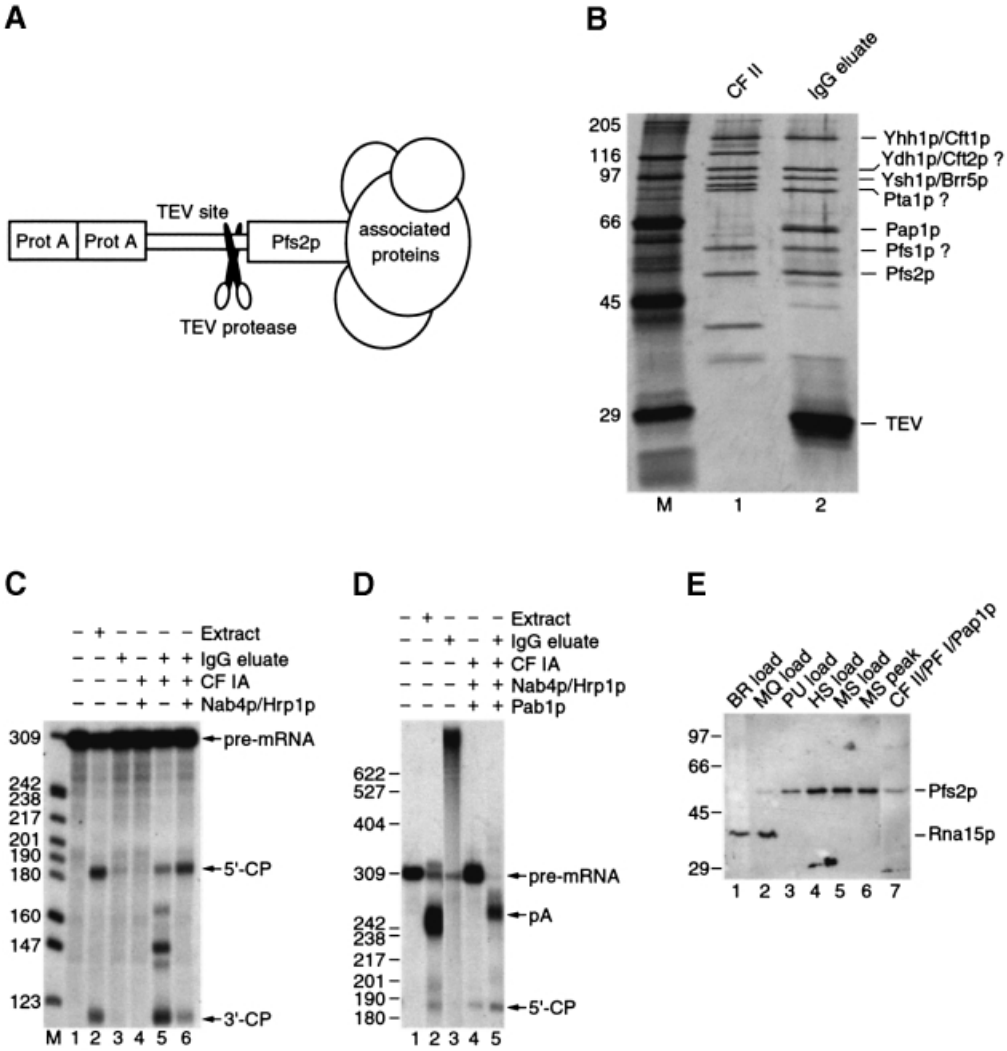

Fig. 4. Affinity purification of proteins bound to Pfs2p yields a CF II–PF I complex. (**A**) Schematic representation of the protein A–TEV cleavage site–Pfs2p fusion protein. (**B**) A multiprotein complex associated with protein A–TEV-tagged Pfs2p was isolated by batch absorption to IgG–agarose from an extract prepared from strain MO20. The complex was eluted by site-specific proteolytic cleavage with TEV protease (Materials and methods). A 14.4 µl aliquot of IgG–agarose eluate was separated on a 10% SDS–polyacrylamide gel and stained with silver (lane 2). For comparison, 8 µl of a partially purified CF II fraction (Minvielle-Sebastia *et al*., 1998) were run on the gel (lane 1). The position of TEV protease and polypeptides that were identified in the IgG–agarose eluate by immunodetection are indicated on the right. Bands that could not be identified by immunodetection but show electrophoretic mobilities expected for known 3′-end-processing proteins are indicated with a question mark. The sizes of the marker bands (in kDa) are indicated on the left. (**C**) Specific *in vitro* cleavage assay in which the IgG–agarose eluate was tested for CF II activity. A 2 µl aliquot of wild-type yeast extract or combinations of 0.2 µl of IgG–agarose eluate, 1.5 µl of purified CF IA (Minvielle-Sebastia *et al*., 1997) and 10 ng of recombinant Nab4p/Hrp1p were added as indicated on the top. The position of the cleavage substrate (pre-mRNA), the 5′ cleavage product (5′-CP) and the 3′ cleavage product (3′-CP) are indicated on the right. The position and size of the marker bands are indicated on the left. (**D**) Specific *in vitro* cleavage and polyadenylation assay in which the IgG–agarose eluate was tested for CF II–PF I activity. A 2 µl aliquot of wild-type yeast extract or combinations of 3 µl of IgG–agarose eluate, 1.5 µl of purified CF IA (Minvielle-Sebastia *et al*., 1997), 10 ng of recombinant Nab4p/Hrp1p and 80 ng of Pab1p were added as indicated on the top. The positions of the substrate (pre-mRNA), the 5′ cleavage product (5′-CP) and the polyadenylated cleavage products (pA) are indicated on the right. The position and size of the marker bands are indicated on the left. (**E**) Pfs2p co-purifies with CF II over six chromatographic steps. CF II had been purified previously by column chromatography (Minvielle-Sebastia *et al*., 1998) over Macro Prep Q, Bio Rex (BR), Mono Q (MQ), poly(U)–Sepharose (PU), heparin–Sepharose (HS) and Mono S (MS). Aliquots (5 µl) of the different CF II fractions were separated on a 10% SDS–polyacrylamide gel (lanes 1–6). For comparison, 0.5 µl of a partially purified CF II–PF I fraction were loaded (lane 7). An immunoblot of the protein gel was probed with antibodies directed against Pfs2p and Rna15p. The positions of Pfs2p and Rna15p are indicated on the right. The position and sizes of the marker bands (in kDa) are indicated on the left.

that described poly(A) polymerase as an independent factor (Lingner *et al*., 1991; Chen and Moore, 1992).

