MOLECULAR AND CELLULAR BIOLOGY, Mar. 1997, p. 1102–1109 Vol. 17, No. 3
0270-7306/97/$04.00+0
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PCF11 Encodes a Third Protein Component of Yeast Cleavage and Polyadenylation Factor I

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Received 9 September 1996/Returned for modification 11 October 1996/Accepted 3 December 1996

Cleavage and polyadenylation factor I (CF I) is one of four factors required in vitro for yeast pre-mRNA 3′-end processing. Two protein components of this factor, encoded by genes *RNA14* and *RNA15*, have already been identified. We describe here another gene, *PCF11* (for protein 1 of CF I), that genetically interacts with *RNA14* and *RNA15* and which presumably codes for a third protein component of CF I. This gene was isolated in a two-hybrid screening designed to identify proteins interacting with Rna14 and Rna15. *PCF11* is an essential gene encoding for a protein of 626 amino acids having an apparent molecular mass of 70 kDa. Thermosensitive mutations in *PCF11* are synergistically lethal with thermosensitive alleles of *RNA14* and *RNA15*. The *Pcf11-2* thermosensitive strain shows a shortening of the poly(A) tails and a strong decrease in the steady-state level of actin transcripts after a shift to the nonpermissive temperature as do the thermosensitive alleles of *RNA14* and *RNA15*. Extracts from the *pcf11-1* and *pcf11-2* thermosensitive strains and the wild-type strain, when Pcf11 is neutralized by specific antibodies, are deficient in cleavage and polyadenylation. Moreover, fractions obtained by anion-exchange chromatography of extracts from the wild-type strain contain both Pcf11 and Rna15 in the same fractions, as shown by immunoblotting with a Pcf11-specific antibody.

In eucaryotic cells, most newly synthesized mRNA molecules have a poly(A) tail added onto their 3′ ends as an early step in the maturation of the primary transcript. This process requires specific endonucleolytic cleavage of a precursor mRNA followed by polymerization of adenosine residues to a tail length ranging from about 75 residues in the yeast *Saccharomyces cerevisiae* to nearly 300 residues in humans (11, 43). The poly(A) tail is an important component in determining mRNA stability (6) and efficient nuclear-cytoplasmic transport (26). Recent work has shown the importance of poly(A) tails in the initiation of translation by the cytoplasmic poly(A)-binding protein 1 (Pab1) (18, 21, 34); for a review, see reference (16). Shortening of the poly(A) tail by the cytoplasmic poly(A) nuclease probably plays an important role in the control of gene expression. This process probably results in a decrease in translation and a stimulation of degradation (8). In yeast, a poly(A)-specific RNase was identified by its requirement for poly(A)-binding protein stimulated poly(A) nuclease activity (35, 36). In mammalian cells, a poly(A)-specific RNase has been partially purified (2), but it does not appear to require any RNA-binding protein for its function (3).

Although cleavage and polyadenylation are normally tightly coupled in vivo, polyadenylation can be uncoupled from cleavage in vitro by the use of precleaved RNA substrates that end at their natural polyadenylation sites (10, 45). In addition to an endonuclease and a poly(A) polymerase, the biochemistry of mRNA polyadenylation appears to require several other enzymatic activities which enhance the specificity and the rate of each step (12–14, 25). The mechanisms and the proteins involved in these process are beginning to be elucidated in mammalian cells as well as in yeast (22, 42). The yeast *RNA14* and *RNA15* gene products, first identified through their involvement in polyadenylation (7, 28), have recently been shown to have a direct role in the endonucleolytic cleavage of the primary transcript and to be a component of yeast cleavage and polyadenylation factor I (CF I) (27). To discover new components of the polyadenylation complex, we have used the double-hybrid technique (17) to screen for proteins that interact with the Rna14 and the Rna15 proteins. We report here the identification of a new gene which codes for a component of CF I similarly to the *RNA14* and *RNA15* genes.

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MATERIALS AND METHODS

Strains, media, and genetic techniques. Yeast strains (Table 1) were maintained on YPD (1% yeast extract, 2% peptone, 2% glucose) or on a selective YNB medium (0.67% yeast nitrogen base without amino acids, 2% glucose) supplemented with the appropriate nutritional ingredients (38). *Escherichia coli* JM 103 *endA1 thi-1 hsdR supE sbcBC strA* Δ*(lac-pro)* [F′ *traD36 proAB lacI*^{q} *ZDM15.]* and BL21 [F$^-$ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)] were grown on Luria-Bertani medium supplemented with ampicillin (50 μg/ml) or carbenicillin (100 μg/ml). All temperature-sensitive (Ts) mutants were grown at 24°C (permissive temperature) to the mid-log phase and shifted to 37°C for studies at the nonpermissive temperature. Yeast cells were transformed by treatment with lithium acetate and polyethylene glycol (19). The sporulation and analysis of tetrads were done as described previously (29).

Isolation of Rna14- and Rna15-interacting protein in the GAL4 two-hybrid system. Plasmids pAS2Δ-*RNA14* and pGBT-*RNA15* (kind gift of A. Petitjean) were constructed by fusing the corresponding open reading frames (ORFs) with the *GAL4* DNA-binding domain (GBD) encoded by pAS2Δ (kind gift of M. Frommont) and pGBT9 (4), respectively (Table 2). pGBT9 was chosen instead of pAS2Δ for the *RNA15* fusion because the pAS2Δ-*RNA15* fusion activates reporter transcription on its own. pAS2Δ is a pAS2 plasmid (kind gift of S. Elledge) from which *CYH2* and the influenza virus (Flu) epitope sequences have been deleted. *RNA14* was amplified by PCR using oligonucleotides 2592 (5′-G CAAGATCTCCAGCTCTACGACTCCTG-3′) and 5145 (5′-CGCGGATCCG CGTTAACCTGACTTGGTGCTCTC-3′) containing, respectively, *Bgl*II and *Bam*HI sites. The PCR product was cloned into the *Bam*HI site of pAS2Δ. Similarly, *RNA15* was PCR amplified by using oligonucleotides 2594 (5′-GCAA GATCTTTATGAATAGGCAGAGCGGTG-3′) and 2596 (5′-CTCAGATCTT CAAAATGCACCAAATTCTCC-3′) containing *Bgl*II sites. The PCR product was cloned into the *Bam*HI site of pGBT9. The expression of the fusion proteins was verified by Western blotting.

To select for proteins interacting with Rna15, strain J 693 was transformed first with plasmid pGBT-*RNA15* and then by the FRYL library (kind gift of M. Frommont) or by a similar library constructed in our laboratory. Both libraries consist of fusions of yeast genomic fragments (strain YM 955) and the *GAL4*

TABLE 1. Yeast strains used

Strain	Genotype or description	Source
W303-1B[a]	*MAT***a** *ura3-1 trp1-1 ade2-1 leu2-3,112 his3-11,15*	R. Rothstein
NA50[a]	*MAT***a**/*MATa ura3-1/ura3-1 trp1Δ/trp1Δ ade2-1/ade2-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 PCF11/pcf11-Δ::TRP1*	This study
NA52[a]	*MAT***a** *ura3-1 trp1Δ ade2-1 leu2-3,112 his3-11,15 pcf11-Δ::TRP1*/pFL38-*PCF11*	This study
NA53[a]	*MATα ura3-1 trp1Δ ade2-1 leu2-3,112 his3-11,15 pcf11-Δ::TRP1*/pFL38-*PCF11*	This study
NA54[a]	*MAT***a** *ura3-1 trp1Δ ade2-1 leu2-3,112 his3-11,15 pcf11-Δ::TRP1*/pYeF1-*PCF11*	This study
NA57[a]	*MAT***a** *ura3-1 trp1Δ, ade2-1 leu2-3,112 his3-11,15 pcf11-Δ::TRP1*/pFL36-*pcf11-1*	This study
NA64[a]	*MAT***a** *ura3-1 trp1Δ ade2-1 leu2-3,112 his3-11,15 pcf11-Δ::TRP1*/pFL36-*pcf11-2*	This study
NA65[a]	*MAT***a** *ura3-1 trp1Δ ade2-1 leu2-3,112 his3-11,15 pcf11-2*	This study
NA66[a]	*MAT***a** *ura3-1 tp1Δ ade2-1 leu2-3,112 his3-11,15 pcf11-3*	This study
NA67[a]	*MAT***a** *ura3-1 trp1Δ ade2-1 leu2-3,112 his3-11,15 pcf11-9*	This study
rna15-2 W[a]	*MATα ura3-1 trp1-1 ade2-1 leu2-3,112 his3-11,15 rna15-2*	This study
rna14-1 W[a]	*MATα ura3-1 trp1-1 ade2-1 leu2-3,112 his3-11,15 rna14-1*	This study
YM 955	*MAT***a** *ura3-52 ade2-101 lys2-801 leu2-3,112 tyr1-501 his3Δ trp1Δ gal4Δ gal80Δ*	M. Johnston
Y 190	*MAT***a** *trp1-901 ade2-101 leu2-3,112 his3-11,15 URA3::UAS GAL1-lacZ gal4Δ gal80Δ LYS2::UASGAL1-HIS3 cych2*[r]	R. Rothstein
J 693	*MATα trp1-1 ade2-1 leu2-3,112 his3-11,15 URA3::UAS GAL1-lacZ gal4Δ gal80Δ LYS2::UASGAL1-HIS3 cych2*[r]	R. Rothstein
FY 1679	*MAT***a**/*MATα ura3-52/ura3-52 TRP1/trp1Δ63 LEU2/leu2Δ1 HIS3/his3Δ200*	F. Winston
BMA64-2N[a]	*MAT***a**/*MATα ura3-1/ura3-1 trp1Δ/trp1Δ ade2-1/ade2-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15*	A. Baudin

[a] Isogenic to W303-1B.

activating domain (GAD) of plasmid pACTII (kind gift of S. Elledge). The same protocol was followed for Rna14 except that the bait plasmid was pAS2Δ-*RNA14* and the recipient strain was Y 190. His$^+$ clones were selected on a medium containing 50 mM 3-aminotriazole and scored for *lacZ* expression. After recovery of the pACTII clones, their interaction with the cognate bait was verified by a new transformation. The sequence adjacent to the fusion site of each plasmid was determined to identify the corresponding yeast insert.

Plasmids pACT-*RNA14* and pACT-*RNA15* were obtained by switching the respective ORFs from the bait plasmids to plasmid pACTII.

Cloning and disruption of the *PCF11* gene. A 3,024-bp genomic fragment containing the *PCF11* gene was amplified by PCR, starting 458 bp upstream of the initiation ATG codon and ending 686 bp downstream of the stop codon. Genomic DNA was purified from strain FY 1679 (44). Amplification by PCR was carried out with oligonucleotides 6880 (5′-AGCTATACAGAACTTTCTCAGTC-3′) and 6882 (5′-CTAGTGTTGACGTTTTAGGAACAC-3′). The PCR product was cloned into plasmid pTA (TA cloning kit; Invitrogen) to generate plasmid pTA-*PCF11*. The *PCF11* insert was subcloned by directional cloning between the *Bam*HI and *Xba*I sites of pFL38 to generate the *URA3 PCF11* plasmid pNA39. Similarly, a directional *Sac*I-*Xba*I cloning into pFL36 and into pFL36SX (a pFL36 derivative in which the *Sph*I and *Xho*I sites have been eliminated by digestion and blunt-end ligation) generated the *LEU2 PCF11* plasmids pNA43 and pNA46, respectively.

One chromosomal copy of the *PCF11* gene was replaced with a complete *pcf11-Δ::TRP1* deletion by the one-step gene disruption method (5) in yeast strain BMA64 to create the heterozygous diploid strain NA50 (Table 1). The DNA fragment used for transformation contains the *TRP1* gene flanked by short sequences starting 28 bp upstream and ending 165 bp downstream of the *PCF11* initiation and stop codons, respectively. This DNA fragment was generated in a one-step PCR amplification using oligonucleotides 6879 (5′-GGTCTATTTGTAATACTCTCTTTTCATTATGGATCACGACACAGAAGTTATAGTCAAGGGGCCAAGAGGGAGGGC-3′) and 6881 (5′-GGGCTTTTGGCTACATATGTAAAATTACATATAATATAATGGACACATAAGCCTTAAATAAATACTACTC-3′). The sequences allowing *TRP1* amplification are underlined. Trp$^+$ transformants were checked by Southern blotting for integration into *PCF11*. Diploid strain NA50 bearing the deletion of *PCF11* was transformed with pNA39 (*CEN URA3 PCF11*) and was further sporulated and dissected to obtain the isogenic haploid strains NA52 and NA53 (Table 1), from the same ascus. These strains are deleted for genomic *PCF11* and complemented by the *URA3 PCF11* plasmid.