The CPSF homologues Yhh1p/Cft1p, Ydh1p/Cft2p and Ysh1p/Brr5p found associated with PF I–Pap1p activity (Preker *et al*., 1997) were also reported to be present in CF II (Zhao *et al*., 1997). In fact, CF II appeared to consist of a subset of the proteins that co-purified with PF I–Pap1p activity. This suggested that the complex isolated by Preker *et al*. (1997) also contains CF II activity.

To verify this hypothesis, we assayed the IgG–agarose eluate for PF I–Pap1p and CF II activity. Figure 4C shows a specific *in vitro* cleavage assay in which the IgG–agarose eluate was tested for CF II activity. The cleavage reaction was reconstituted when the eluate was combined with purified CF IA and recombinant Nab4p/Hrp1p (Figure 4C, compare lane 6 with lane 2). Omission of Nab4p/Hrp1p led to the production of additional cleavage bands (Figure 4C, lane 5) as observed previously (Minvielle-Sebastia *et al*.,

1998). From these results, we conclude that the isolated multiprotein complex had CF II activity. Very weak cleavage activity was observed without addition of CF I (Figure 4C, lane 3), suggesting that the eluted material also contained traces of CF I.

Correct cleavage and polyadenylation of the CYC1 RNA were obtained when the eluate was combined with CF IA, Nab4p/Hrp1p and Pab1p (Figure 4D, compare lane 5 with lane 2). Reconstitution of the reaction did not require addition of recombinant Pap1p, confirming that the material eluted from IgG–agarose also contained poly(A) polymerase activity. Taken together, these results demonstrate that a functional CF II–PF I complex can be isolated from a yeast extract in a one-step affinity purification procedure and that Pfs2p is part of this complex.

Besides the CF II purification reported by Zhao *et al.* (1997), another preparation of CF II has been described recently (Minvielle-Sebastia *et al.*, 1998). In this case, the purification procedure included six chromatographic separation steps and the isolated material was found to be free of PF I activity (Minvielle-Sebastia *et al.*, 1998). The protein gel depicted in Figure 4B shows that this CF II preparation contained several proteins of the same size as the bands present in the IgG–agarose eluate that showed CF II–PF I activity (compare lanes 1 and 2). This was not surprising since co-purification of Yhh1p/Cft1p, Ydh1p/Cft2p and Ysh1p/Brr5p with CF II and CF II–PF I had been expected. Interestingly, this CF II fraction also contained a protein that migrated as Pfs2p. Therefore, we tested for the presence of Pfs2p in this CF II preparation. Figure 4E shows an immunoblot on which fractions from several CF II purification steps were probed with antibodies directed against Pfs2p and, as a control, with antibodies against the CF IA subunit Rna15p. Rna15p was separated from CF II during chromatography on the Mono Q column, whereas Pfs2p was still present in the purest CF II fractions (Figure 4E, lane 6). Thus, Pfs2p co-purified with CF II over six fractionation steps. These results suggest that Pfs2p is not only part of the CF II–PF I complex but is also associated with CF II.

Isolation of the CF II–PF I complex from a pfs2 mutant strain

To elucidate the molecular mechanism of the *in vitro* processing defects of the *pfs2* mutant extracts, we decided to isolate the CF II–PF I complex from *pfs2* mutants. For this purpose, we generated a strain that expressed a mutant protein A–TEV site fusion protein derived from Pfs2-2p instead of the wild-type protein. Strains producing protein A-tagged versions of Pfs2-1p or Pfs2-3p instead of the wild-type protein could not be isolated because these mutant fusion proteins did not support viability in the null mutant background. As expected, the strain expressing the mutant protein A–TEV site fusion protein derived from Pfs2-2p showed temperature–formamide sensitivity (results not shown). We isolated the CF II–PF I complex of this strain by affinity purification as described above. Figure 5 shows a protein gel in which the IgG–agarose eluates from the wild-type and the mutant strain were analysed. Since the mutant protein appeared to run slightly higher than wild-type Pfs2p (Figure 5, compare lanes 1 and 2), its presence in the eluate was confirmed by immunodetection (results not shown). Interestingly,

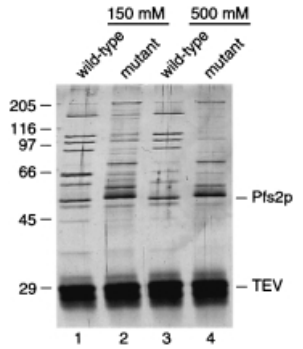

Fig. 5. Affinity purification of proteins associated with wild-type and mutant Pfs2p. IgG–agarose batch absorption of proteins from extracts prepared from a strain expressing protein A–TEV–Pfs2p (strain MO20, lanes 1 and 3) and a strain expressing a mutant protein A–TEV fusion derived from Pfs2-2p (strain MO21, lanes 2 and 4) was carried out as described in Materials and methods. The washing buffer contained either 150 or 500 mM NaCl (as indicated on the top). Bound material was eluted upon cleavage with TEV protease and 10% of each eluate was run on a 10% SDS–polyacrylamide gel, which was subsequently stained with silver. The positions of Pfs2p and TEV protease are indicated on the right. The sizes of the marker bands (in kDa) are indicated on the left.

most proteins of the CF II–PF I complex were reduced in levels in the eluate prepared from the mutant extract (Figure 5, compare lanes 1 and 2). This effect was even more pronounced when the IgG–agarose-bound complexes were subjected to high stringency washes (Figure 5, compare lanes 3 and 4). To exclude the possibility that the CF II–PF I complex is degraded preferentially in the mutant extract, the level of Yhh1p/Cft1p, Ysh1p/Brr5p and Fip1p in wild-type and mutant extract was compared by immunodetection. We did not observe increased degradation in the mutant extract (results not shown). It is also conceivable that the mutant protein interacts preferentially with polypeptides that do not belong to the CF II–PF I complex. This view is supported by the presence of proteins in the IgG eluate from the mutant extract that appear to be absent or at least under-represented in the wild-type preparation (Figure 5, compare lanes 3 and 4).

In summary, the results presented in Figure 5 indicate that the 3′-end-processing defect of the *pfs2-2* mutant extract is caused by the reduced stability or efficiency of formation of an active CF II–PF I complex.

Pfs2p bridges CF II–PF I and CF IA

To identify direct interaction partners of Pfs2p among known subunits of 3′-end-processing factors, we performed pull-down experiments (see Materials and methods). ^{35}S-labelled *in vitro* translated Rna14p, Rna15p, Clp1p, Pcf11p, Ysh1p/Brr5p, Yth1p, Fip1p and Pap1p were incubated individually with GST-tagged Pfs2p and interacting proteins were isolated with glutathione–Sepharose. Figure 6 shows the autoradiogram of a protein gel on which the bound material was separated. Interaction with GST–Pfs2p was observed reproducibly only for Rna14p, Ysh1p and Fip1p (Figure 6, compare lane 3 with lane 2, lane 15 with lane 14, and lane 21 with lane 20). Rna15p and Yth1p did not bind GST–Pfs2p above background (Figure 6, compare lane 6 with lane 5, and lane 18 with lane 17), while Pcf11p interacted with GST unspecifically (see lanes 11 and 12). In the case of Clp1p,

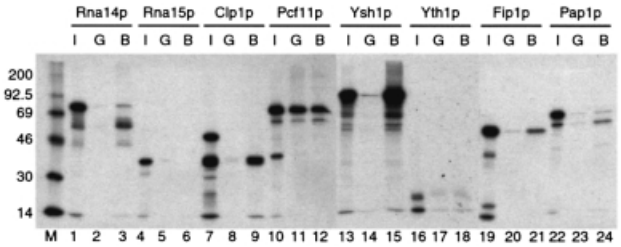

Fig. 6. Pfs2p physically interacts with subunits of CF II–PF I and CF IA *in vitro*. GST-tagged Pfs2p was tested for interaction with either of the following *in vitro* translated, ^{35}S-labelled proteins: Ysh1p/Brr5p, Yth1p, Fip1p, Pap1p, Rna14p, Rna15p, Clp1p or Pcf11p. Pfs2p and proteins associated with it were isolated by absorption to glutathione–Sepharose and analysed on an SDS gel. The protein tested for interaction with Pfs2p is indicated on the top of each lane. I, 5% of the input of *in vitro* translated protein; G, control in which GST instead of GST–Pfs2p was used; B, bound material. The sizes of the marker bands (in kDa) are indicated on the left.

no full-length protein could be detected in the fraction of bound material (Figure 6, compare lane 9 with lane 7). The signal above background obtained with Pap1p (Figure 6, lanes 24 and 23) was not reproducible and could not be confirmed by co-immunoprecipitation with *in vitro* translated Pfs2p and α Pfs2 antibodies. In contrast, Rna14p, Fip1p and Ysh1p were co-immunoprecipitated with Pfs2p (results not shown). In addition, Pfs2p and Rna14p were found to interact in the yeast two-hybrid system (results not shown). The direct interaction of Pfs2p with Rna14p may account for the presence of small amounts of CF IA in the CF II–PF I preparation obtained by affinity purification (see Figure 4C).

The results presented in Figure 6 show that Pfs2p interacts not only with CF II–PF I subunits but also with the CF IA component Rna14p. This indicated that Pfs2p provides a physical link between CF II–PF I and CF IA, and is consistent with the idea that this WD-repeat protein functions in the assembly of the 3′-end-processing complex. Furthermore, the direct interaction of Pfs2p with Ysh1p provided an explanation for its co-purification with CF II (see Figure 4E).

Discussion

Pfs2p is required for 3′-end processing of pre-mRNA

Preker *et al*. (1997) previously reported the purification of a multiprotein complex that included PF I–Pap1p activity. The isolation of the active complex was facilitated by a tagged version of Fip1p and purification was monitored by complementation of *fip1* mutant extracts. Besides Fip1p, Pap1p, Pfs1p, Pta1p and the four putative yeast homologues of the mammalian CPSF subunits, Yhh1p/Cft1p, Ydh1p/Cft2p, Ysh1p/Brr5p and Yth1p, this complex included the 53 kDa polypeptide Pfs2p.

Here, we report the characterization of the WD-repeat protein Pfs2p. WD-repeat proteins participate in diverse cellular processes in eukaryotes, e.g. signal transduction, transcriptional regulation and pre-mRNA splicing (Neer *et al*., 1994). The participation in dynamic protein–protein interactions and in the assembly of large complexes has been suggested to be the common function of these proteins (Neer *et al*., 1994). The crystal structure of bovine β-transducin, which has seven WD repeats, revealed a circularized propeller-like structure (for a review, see Smith *et al*., 1999). Since Pfs2p has the same number of repeating WD units, it may form a similar structure. In this case, removal of any part of the WD-repeat portion would be expected to disrupt the propeller structure and consequently destroy the biological activity of the protein.

Indeed, the C-terminal non-WD-repeat portion of Pfs2p was found to be dispensable, whereas any deletion of parts of the WD-repeat region of the protein caused lethality. Thus, the inability of mutant forms lacking part of the WD-repeat region to support viability is consistent with a propeller-like structure of Pfs2p.

To analyse the function of Pfs2p, we isolated conditionally lethal *pfs2* mutant strains and tested their ability to cleave and polyadenylate synthetic pre-mRNA *in vitro*. The cleavage and polyadenylation defect of the *pfs2-1* mutant extract indicated the participation of Pfs2p in both processing steps. Moreover, it is striking that none of the *pfs2* mutants we isolated lost polyadenylation activity while keeping full cleavage activity. The *pfs2* mutant *in vitro* cleavage phenotype clearly differs from that of mutants in factors that are thought to be required for polyadenylation only, such as Fip1p and Pap1p (Patel and Butler, 1992; Minvielle-Sebastia *et al*., 1994; Preker *et al*., 1995).

Additional support for the involvement of Pfs2p in cleavage and polyadenylation came from the immunodepletion and immunoinactivation of 3′-end-processing activity with antibodies directed against Pfs2p. Taken together, these results show that Pfs2p plays an essential role in both steps of 3′-end processing.