Generation of thermosensitive alleles. *PCF11* was mutagenized by the PCR method of Muhlrad et al. (30). The *PCF11* ORF was amplified under mutagenic PCR conditions, using oligonucleotides 6886 and 6943 (see below). pNA46 (*CEN LEU2 PCF11*) was digested with *Xho*I and *Sph*I (in positions 66 and 1527, respectively, of the ORF) to remove the major part of the *PCF11* gene. This gapped plasmid and the mutagenized PCR product were cotransformed directly into yeast strain NA52 (*pcf11-Δ::TRP1/CEN URA3 PCF11*), and Leu$^+$ colonies were selected. The endogenous *URA3 PCF11* plasmid was chased in the presence of 5-fluoro-orotic acid (5-FOA) (9), and the resulting colonies were tested for thermosensitivity.

DNA and RNA procedures. Total yeast DNA and RNA were extracted as described by Sherman et al. (38). For DNA and RNA blotting, the techniques used were as described by Sambrook et al. (37). The ECL (enhanced chemiluminescence) nucleic acid labeling and detection system (Amersham Corporation) was used to probe the Southern blots. For the Northern blot, the probes were radiolabeled with [^{32}P]dCTP at 3,000 Ci/mmol (ICN). For study of the poly(A) tail, the RNA 3′ end was labeled as described previously (1). Cloned

TABLE 2. Plasmid constructions

Plasmid	Construction
pAS2Δ-*RNA14*	In-frame cloning of the PCR-amplified *RNA14* ORF into the GBD of pAS2Δ, using the 5′-end oligonuclotide 2592 and the 3′-end oligonucleotide 5145, containing *Bgl*II and *Bam*HI restriction sites, respectively
pGBT-*RNA15*	In-frame cloning of the PCR-amplified *RNA15* ORF into the GBD of pGBT9, using the 5′-end oligonucleotide 2594 and the 3′-end oligonucleotide 2596 containing *Bgl*II restriction sites
pTA-*PCF11*	PCR-amplified *PCF11* (3,024 bp) cloned into pTA (Invitrogen), using the 5′-end oligonucleotide 6880 and the 3′-end oligonucleotide 6882
pNA39	A PCR genomic fragment containing the 3,024-bp full-length *PCF11* gene insert from pTA-*PCF11* was cloned into the *Bam*HI and *Xba*I cloning sites of pFL38, a *URA3* yeast *CEN ARS*-based plasmid
pNA42	By using the 5′-end oligonucleotide 6990 and the 3′-end oligonucleotide 6991, containing *Not*I and *Bsu*36I restriction sites, respectively, the *PCF11* ORF was PCR amplified and cloned in phase with Flu epitope in pYeF1, a *URA3* plasmid
pNA43	A PCR genomic fragment containing the 3,024-bp full-length *PCF11* gene was cloned in the *Sac*I-*Xba*I cloning site of pFL36, a *LEU2* yeast *CEN ARS*-based plasmid
pFL36SX	*Sph*I and *Xho*I restriction sites were removed from the polylinker of pFL36, a *URA3* yeast *CEN ARS*-based plasmid
pNA46	A PCR genomic fragment containing the 3,024-bp full-length *PCF11* gene was cloned in the *Sac*I-*Xba*I cloning site of pFL36SX, a *LEU2* yeast *CEN ARS*-based plasmid
pNA47	By using the 5′-end oligonucleotide 6886 and the 3′-end oligonucleotide 6943, containing *Nco*I and *Bam*HI restriction sites, respectively, the *PCF11* ORF was PCR amplified and cloned in phase with polyhistidine in pET-22.

PCR DNAs were sequenced by the dideoxy method, using a Sequenase kit (U.S. Biochemical), and the sequence reaction products were run on 6% polyacrylamide gels.

In vitro 3′-end processing assay. The excised and eluted *CYC1* precursor was used for an in vitro cleavage reaction as previously described (27). For the in vitro polyadenylation reaction, synthetic radiolabeled *CYC1* pre-mRNA was incubated in wild-type (W303-1B) extract to obtain the precleaved precursor, after phenol-chloroform-isoamyl alcohol (25:24:1) purification and ethanol precipitation. The yeast extracts were prepared either as described by Lin et al. (24) or according to a modified protocol described by Chen and Moore (12). All buffers included the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 0.4 μg of leupeptin per ml, and 1.4 μg of pepstatin per ml. A standard in vitro processing reaction was carried out at 23 or 35°C as needed in a 25-μl reaction volume containing 2 μl of extract. For separation of cleavage factors from poly(A) polymerase activity, chromatography on a 1-ml Mono Q ion-exchange column (5 by 50 mm; Pharmacia) was performed essentially as described previously (12). For separation of CF I and CF II, fractions containing cleavage activity obtained from two Mono Q separations were pooled, dialyzed, and rechromatographed on the same column with a shallower salt gradient as previously described (12).

Protein procedures and antibody production. For epitope-tagged Pcf11, we used the technique developed by Cullin and Minvielle-Sebastia (15). The *PCF11* ORF was amplified by PCR with oligonucleotides 6990 (5′-GGGGCGGCCGCCGATCACGACACAGAAG-3′) and 6991 (5′-GGGCCTTAGGGTTATTTTGTGACCAATTTC-3′), which introduce *Not*I and *Bsu*36I restriction sites at the 5′ and 3′ ends of the gene, respectively. The resulting cassette was cloned into the same restriction sites of pYeF1 vector, to generate the plasmid pNA42. This cloning gives an in-frame fusion of the *PCF11* gene with the sequence encoding the Flu epitope (YPYDVPDYA), directing the expression of a Pcf11 hybrid protein tagged at the N terminus. Monoclonal antibody (MAb) 12CA5, which recognizes the Flu epitope sequence, was used at a dilution of 1/50,000, and proteins were revealed with an anti-mouse antibody coupled to horseradish peroxidase (Amersham). Labeled bands were visualized following processing with the Amersham ECL detection kit.

Heterologous expression of Pcf11 in *E. coli*, to be used for the production of antibodies, was obtained by using plasmid pNA47, which was constructed as follows. Primers 6886 (5′-CATGCCATGGATCACGACACAGAAGTTATAG-3′) and 6943 (5′-CCGGATCCCGTTTTGTGACCAATTTCTTTAAGTC-3′), which introduce *Nco*I and *Bam*HI restriction sites at the 5′ and 3′ ends of the gene, respectively, were used in PCR amplification to generate the *PCF11* ORF. The resulting cassette was cloned into the *Nco*I and *Bam*HI restriction sites of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible His_6-tagged vector pET-22b (Novagen Corp.) in an orientation such that the *PCF11* ORF was expressed as a translational fusion to polyhistidine. *E. coli* BL21(DE3) cells were transformed with pNA47, and protein expression was induced with IPTG. The Pcf11 fusion protein, which contains six consecutive histidine residues encoded by the vector sequence following the 626 amino acids (aa) of Pcf11, was purified on a nickel-agarose column as specified by the supplier of the QIAexpressionist kit (Qiagen). A total of 3 mg of the purified Pcf11 fusion protein was obtained from 500 ml of bacteria which had been induced for 3 h at 37°C with IPTG. Rabbits were injected with 1 mg of the protein three times over a 9-week period.