Pfs2p is a subunit of the CF II–PF I complex

We have used a yeast strain expressing protein A-tagged Pfs2p to purify polypeptides that are associated with this protein. This resulted in the isolation of a complex that included Yhh1p/Cft1p, Ysh1p/Brr5p, Pap1p, Pfs2p, Fip1p and Yth1p, as shown by Western blotting. The electrophoretic mobility of the other proteins of the complex is consistent with the assumption that they are Ydh1p/Cft2p, Pta1p and Pfs1p. All these nine proteins were also found in the complex that had been purified as PF I–Pap1p (Preker *et al*., 1997). Furthermore, we have shown that the isolated complex has CF II–PF I activity.

To the best of our knowledge, this is the first example of a one-step purification of a biochemically active polyadenylation factor from the original organism. It is likely that the purification method we used here will prove to be applicable for the isolation of other biochemically active multicomponent complexes as well as for the study of complexes that contain mutant proteins (Senger *et al*., 1998).

In the protein A-mediated affinity purification, Pfs2p was depleted up to 80% from the extract (results not shown). Thus, it appears that Pfs2p exists predominantly as a component of the CF II–PF I complex in yeast extracts. This is in agreement with a primary function for Pfs2p in mRNA 3′-end formation. Interestingly, a mutant form of Pfs2p that was derived from Pfs2-2p appears to be less tightly associated with the CF II–PF I complex than is the wild-type protein. Furthermore, the *pfs2-2* mutant extract exhibits a strong reduction of cleavage and polyadenylation activity. This indicates that Pfs2p needs

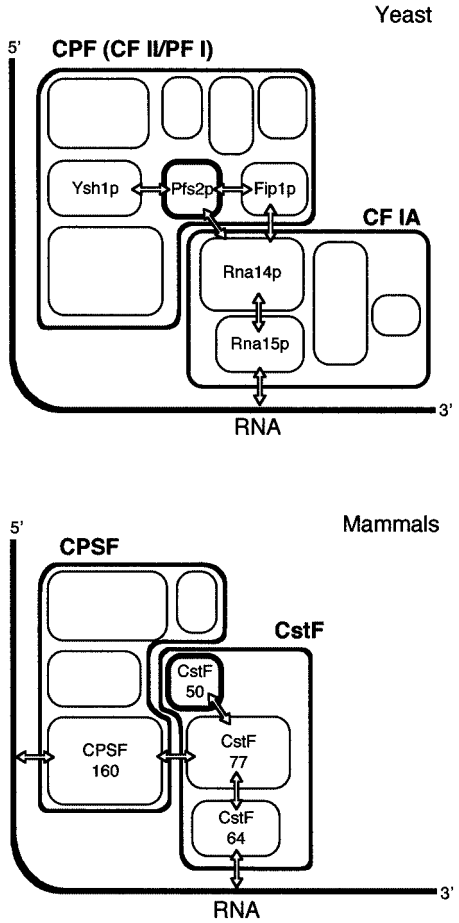

Fig. 7. Schematic representation of the mammalian and yeast 3′-end-processing complex. For simplicity, several processing factors are omitted and only a subset of all known protein–protein and protein–RNA interactions are indicated by double-headed arrows. CPF includes PF I and CF II. The WD-repeat proteins Pfs2p and CstF-50 are highlighted by thick borders.

to be associated with the complex to fulfil its function in 3′-end processing.

The WD-repeat protein Pfs2p bridges two 3′-end-processing factors

Information about the direct interactions of Pfs2p with other 3′-end-processing factor subunits was obtained from pull-down experiments with GST–Pfs2p and *in vitro* translated proteins. This has revealed interactions of Pfs2p with Ysh1p, Fip1p and Rna14p. Therefore, Pfs2p interacts with components of at least two different processing factors, CF II–PF I and CF IA (see also Figure 7).

Our results on Pfs2p are consistent with the proposed role of WD-repeat proteins as an assembly platform for multiprotein complexes: its association with CF II and the CF II–PF I–Pap1p complex as well as the interaction with a subunit of CF IA make Pfs2p a good candidate for a protein around which the 3′-end-processing complex can assemble.

WD-repeat proteins interact with HAT-repeat proteins in the 3′-end-processing complex of mammals and yeast

Like yeast Pfs2p, the mammalian CstF 50 kDa subunit is a WD-repeat protein that participates in 3′-end formation (Takagaki and Manley, 1992). Although sequence alignments of Pfs2p and CstF-50 do not support the idea that they are homologues (20.5% identity), structural similarity is conceivable and common features exist in the interactions of the two WD-repeat proteins. In addition to the 50 kDa protein, CstF contains polypeptides of 77 and 64 kDa (Takagaki *et al*., 1990). In the mammalian 3′-end-formation complex, CstF-50 binds to CstF-77 (Takagaki and Manley, 1994). In yeast, Pfs2p interacts with Rna14p, which is the yeast homologue of CstF-77 (see Figure 7). Both Rna14p and CstF-77 contain so-called HAT repeats. The HAT motif (for half a TPR) consists of repeats that are predicted to form amphipathic α-helices (Preker and Keller, 1998) and is similar to the TPR motif that forms repeats of 34 amino acids (Lamb *et al*., 1995). Recent determination of the crystal structure of a member of the TPR protein family confirmed the predicted α-helical structure (Das *et al*., 1998). There are some cases in which a WD-repeat protein was found in a complex together with a TPR protein (Williams *et al*., 1991; Fabrizio *et al*., 1994; Lim *et al*., 1998). This has led to the proposal that members of the WD family can function together with members of the TPR family (van der Voorn and Ploegh, 1992). Because the HAT motif resembles the TPR motif, the interaction between Pfs2p and Rna14p is consistent with this idea.

The fact that the 3′-end-processing complex of both mammals and yeast includes a WD-repeat protein that interacts with a HAT-motif protein suggests that Pfs2p and CstF-50 fulfil similar tasks. If a common function of WD-repeat proteins in 3′-end formation is to bridge different processing factors, an interaction of CstF-50 with a processing factor different from CstF would be expected. Since the yeast CF II–PF I complex to which Pfs2p belongs includes homologues of all four mammalian CPSF subunits, it is tempting to speculate that CstF-50 may interact with a component of the cleavage and polyadenylation specificity factor. An interaction between these two factors has been demonstrated (see Figure 7) to occur between CstF-77 and CPSF-160 (Murthy and Manley, 1995). However, additional protein–protein contacts may be required to stabilize the interaction between CstF and CPSF. An involvement of CstF-50 would support the idea that an important function of WD-repeat proteins in 3′-end formation is the assembly and/or stabilization of the processing complex.