Purified Pcf11 protein was immobilized on an agarose Amminolink column by using an ImmunoPure Antigen/Antibody Immobilisation kit as specified by the supplier (Pierce). Three milliliters of crude serum was applied to the column, and specific immunoglobulin G was eluted by using the Pierce protocol.

For antibody neutralization studies, 1 and 2 μl of purified polyclonal anti-Pcf11 antibody were incubated with 2 μl of wild-type extract in a standard processing reaction assay. The mixtures were incubated for 45 min at 4°C before addition of the RNA precursor for the processing reaction. Two microliters of wild-type extract without antibody was treated under the same conditions.

Immunological techniques. Pcf11 was detected by immunoblot analysis of the fractions obtained following anion-exchange chromatography on a Mono Q column. Samples were separated on a sodium dodecyl sulfate (SDS)–polyacrylamide gel and electrotransferred to a nitrocellulose filter in a Bio-Rad transblot apparatus (41). The blot was blocked with 5% nonfat milk powder dissolved in Tris-buffered saline–Tween 20 buffer (37) and was processed for immunological detection of antigens by using a polyclonal immune serum containing a specific antibody, either anti-Pcf11, anti-Rna14, or anti-Rna15, at a dilution of 1/5,000, 1/2,000 or 1/20,000, respectively. The protein bands were detected by using the Amersham ECL detection kit after incubation of the blot with peroxidase-labeled antibodies to rabbit immunoglobulin G at a dilution of 1/2,000 (Amersham). Five-microliter aliquots of samples without loading buffer were used for the determination of the extract protein concentrations, using the Pierce bicinchoninic acid protein assay reagent kit.

Nucleotide sequence accession number. The GenBank accession number for *PCF11* is YD9934_13c.

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  1 MDHDTEVIVK DFNSILEELT FNSRPIITTL TKLAEENISC AQYFVDAIES

 51 RIEKCMPKQK LYAFYALDSI CKNVGSPYTI YFSRNLFNLY KRTYLLVDNT

101 TRTKLINMFK LWLNPNDTGL PLFEGSALEK IEQFLIKASA LHQKNLQAML

151 PTPTVPLLLR DIDKLTCLTS ERLKNQPNDE KLKMKLLVLS QLKQELKREK

      •                              •
201 LTLNALKQVQ MQLRQVFSQD QQVLQERMRY HELQQQQQQQ QQQQQQQQQQ

                         *
                         •                •         *
251 QQQYHETKDM VGSYTQNSNS AIPLFGNNSD TTNQQNSLSS SLFGNISGVE

301 SFQEIEKKKS LNKINNLYAS LKAEGLIYTP PKESIVTLYK KLNGHSNYSL

351 DSHEKQLMKN LPKIPLLNDI LSDCKAYFAT VNIDVLNNPS LQLSEQTLLQ

401 ENPIVQNNLI HLLYRSKPNK CSVCGKRFGN SESEKLLQNE HLDWHFRINT

451 RIKGSQNTAN TGISNSNLNT TTTRKNIQSR NWYLSDSQWA AFKDDEITST

501 KHKNDYTDPH ANKNIDKSAL NIHADENDEG SVDNTLGSDR SNELEIRGKY

551 VVVPETSQDM AFKCPICKET VTGVYDEESG EWVWKNTIEV NGKYFHSTCY

601 HETSQNSSKS NSGKVGLDDL KKLVTK  626
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FIG. 1. Predicted amino acid sequence of the *PCF11* product and positions of the Pcf11-GAD fusions. The position of the first amino acid of each Pcf11-GAD fusions is indicated above the Pcf11 sequence by a dot when detected by the Rna14 bait and by an asterisk when isolated by the Rna15 bait. The glutamine stretch (aa 234 to 253) is underlined.

RESULTS

Isolation of the *PCF11* gene. During screening of yeast genomic libraries in a two-hybrid assay using *RNA14* and *RNA15* fusions as baits, five different in-frame fusions of an ORF, hereafter called *PCF11*, were isolated (four times with *RNA14* and twice with *RNA15*). *PCF11* corresponds to ORF YDR228C on chromosome IV, encoding a protein of 626 aa with a predicted molecular mass of 71,853 Da. The Pcf11-GAD fusions isolated from the pACTII libraries start at codons 203, 232, 271, 288, and 294 of the *PCF11* ORF (Fig. 1). The corresponding plasmids were called pACT-*PCF11*(203) to pACT-*PCF11*(294). No homologous sequences or known sequence motifs were found by using the Blastn and Tblastn programs to search databases with the Pcf11 sequence. In the middle of Pcf11 was found a stretch of 20 glutamines which could be a hinge between two functional domains of the protein, although the functional meaning of such stretches remains an open question. All of the Pcf11-GAD fusions contained the COOH part of Pcf11, and the hinge is clearly not involved in the interaction of Pcf11 with Rna14 and Rna15 since three of the Pcf11-GAD fusions began after the polyglutamine stretch. Plasmid pACT-*PCF11*(271), found in both screenings, contained a *PCF11* fragment extending from codons 271 to 452.

Interactions between Rna14, Rna15, and Pcf11 were quantitated by measuring β-galactosidase activity for various combinations of fusion proteins (Table 3). Plasmid pACT-*PCF11* (271) gives the same β-galactosidase activity values as plasmid pACT-*PCF11*(288) in all combinations. This analysis defines a region of Pcf11 sufficient for interaction with Rna14 and Rna15, and extending from aa 288 to 400.

Specificity of interaction was supported by the fact that no interaction was found between Pcf11 and four other baits used as false-positive detectors (data not shown).

***PCF11* is an essential gene.** The entire coding region of the *PCF11* gene was replaced by a *TRP1* gene insertion, using a PCR strategy (5). A DNA fragment, in which the *TRP1* gene is flanked by short sequences homologous to the 5′ and 3′ sequences of the *PCF11* gene, was generated in a one-step PCR amplification. This DNA fragment was used to transform diploid strain BMA64-2N, which is homozygous for a deletion of the *TRP1* gene (5a). Three independent Trp^+ transformants

TABLE 3. Interactions between Rna14, Rna15, and Pcf11 in the two-hybrid system

GAD-expressing plasmid	GBD-expressing plasmid	Colony color[a]	β-Galactosidase activity[b]
pACT-*PCF11*(271)	pAS2Δ-*RNA14*	Dark blue	81
pACT-*PCF11*(271)	pGBT-*RNA15*	Dark blue	45
pACT-*PCF11*(271)	pAS2Δ	White	1.7
pACT-*PCF11*(271)	pGBT9	White	1.6
pACTII	pAS2Δ-*RNA14*	White	1
pACTII	pGBT-*RNA15*	White	1.9
pACT-*RNA14*	pGBT-*RNA15*	Dark blue	310
pACT-*RNA15*	pAS2Δ-*RNA14*	Dark blue	420
pACT-*RNA14*	pGBT9	White	1
pACT-*RNA15*	pAS2Δ	White	1.7

[a] Determined by filter assay.
[b] Quantitative β-galactosidase assays were performed with strain J 693 expressing the designed constructs. Assays were done as described previously (23). β-Galactosidase activity is expressed in Miller units. Values are the averages for two different mixtures of transformants. Standard errors were <20%.

were analyzed by Southern blotting. This analysis confirmed that for each of them, the integration of the *pcf11*-Δ*::TRP1* deleted allele was at one of the two *PCF11* loci (data not shown). One of these Trp^+ transformants was subjected to sporulation and to tetrad analysis, which showed a Mendelian 2:2 segregation of two viable and two nonviable spores. The viable spores were invariably Trp^-, showing the lethality of the spores containing the *pcf11*-Δ*::TRP1* deleted allele. The deletion was rescued in the haploid strains when *PCF11* was supplied on a centromeric plasmid. This result shows that *PCF11* is an essential gene.

Isolation of *pcf11* thermosensitive alleles. Mutagenic PCR conditions were used to generate thermosensitive mutations in the *PCF11* gene. Yeast strain NA52 (*pcf11*-Δ*::TRP1/CEN URA3 PCF11*) was cotransformed with PCR-mutagenized *PCF11* ORF and the gapped plasmid pNA46 (*CEN LEU2 PCF11*) (see Materials and Methods). Leu^+ colonies were selected. The endogenous *CEN URA3 PCF11* plasmid was then counterselected by replica plating the Leu^+ transformants on 5-FOA medium, and the 5-FOA plates were replica plated on YNB plates incubated at 37°C to identify thermosensitive colonies. Eleven independent colonies showed inhibition of growth at 37°C; the mutant alleles which they carried are all recessive, and they were named *pcf11-1* through *pcf11-11*. The *pcf11-2*, *pcf11-3*, and *pcf11-9* alleles were integrated at the chromosomal *PCF11* locus to create thermosensitive strains NA65, NA66, and NA67, respectively. This was achieved by transforming strain NA52 (*pcf11*-Δ*::TRP1/CEN URA3 PCF11*) with *Bam*HI-*Eco*RV fragments bearing the *pcf11* mutations and then selecting strains able to lose the *URA3 PCF11* plasmid on 5-FOA at 24°C. Among them, some Leu^+ Trp^+ cells corresponded to a background of undigested *LEU2 pcf11* plasmids, but the Leu^- Trp^- cells corresponded to the integration of the Ts alleles at the original locus.

Synthetic lethality of the *pcf11* mutation and the *rna14* or *rna15* mutations. To confirm the apparent in vivo association of Pcf11 with Rna14 and Rna15, the strains containing the *pcf11-2*, *pcf11-3*, or *pcf11-9* allele were crossed with either the *rna14-1* or *rna15-2* strain. Tetrads were analyzed from the corresponding diploids, and for each tetratype which contained a wild-type recombinant, one spore was nonviable even at the permissive temperature, showing synthetic lethality between the different *pcf11* Ts alleles and the *rna14* or *rna15* Ts allele. At least eight tetratypes were obtained in each cross. To obtain further confirmation of the synthetic lethality, the diploids formed by crossing a *pcf11-2* strain with an *rna14-1* strain or an *rna15-2* strain were transformed by a *CEN PCF11 URA3* plasmid and then submitted to tetrad analysis. In tetratypes presenting a wild-type recombinant, one of the spores was unable to grow on 5-FOA, showing its inability to lose the plasmid and therefore the necessity of the *PCF11* wild-type gene for the survival of the *pcf11-2 rna14-1* and *pcf11-2 rna15-2* double mutants.

Effects of the *pcf11-1* and *pcf11-2* mutations on in vivo polyadenylation. The double-hybrid system and the synthetic lethality establish the in vivo physical and functional interactions of the Pcf11 protein with Rna14 and Rna15. We therefore decided to examine whether the Ts alleles of *pcf11* created some defect in the cleavage or polyadenylation of mRNAs as do *rna14* and *rna15* Ts alleles (28). The ability of strains possessing the *pcf11-1* and *pcf11-2* alleles to perform 3′-end mRNA processing at the nonpermissive temperature, either in vivo or in vitro, was examined. To investigate the processing of the poly(A) tail in vivo, total mRNAs were extracted from the wild-type strain and from the *pcf11-1* and *pcf11-2* mutant strains either grown at 24°C or grown at 24°C and then shifted to 37°C for 30 min. We first compared the lengths of the poly(A) tails of the mRNAs extracted from the different strains. RNAs were labeled in vitro at their 3′ ends and subjected to RNase degradation to leave only their poly(A) tails intact. As shown in Fig. 2, there was no change in the poly(A) tail length in the wild-type and mutant strains grown at 24°C (lanes 1, 3, and 5). After the 37°C shift, the *pcf11-1* mutant strain showed a discrete increase in poly(A) tail length compared to the wild type (compare lanes 2 and 4), whereas the *pcf11-2* mutant strain displayed a significant reduction in the poly(A) tail length (compare lane 2 and lane 6).

The amount of actin mRNA in the *pcf11-1* and *pcf11-2* mutant strains shifted to 37°C was quantitated by Northern blotting. As shown in Fig. 3, both mutant strains displayed a rapid reduction in the steady-state level of the *ACT1* mRNA after a shift to 37°C (lanes 4 and 6).