CF II and PF I form a cleavage and polyadenylation factor (CPF)

Employing a one-step affinity purification method, we showed that a multiprotein complex that carries CF II–PF I activity can be purified with protein A–Pfs2p from yeast extracts. A subset of the proteins of this complex has been co-purified with CF II activity in a different approach (Zhao *et al*., 1997), and the purified material was not reported to contain PF I activity. Thus, the CF II–PF I holoenzyme complex can be disrupted *in vitro*.

Originally, CF II was defined as a factor that acts together with CF I to accomplish the site-specific endonucleolytic cleavage but is not involved in the subsequent polyadenylation reaction. In contrast, PF I has been described as a polyadenylation factor that is not needed for cleavage (Chen and Moore, 1992). This may suggest

that CF II and PF I act independently without physical interaction. However, the results presented here demonstrate that CF II and PF I occur together in a stable complex. This observation strongly suggests that the two factors also interact during the processing reaction.

The interaction between CF II and PF I may be very important for 3′-end processing. Engagement of CF II in the cleavage reaction prior to PF I binding could lead to the accumulation of cleaved but still unpolyadenylated pre-mRNA. In contrast, binding of a pre-formed CF II–PF I complex would ensure more efficient coupling of cleavage and polyadenylation and thus help to increase the processivity of 3′-end formation.

We therefore propose that CF II and PF I enter the 3′-end-processing reaction together as a cleavage and polyadenylation factor (CPF) that should be considered as a functional unit. Thus, CPF includes proteins that are possibly involved in cleavage site selection (Zhao *et al*., 1997), and at least one protein (Fip1p) that interacts directly with poly(A) polymerase and is thought to hold the polymerase close to its RNA substrate during polyadenylation (Preker *et al*., 1995). Thus, CPF not only contains homologues of the subunits of mammalian CPSF, but also appears to be closely related to it with regard to function.

Materials and methods

Peptide sequencing and sequence analysis

Approximately 50 µg of the 53 kDa protein that co-purified with PF I–Pap1p activity (Preker *et al*., 1997) were used for peptide sequencing (TopLab, München, Germany).

Sequence analysis was performed with the University of Wisconsin Genetics Computer Group programs, version 8.1 (Devereux *et al*., 1984). The amino acid sequence of Pfs2p was compared with the EMBL nucleotide sequence database and the SwissProt protein database with the TFASTA program (Pearson and Lipman, 1988). Secondary structure prediction was done with the e-mail server at the EMBL (Rost and Sander, 1993, 1994).

Isolation of a genomic clone of PFS2

A 0.9 kb fragment of the coding region of *PFS2* was PCR amplified from genomic *S.cerevisiae* DNA with the following oligonucleotides: 5′-GGAATTCCATATGGACGGGCATAATCAG-3′ and 5′-GGTGCC-ATTCTAGTGTCA-3′. A random primer-labelled probe derived from the PCR fragment was used to screen a genomic yeast DNA plasmid library (vector: pRS413). The library was a gift from Horst Pick (Biozentrum, Basel). A total of 1.5×10^5 *Escherichia coli* colonies were screened on nitrocellulose filters according to standard procedures with minor modifications (Sambrook *et al*., 1989). A clone containing the complete *PFS2* gene was isolated (pRS413-PFS2). From this clone, a 2 kb *Hpa*I fragment that spanned the complete coding region of *PFS2* and included 250 bp upstream of the start ATG as well as 300 bp of the 3′-untranslated region was subcloned into the *Eco*RV site of the Bluescript KS vector (pBS). From the resulting plasmid (pBS-PFS2), the 2 kb insert was cloned into the *Pst*I–*Hin*dIII site of pFL36 and pFL38 (Bonneaud *et al*., 1991) to yield pFL36-PFS2 (*LEU2*, CEN) and pFL38-PFS2 (*URA3*, CEN), respectively.

Yeast strains, genetic methods and media

Most yeast media were prepared as described previously (Guthrie and Fink, 1991). When strains were tested for formamide sensitivity, 3% formamide (v/v) was added (Aguilera, 1994). Yeast cells were transformed using the lithium acetate method (Gietz *et al*., 1992). The *S.cerevisiae* strains used in this work are listed in Table I.

PCR-mediated gene replacements

The gene deletion mutant MO1 (see Table I) was generated by replacement of 98% of the *PFS2* coding region (amino acids 9–463) with a *Bgl*II *TRP1* cassette (846 nucleotides) in the diploid strain BMA41 according to the PCR-mediated method described previously (Baudin *et al*., 1993). The following primers were used for PCR amplification of the *TRP1* cassette: 5′-AGCTCAACATAGACACGTTATGGA-CGGGCATAATCAGAACCAAGATCTGAATTAATTCG-3′ and 5′-GTA-TCCTAAAATCTCGTCAAAGCGCACTTTTATATGCTTAAAAGATC-TGGGCAAGTGCA-3′. The correct integration of the *TRP1* marker cassette at the *PFS2* locus was verified on a genomic Southern blot.

Isolation of temperature–formamide-sensitive pfs2 mutants

The plasmid pFL38-PFS2 was used as template for error-prone PCR. The amplification of *PFS2* was done with the oligonucleotides 5′-GGGATGTGCTGCAAGGCG-3′ and 5′-TCATTAGGCACCCCA-GGC-3′, which anneal to flanking vector sequences in pFL38-PFS2. PCR was carried out with *Taq* DNA polymerase (Boehringer Mannheim) in the reaction buffer provided by the manufacturer. The reaction mixture contained 50 pmol of each primer, 200 µM dCTP, 200 µM dGTP, 200 µM dTTP and 25 µM dATP. The PCR products were co-transformed with linearized pFL36 (*LEU2*, CEN) into MO7 (see Table I). Overlapping stretches of PCR products and vector allowed homologous recombination *in vivo* (Muhlrad *et al*., 1992). LEU^+ transformants were forced to lose the *URA3*-marked plasmid pFL38-PFS2 by growth on medium containing 5-FOA (Boeke *et al*., 1987). Clones were selected that could grow at 24°C but not at 35°C in the presence of 3% formamide. From four transformants, the respective plasmid causing the temperature–formamide sensitivity was isolated and re-introduced into the *pfs2* null mutant background. This resulted in the isolation of the temperature–formamide-sensitive strains MO13, MO14, MO15 and MO17 (see Table I).