The *pcf11-1* and *pcf11-2* mutations alter the 3′-end processing of the *CYC1* pre-mRNA in vitro. Extracts from the wild-type strain and the mutant *pcf11-1* and *pcf11-2* strains, grown at the permissive temperature, were tested for in vitro 3′-end processing at 23 and 35°C, using a synthetic radiolabeled *CYC1* precursor mRNA (Fig. 4). We first analyzed the cleavage reaction under conditions that allowed only the cleavage of the labeled *CYC1* precursor (Fig. 4A). With the wild-type extract, the *CYC1* precursor was cleaved, generating 5′ and 3′ fragments (185 and 116 nucleotides, respectively), with the same efficiency at 23 and 35°C (Fig. 4A, lanes 2 and 6). At 23°C, the *pcf11-1* and *pcf11-2* extracts produce about four times less cleavage product than the wild-type extract (compare lanes 2 to 4). At 35°C, very little (*pcf11-1*) or no (*pcf11-2*) cleavage product was detected (lanes 7 and 8). The polyadenylation reaction was performed with a precleaved *CYC1* precursor (see Materials and Methods) (Fig. 4B). In a wild-type extract, the 5′ *CYC1* precursor fragment was polyadenylated with about 70 adenosine residues at both 23 and 35°C (Fig. 4B, lanes 2 and 6). At 23°C, in *pcf11-1* and *pcf11-2* extracts, we observed the formation of a very short poly(A) tail on the precleaved *CYC1* (Fig. 4B, lanes 3 and 4). At 35°C, no poly(A) tail was detected in either mutant extract (Fig. 4B, lanes 7 and 8). Thus, in vitro, *pcf11* mutants are defective in both 3′-end cleavage and polyadenylation.

Neutralization of Pcf11 in wild-type extract. Treatment of wild-type extract with purified anti-Pcf11 antibody completely abolished the activities of *CYC1* cleavage and polyadenylation (Fig. 5, lanes 3, 4, 7, and 8). As a control to show that inhibition

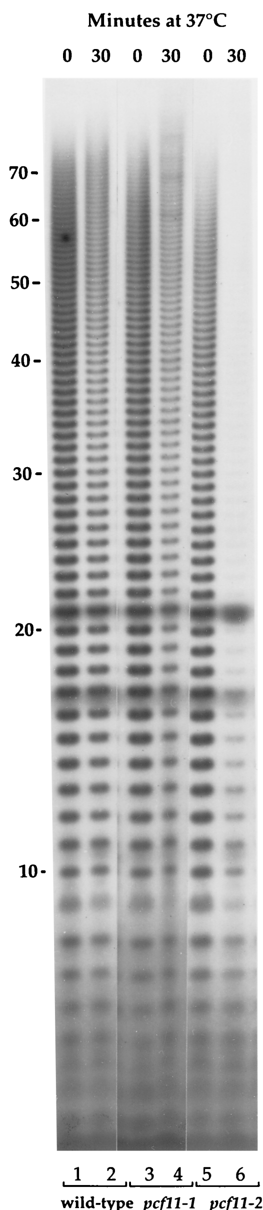

FIG. 2. Lengths of poly(A) tails in the wild-type strain and in the *pcf11-1* and *pcf11-2* mutant strains after a shift to 37°C. Cells were grown at 24°C and then shifted to 37°C for 30 min. One microgram of total RNA was 3′-end labeled with RNA ligase and [5′-^{32}P]pCp and then digested with RNase A and RNase T_1 to yield only intact labeled poly(A). After electrophoresis through a 15% polyacrylamide–8 M urea gel, the poly(A) tails were visualized by autoradiography. Markers indicate the sizes (in nucleotides) of the poly(A) tails.

was not due to a nonspecific effect resulting from the addition of antibodies, we used anti-yeast Pab protein antibodies which were purified by using the same conditions. We observed no cleavage inhibition when equivalent amounts of these purified polyclonal antibodies were used (data not shown). The preimmune antibodies could not be used as a control because they completely degraded the precursor. Pcf11 immunoneutralization of a wild-type extract causes exactly the same deficiency in *CYC1* processing as seen with *pcf11* mutant extracts. This result provides additional evidence that Pcf11 is necessary for cleavage and polyadenylation of pre-mRNA.

Copurification of the Pcf11 protein within the CF I complex. To test whether the Pcf11 protein is a component of CF I, we constructed an epitope-tagged Pcf11 protein in order to monitor its copurification with the other components of the polyadenylation complex. The *PCF11* ORF generated by PCR amplification was cloned into the pYeF1 vector (see Materials and Methods). The fusion of *PCF11* with the Flu epitope in plasmid pNA42 was shown to be functional by complementation of the *pcf11-Δ::TRP1* deletion after transformation of the *PCF11/pcf11-Δ::TRP1* diploid strain NA50 (Table 1). Transformants were plated on a selective medium, and sporulation was induced to obtain, among the haploids, strain NA54; this *pcf11-Δ::TRP1* strain carries plasmid pNA42, which produces the epitope-tagged Pcf11 protein.

Whole-cell extracts were prepared from NA54 and used as the starting material for the fractionation studies. The crude extract was applied to a Mono Q anion-exchange column, and the proteins were eluted by an increasing salt gradient (12). Fractions were then analyzed by SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and probed with MAb 12CA5 (which recognizes the Flu epitope) and anti-Rna14 and anti-Rna15 antibodies. As shown in Fig. 6, the epitope-tagged Pcf11 protein coeluted with Rna14 and Rna15. Fraction 12 shows an intense signal for Pcf11, Rna14, and Rna15. Pcf11 was reproducibly eluted at 445 mM KCl, which corresponds to the ionic strength at which the cleavage activity factors CF I and CF II elute (12).

A further purification separating CF I from CF II was used to localize Pcf11 to one of these two complexes. Cleavage activity-containing fractions obtained from two Mono Q separations were pooled, dialyzed, reloaded on a Mono Q column (5 by 50 mm), and eluted with a shallow salt gradient as described by Chen and Moore (12). The low efficiency of detection of the epitope-tagged Pcf11 by MAb 12CA5 did not allow detection of Pcf11. We therefore undertook the production of a polyclonal antibody against Pcf11. A *PCF11* ORF was introduced into a T7 expression vector, and large quantities of histidine-tagged protein were obtained. This material was purified on a nickel column and injected into rabbits for antibody production. As can be seen in Fig. 7, Pcf11 was specifically recognized by the antiserum used at a 1/5,000 dilution. Its apparent molecular mass is nearly 70 kDa, as was also observed in assays using MAb 12CA5 against the epitope-tagged Pcf11 (Fig. 6). Western blotting analysis of the purified CF I and CF II complexes in the Mono Q eluates with the anti-Pcf11 antibody showed the presence of Pcf11 in the same fractions that contained Rna15 (Fig. 7) and Rna14 (data not shown).