Recombinant proteins and purified processing factors

Pfs2p was expressed as a His_6 fusion protein and as a GST fusion protein. The recombinant proteins were expressed in *E.coli* BL21 (DE3) pLysS (Studier, 1991). Growth of the bacteria, induction of expression, homogenization of the cells, binding of proteins to nickel–nitrilotriacetic acid–agarose (Ni-NTA, Qiagen) or glutathione–Sepharose (Pharmacia), washing steps and elution of the bound material were done according to the manufacturers' instructions.

Purified CF IA and CF II as well as recombinant Nab4p/Hrp1p were gifts from Lionel Minvielle-Sebastia (Minvielle-Sebastia *et al*., 1997, 1998). Recombinant Pab1p was kindly provided by Alan B.Sachs (University of California, Berkeley).

Affinity purification of CF II–PF I–Pap1p

The CF II–PF I complex was affinity purified from an extract of a yeast strain that expresses a tagged version of Pfs2p. The tag included two IgG-binding domains of the *Staphylococcus aureus* protein A followed by the recognition sequence of the site-specific TEV protease (Senger *et al*., 1998). To generate an expression construct for the protein A–TEV–Pfs2p fusion protein, the coding part of *PFS2* was amplified by PCR and cloned into the *Nco*I–*Sma*I site of pRS315-LEU2-ProtA-TEV (Senger *et al*., 1998). The insert of the construct was sequenced and found to contain a single mutation that did not alter the coding capacity. The resulting plasmid was introduced in the *pfs2* null mutant background to yield MO20 (see Table I).

Batch absorption to IgG–agarose was done to isolate protein A–Pfs2p and associated proteins from an extract prepared from strain MO20. All incubation steps described below up to the proteolytic cleavage were done at 4°C with gentle mixing. A 30 µl aliquot of IgG–agarose (Sigma), 100 µl of the extract, 50 µl of 80% glycerol and 300 µl of buffer IPPD-150 [10 mM Tris–HCl pH 7.9, 150 mM NaCl, 0.1% NP-40, 0.5 mM dithiothreitol (DTT)] including 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.4 µg/ml leupeptin and 0.7 µg/ml pepstatin were mixed in a tube and incubated for 2 h. After centrifugation, the supernatant was removed and the matrix was washed four times for 20 min with 1 ml of IPPD-150 buffer. Alternatively, the matrix was washed with IPPD buffer containing 250 or 500 mM NaCl to increase stringency. After washing, the beads were incubated for 5 min with 1 ml of buffer T [50 mM Tris–HCl pH 8.0, 10% glycerol, 50 mM potassium acetate, 0.5 mM EDTA, 1 mM DTT, 0.02% NP-40, 0.4 µg/ml leupeptin]. For elution of the bound material, the beads were incubated with 50 µl of buffer T containing 6 U of recombinant TEV protease (Gibco-BRL) at 14°C for 90 min. The site-specific proteolytic cleavage released Pfs2p and associated proteins from the protein A tag and thereby from the IgG–agarose matrix. After centrifugation, the supernatant was collected in a fresh tube and the beads were washed with 100 µl of buffer T. This wash solution was then combined with the eluate.

Co-elution experiments were carried out accordingly with a protein A-tagged mutant version of Pfs2p. For this purpose, the coding sequence

of *pfs2-2* was amplified by PCR and cloned into the *Nco*I–*Sma*I site of pRS315-LEU2-ProtA-TEV. A mutation that caused a C-terminal extension of Pfs2-2p by eight amino acids was revealed by DNA sequencing. The resulting plasmid was introduced in the *pfs2* null mutant background.

In vitro 3′-end-processing assays

Yeast cultures were grown in 1 l of YPAD to an OD_{600} of 2–6. The harvested cells were frozen in liquid nitrogen and homogenized in a mortar as described elsewhere (Ansari and Schwer, 1995). The subsequent centrifugation steps, the ammonium sulfate precipitation and the dialysis were carried out essentially as described previously (Butler *et al*., 1990).

^{32}P-labelled CYC1 RNA and CYC1 pre-cleaved RNA were prepared as run-off transcripts and gel purified as described previously (Minvielle-Sebastia *et al*., 1994; Preker *et al*., 1995).

In vitro polyadenylation assays were carried out in the presence of 2 mM ATP and 1.8 mM magnesium acetate as described previously (Minvielle-Sebastia *et al*., 1994). When only cleavage was assayed, magnesium acetate was replaced by EDTA, resulting in inhibition of poly(A) polymerase activity.

When mutant extracts were used in the assay, the reactions were carried out at 32 instead of 30°C.

Immunoblots

For immunoblots, protein samples were separated on either 10 or 12% SDS–polyacrylamide gels (Laemmli, 1970). After transfer of proteins to nitrocellulose membranes, the blots were processed according to standard procedures. Peroxidase-conjugated swine anti-rabbit immunoglobulins (DAKO) served as secondary antibody. Chemiluminescence (ECL kit, Amersham) was used for detection.

Antibodies and immunodepletion

Polyclonal anti-Pfs2 antiserum was elicited by immunizing a rabbit with 100 µg of recombinant histidine-tagged Pfs2 protein (seven injections).

Yeast wild-type extract (VDH2) was immunodepleted twice with either protein A–Sepharose-bound anti-Pfs2p antibodies or pre-immune serum as described elsewhere (Barabino *et al*., 1997).