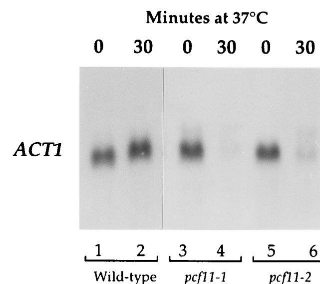

FIG. 3. Amount of *ACT1* mRNA left after heat inactivation of Pcf11. The mRNA levels were determined by Northern blot analysis; 10 μg of total RNA was resuspended in RNA sample buffer, and the samples were fractionated on a 1% agarose gel in 1× morpholine propanesulfonic acid buffer. The RNA blot was hybridized with a radioactive *ACT1* ORF fragment.

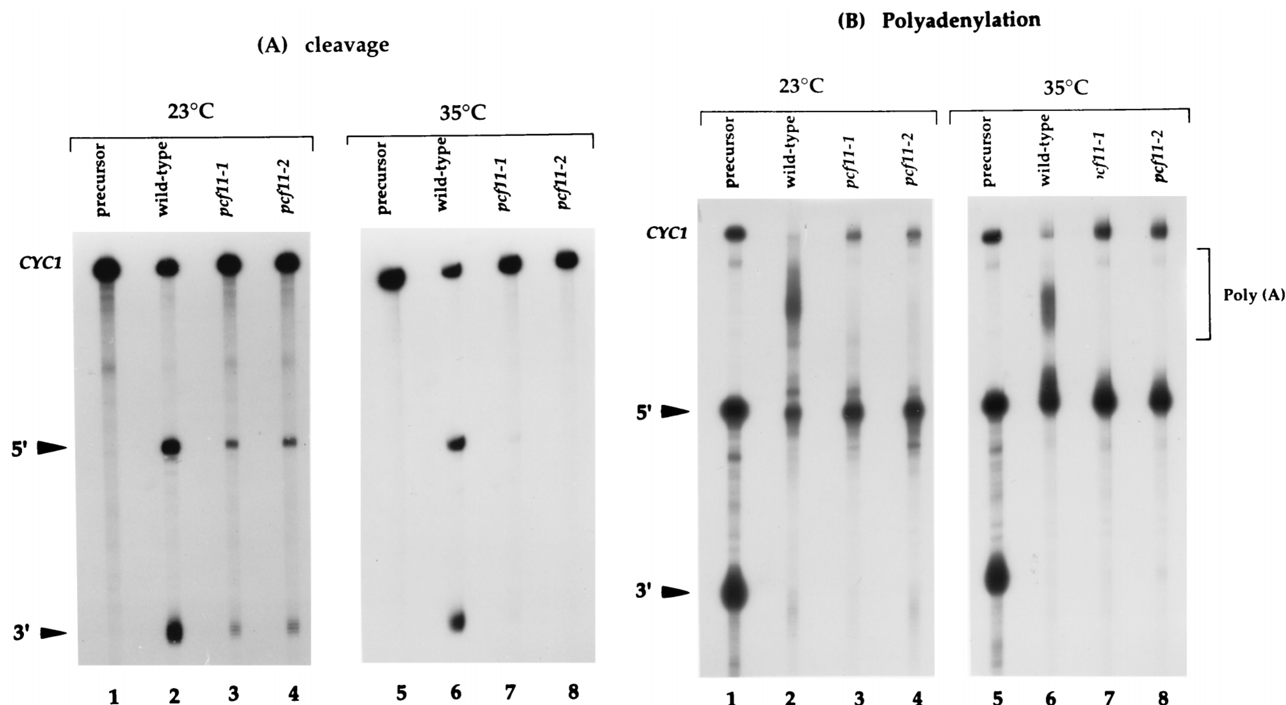

FIG. 4. Temperature dependence of cleavage and polyadenylation by wild-type and *pcf11* mutant extracts in vitro. (A) For the cleavage reaction, synthetic radiolabeled *CYC1* pre-mRNAs were incubated in extracts of wild-type (lanes 2 and 6), *pcf11-1* (lanes 3 and 7), and *pcf11-2* (lanes 4 and 8) strains for 60 min at 23 or 35°C as indicated. (B) For the polyadenylation reaction, *CYC1* mRNAs precleaved by a wild-type extract were assayed under standard reaction conditions for 60 min in whole-cell extracts made from wild-type (lanes 2 and 6), *pcf11-1* (lanes 3 and 7), and *pcf11-2* (lanes 4 and 8) strains at 23 or 35°C as indicated. The products were analyzed on a 6% polyacrylamide–7 M urea electrophoresis gel.

These data demonstrate clearly that Pcf11 copurifies with CF I, which participates in both cleavage and polyadenylation steps.

DISCUSSION

In this study, we have identified, by both genetic and biochemical criteria, *PCF11* as a new yeast gene whose product is involved in the polyadenylation of mRNAs. The genetic identification relies on two lines of evidence. First, in the double-hybrid system, Pcf11 interacts with both Rna14 and Rna15, two proteins which are part of the polyadenylation machinery (27, 28). Second, thermosensitive *pcf11* alleles present a synthetic lethality with thermosensitive alleles of *RNA14* or *RNA15*. These genetic observations suggest that Pcf11, like Rna14 and Rna15, is functionally involved in mRNA 3′-end processing. Biochemical analysis of the *pcf11* mutants confirms the genetic results, as it reveals defects very similar to those observed in the *rna14-1* and *rna15-2* Ts mutants (27, 28). Under restrictive growth conditions, there is, in vivo, a shortening of the mRNA poly(A) tails and also a strong decrease in the steady-state level of the *ACT1* mRNA. In vitro, extracts from the *pcf11* Ts mutant as well as Pcf11 neutralized wild-type extracts are deficient in both the cleavage and the polyadenylation of the *CYC1* transcript, suggesting that like Rna14 and Rna15, Pcf11 is a component of CF I. Other data strongly support this assumption, as Pcf11 copurifies with Rna15 under the same nondenaturing conditions which allow the purification of a functional CF I complex (12). These results, taken together, strongly suggest that Pcf11 is a third, previously unknown component of CF I in yeast.