In vitro protein-binding assays

For the *in vitro* expression of Ysh1/Brr5, Yth1, Fip1, Pap1, Rna15, Rna14, Clp1 and Pcf11 proteins, plasmid DNAs were transcribed and translated with the TNT coupled transcription–translation system (Promega) in a total volume of 50 µl according to the manufacturer's instructions. Purified GST or GST–Pfs2p fusion protein (0.2–0.5 µg) was incubated with the *in vitro* translated, ^{35}S-labelled proteins (2–5 µl of the *in vitro* translation reaction corresponding to ~1–2 fmol) in a total volume of 25 µl for 1 h at 25°C. A 500 µl aliquot of binding buffer [phosphate-buffered saline (PBS), 0.05% NP-40, 0.5 mg/ml bovine serum albumin (BSA)] was added together with 15 µl of glutathione–Sepharose. After incubation for 1.5 h at 4°C, the resin was pelleted and washed three times with 1 ml of IPP 250 (20 mM Tris–HCl pH 7.9, 250 mM NaCl, 0.05% NP-40). Proteins were eluted in 20 µl of SDS–PAGE sample buffer and resolved by 10% SDS–PAGE. The signal was enhanced by treatment with Amplify solution (Amersham).

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References

Aguilera,A. (1994) Formamide sensitivity: a novel conditional phenotype in yeast. *Genetics*, **136**, 87–91.

Amrani,N., Minet,M., Le Gouar,M., Lacroute,F. and Wyers,F. (1997a) Yeast Pab1 interacts with Rna15 and participates in the control of the poly(A) tail length *in vitro*. *Mol. Cell. Biol.*, **17**, 3694–3701.

Amrani,N., Minet,M., Wyers,F., Dufour,M.-E., Aggenbeck,L.P. and Lacroute,F. (1997b) PCF11 encodes a third protein component of the yeast cleavage and polyadenylation factor I. *Mol. Cell. Biol.*, **17**, 1102–1109.

Ansari,A. and Schwer,B. (1995) SLU7 and a novel activity, SSF1, act during the PRP16-dependent step of yeast pre-mRNA splicing. *EMBO J.*, **14**, 4001–4009.

Barabino,S.M.L., Hübner,W., Jenny,A., Minvielle-Sebastia,L. and Keller,W. (1997) The 30 kDa subunit of mammalian cleavage and polyadenylation specificity factor and its yeast homologue are RNA-binding zinc finger proteins. *Genes Dev.*, **11**, 1703–1716.

Baudin,A., Ozier-Kalogeropoulos,O., Denouel,A., Lacroute,F. and Cullin,C. (1993) A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. *Nucleic Acids Res.*, **21**, 3329–3330.

Baudin-Baillieu,A., Tollervey,D., Cullin,C. and Lacroute,F. (1997) Functional analysis of Rrp7p, an essential yeast protein involved in pre-rRNA processing and ribosome assembly. *Mol. Cell. Biol.*, **17**, 5023–5032.

Bienroth,S., Wahle,E., Suter-Crazzolara,C. and Keller,W. (1991) Purification of the cleavage and polyadenylation factor involved in the 3′-processing of messenger RNA precursors. *J. Biol. Chem.*, **266**, 19768–19776.

Boeke,J.D., Trueheart,J., Natsoulis,G. and Fink,G.R. (1987) 5-Fluoroorotic acid is a selective agent in yeast molecular genetics. *Methods Enzymol.*, **154**, 164–175.

Bonneaud,N., Ozier-Kalogeropoulos,O., Li,G., Labouesse,M., Minvielle-Sebastia,L. and Lacroute,F. (1991) A family of low and high copy replicative, integrative and single stranded *S.cerevisiae*/*E.coli* shuttle vectors. *Yeast*, **7**, 609–615.

Butler,J.S., Sadhale,P.P. and Platt,T. (1990) RNA processing *in vitro* produces mature 3′-ends of a variety of *Saccharomyces cerevisiae* mRNAs. *Mol. Cell. Biol.*, **10**, 2599–2605.

Carrington,J.C. and Dougherty,W.G. (1988) A viral cleavage site cassette: identification of amino acid sequences required for tobacco etch virus polyprotein processing. *Proc. Natl Acad. Sci. USA*, **85**, 3391–3395.

Chanfreau,G., Noble,S.M. and Guthrie,C. (1996) Essential yeast protein with unexpected similarity to subunits of mammalian cleavage and polyadenylation factor (CPSF). *Science*, **274**, 1511–1514.

Chen,J. and Moore,C. (1992) Separation of factors required for cleavage and polyadenylation of yeast pre-mRNA. *Mol. Cell. Biol.*, **12**, 3470–3481.

Colgan,D.F. and Manley,J.L. (1997) Mechanism and regulation of mRNA polyadenylation. *Genes Dev.*, **11**, 2755–2766.

Das,A.K., Cohen,P.W. and Barford,D. (1998) The structure of the tetratricopeptide repeats of protein phosphatase 5: implications for TPR-mediated protein–protein interactions. *EMBO J.*, **17**, 1192–1199.

Devereux,J., Haeberli,P. and Smithies,O. (1984) A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.*, **12**, 387–395.

Fabrizio,P., Esser,S., Kastner,B. and Lührmann,R. (1994) Isolation of *S.cerevisiae* snRNPs: comparison of U1 and U4/U6·U5 to their human counterparts. *Science*, **264**, 261–265.

Gietz,D., Jean,A.S., Woods,R.A. and Schiestl,R.H. (1992) Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res.*, **20**, 1425.

Guthrie,C. and Fink,G.R., (1991) *Guide to Yeast Genetics and Molecular Biology*. Academic Press, San Diego, CA.

Jenny,A., Minvielle-Sebastia,L., Preker,P.J. and Keller,W. (1996) Sequence similarity between the 73-kilodalton protein of mammalian CPSF and a subunit of yeast polyadenylation factor I. *Science*, **274**, 1514–1517.

Keller,W. and Minvielle-Sebastia,L. (1997) A comparison of mammalian and yeast pre-mRNA 3′-end processing. *Curr. Opin. Cell Biol.*, **9**, 329–336.

Keller,W., Bienroth,S., Lang,K.M. and Christofori,G. (1991) Cleavage and polyadenylation factor CPF specifically interacts with the pre-mRNA 3′ processing signal AAUAAA. *EMBO J.*, **10**, 4241–4249.

Kessler,M.M., Zhao,J. and Moore,C.L. (1996) Purification of the *Saccharomyces cerevisiae* cleavage/polyadenylation factor I. *J. Biol. Chem.*, **271**, 27167–27175.

Kessler,M.M., Henry,M.F., Shen,E., Zhao,J., Gross,S., Silver,P.A. and Moore,C.L. (1997) Hrp1, a sequence-specific RNA-binding protein that shuttles between the nucleus and the cytoplasm, is required for mRNA 3′-end formation in yeast. *Genes Dev.*, **11**, 2545–2556.

Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

Lamb,J.R., Tugendreich,S. and Hieter,P. (1995) Tetratricopeptide repeat interactions: to TPR or not to TPR? *Trends Biochem. Sci*., **20**, 257–259.

Lim,H.H., Goh,P.Y. and Surana,U. (1998) Cdc20 is essential for the cyclosome-mediated proteolysis of both Pds1 and Clb2 during M phase in budding yeast. *Curr. Biol.*, **8**, 231–234.

Lingner,J., Radtke,I., Wahle,E. and Keller,W. (1991) Purification and characterization of poly(A) polymerase from *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **266**, 8741–8746.

Maftahi,M., Gaillardin,C. and Nicaud,J.M. (1998) Generation of *Saccharomyces cerevisiae* deletants and basic phenotypic analysis of eight novel genes from the left arm of chromosome XIV. *Yeast*, **14**, 271–280.

Minvielle-Sebastia,L., Preker,P.J. and Keller,W. (1994) RNA14 and RNA15 proteins as components of a yeast pre-mRNA 3′-end processing factor. *Science*, **266**, 1702–1705.

Minvielle-Sebastia,L., Preker,P.J., Wiederkehr,T., Strahm,Y. and Keller,W. (1997) The major yeast poly(A)-binding protein is associated with cleavage factor IA and functions in pre-messenger RNA 3′-end formation. *Proc. Natl Acad. Sci. USA*, **94**, 7897–7902.

Minvielle-Sebastia,L., Beyer,K., Krecic,A.M., Hector,R.E., Swanson, M.S. and Keller,W. (1998) Control of cleavage site selection during mRNA 3′-end formation by a yeast hnRNP. *EMBO J.*, **17**, 7454–7468.

Muhlrad,D., Hunter,R. and Parker,R. (1992) A rapid method for localized mutagenesis of yeast genes. *Yeast*, **8**, 79–82.

Murthy,K.G.K. and Manley,J.L. (1995) The 160-kD subunit of human cleavage–polyadenylation factor coordinates pre-mRNA 3′-end formation. *Genes Dev.*, **9**, 2672–2683.

Neer,E.J., Schmidt,C.J., Nambudripad,R. and Smith,T.F. (1994) The ancient regulatory-protein family of WD-repeat proteins. *Nature*, **371**, 297–300.

Patel,D. and Butler,J.S. (1992) Conditional defect in mRNA 3′-end processing caused by a mutation in the gene for poly(A) polymerase. *Mol. Cell. Biol.*, **12**, 3297–3304.

Pearson,W.R. and Lipman,D.J. (1988) Improved tools for biological sequence comparison. *Proc. Natl Acad. Sci. USA*, **85**, 2444–2448.

Preker,P.J. and Keller,W. (1998) The HAT helix, a repetitive motif implicated in RNA processing. *Trends Biochem. Sci*., **23**, 15–16.

Preker,P.J., Lingner,J., Minvielle-Sebastia,L. and Keller,W. (1995) The *FIP1* gene encodes a component of a yeast pre-mRNA polyadenylation factor that directly interacts with poly(A) polymerase. *Cell*, **81**, 379–389.

Preker,P.J., Ohnacker,M., Minvielle-Sebastia,L. and Keller,W. (1997) A multisubunit 3′-end processing factor from yeast containing poly(A) polymerase and homologues of the subunits of mammalian cleavage and polyadenylation specificity factor. *EMBO J.*, **16**, 4727–4737.

Rost,B. and Sander,C. (1993) Prediction of protein secondary structure at better than 70% accuracy. *J. Mol. Biol.*, **232**, 584–599.

Rost,B. and Sander,C. (1994) Combining evolutionary information and neural networks to predict protein secondary structure. *Proteins*, **19**, 55–72.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Senger,B., Simos,G., Bischoff,F.R., Podtelejnikov,A., Mann,M. and Hurt,E. (1998) Mtr10p functions as a nuclear import receptor for the mRNA-binding protein Npl3p. *EMBO J.*, **17**, 2196–2207.

Smith,T.F., Gaitatzes,C., Saxena,K. and Neer,E.J. (1999) The WD repeat: a common architecture for diverse functions. *Trends Biochem. Sci*., **24**, 181–185.

Studier,F.W. (1991) Use of bacteriophage T7 lysozyme to improve an inducible T7 expression system. *J. Mol. Biol.*, **219**, 37–44.

Stumpf,G. and Domdey,H. (1996) Dependence of yeast pre-mRNA 3′-end processing on CFT1: a sequence homolog of the mammalian AAUAAA binding factor. *Science*, **274**, 1517–1520.

Takagaki,Y. and Manley,J.L. (1992) A human polyadenylation factor is a G protein β-subunit homologue. *J. Biol. Chem.*, **267**, 23471–23474.

Takagaki,Y. and Manley,J.L. (1994) A polyadenylation factor subunit is the human homologue of the *Drosophila* suppressor of forked protein. *Nature*, **372**, 471–474.

Takagaki,Y., Manley,J.L., MacDonald,C.C., Wilusz,J. and Shenk,T. (1990) A multisubunit factor, CstF, is required for polyadenylation of mammalian pre-mRNAs. *Genes Dev.*, **4**, 2112–2120.

van der Voorn,L. and Ploegh,H.L. (1992) The WD-40 repeat. *FEBS Lett*., **307**, 131–134.

Williams,F.E., Varanasi,U. and Trumbly,R.J. (1991) The CYC8 and TUP1 proteins involved in glucose repression in *Saccharomyces cerevisiae* are associated in a protein complex. *Mol. Cell. Biol.*, **11**, 3307–3316.

Zhao,J., Kessler,M.M. and Moore,C.L. (1997) Cleavage factor II of *Saccharomyces cerevisiae* contains homologues to subunits of the mammalian cleavage/polyadenylation specificity factor and exhibits sequence-specific, ATP-dependent interaction with precursor RNA. *J. Biol. Chem.*, **272**, 10831–10838.

Zhao,J., Hyman,L. and Moore,C. (1999) Formation of mRNA 3′-ends in eukaryotes: mechanism, regulation and interrelationships with other steps in mRNA synthesis. *Microbiol. Mol. Biol. Rev.*, **63**, 405–445.

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