Analysis of the interactions between the three proteins in the two-hybrid system allows the definition of a region of Pcf11 sufficient for an interaction with Rna14 and Rna15. It is a

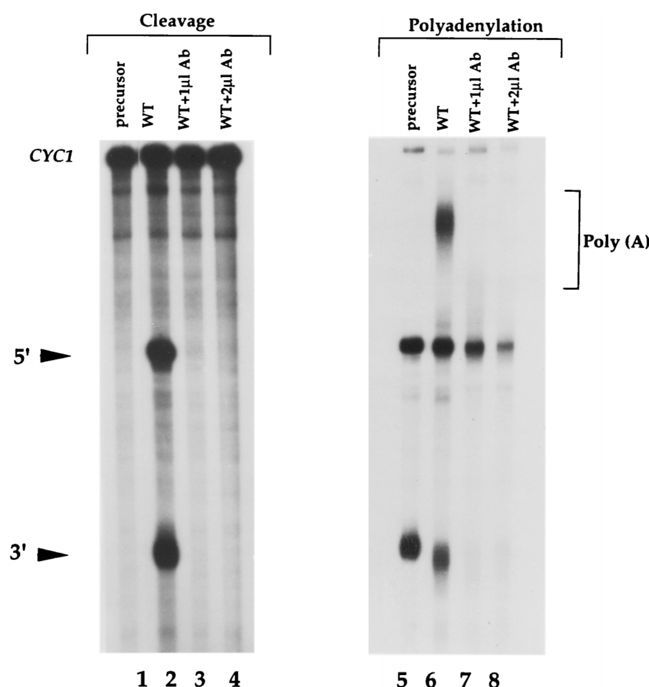

FIG. 5. Inhibition of 3′-end cleavage and polyadenylation activity of a wild-type extract by immunoneutralization with anti-Pcf11 antibody. Cleavage (lanes 1 to 4) and polyadenylation (lanes 5 to 8) were tested separately. The inactivation of processing activity was obtained with 1 µl (lanes 3 and 7) or 2 µl (lanes 4 and 8) of purified anti-Pcf11 antibody.

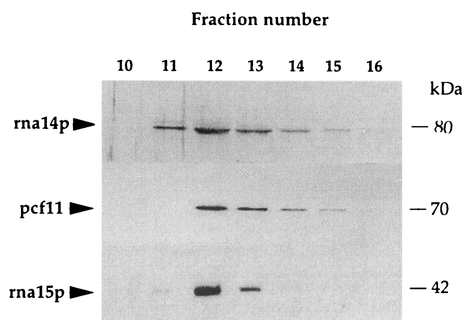

FIG. 6. Western blot analysis of the N-terminal epitope-tagged Pcf11, Rna14, and Rna15 proteins obtained from anion-exchange chromatography on a Mono Q column. Ten microliters of each fraction was loaded on an SDS–10% polyacrylamide gel, and Pcf11, Rna14, and Rna15 were revealed by Western blotting. Detection was done with a 1/20,000 dilution of anti-Rna15 antibody, a 1/50,000 dilution of MAb 12CA5 for tagged Pcf11, or a 1/2,000 dilution of anti-Rna14 antibody. Protein molecular masses are indicated on the right.

113-aa polypeptide whose sequence corresponds to the Pcf11 sequence from aa 288 to 400, located downstream from the glutamine stretch. Two hypotheses may be advanced to account for the positive interaction of Pcf11 with Rna14 and Rna15: (i) there is a direct interaction of Pcf11 with both Rna14 and Rna15; and (ii) Pcf11 interacts directly only with one of these proteins, the positive signal obtained with the other one resulting from the tight association between Rna14 and Rna15, as already suggested (27) and as confirmed by the two-hybrid assays (Table 3).

What is the function of Pcf11? The in vivo phenotypes of thermosensitive *pcf11*, *rna14*, and *rna15* mutants (28) are very similar to each other and even to those of two other mutants bearing Ts mutations in the *PAP1* gene, encoding the poly(A) polymerase (33), or in the *FIP1* gene (32), encoding a component of the polyadenylation factor. Interestingly, this is not true for all *pcf11* mutant alleles, since in vivo, *pcf11-1*, but not *pcf11-2* (Fig. 2), shows a small increase in the length of the poly(A) tails. Despite a clearer differentiation among the functions of *PCF11*, *RNA14*, *RNA15* (27), *PAP1* (31), and *FIP1* (32) by use of the in vitro system, neither the in vitro nor the in vivo phenotype permits the identification of a precise biochemical reaction linked to *PCF11*.

In higher eucaryotes, 77- and 64-kDa proteins homologous to Rna14 and Rna15, respectively (39), have been identified as components of CstF (20, 40). Although the activities and the components of the different fractions of the polyadenylation complexes isolated from higher eucaryotes are difficult to compare with those isolated from yeast (22), it was tempting to look for a homolog of *PCF11* among the genes found to be related to polyadenylation in higher organisms. However, no significant homology of Pcf11 could be found either with products of known genes and cDNAs or in any of the database sequences searched by using the Tblastn program. We are thus left with the following three possibilities: (i) Pcf11 is a fungal specific component, (ii) a homolog of Pcf11 is already in the data banks but the evolutionary conservation of the sequence is too low to be recognized, and (iii) the homolog of Pcf11 exists but has not yet been sequenced in higher organisms.

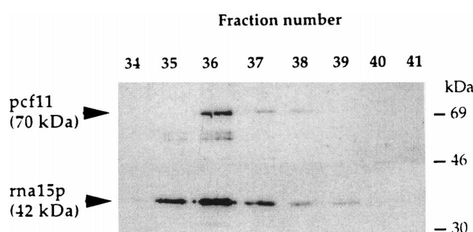

FIG. 7. Copurification of Pcf11 and Rna15. Fractions from anion-exchange chromatography on a Mono Q column which contained cleavage activity were pooled, dialyzed, and reseparated on a Mono Q column with a shallow salt gradient. Fractions were electophoresed on an SDS–10% polyacrylamide gel. The blot was probed with anti-Pcf11 antibody at a dilution of 1/5,000 and with anti-Rna15 antibody at a dilution of 1/20,000. The positions of protein molecular weight markers are indicated on the right.

ACKNOWLEDGMENTS

We thank M. Frommont for providing the FRYL library and plasmid pAS2Δ and A. Petitjean for her gift of plasmid pGBT-*RNA15* and for critical reading of the manuscript. We thank S. Elledge for plasmids pACTII and pAS2 and A. Baudin for strain BMA64-2N. We thank D. Menay for synthesis of all of the oligonucleotides.

This work was supported by the EEC project TAPIR (two-hybrid analysis of proteins involved in RNA metabolism), the Association de la Recherche sur le Cancer, and the Centre National de la Recherche Scientifique.

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