Mol Gen Genet (1997) 255:209–218

ORIGINAL PAPER

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The Uba2 and Ufd1 proteins of Saccharomyces cerevisiae interact with poly(A) polymerase and affect the polyadenylation activity of cell extracts

Received: 6 January 1997 / Accepted: 27 February 1997

Abstract Poly(A) polymerase is responsible for the addition of the adenylate tail to the 3′ ends of mRNA. Using the two-hybrid system, we have identified two proteins which interact specifically with the *Saccharomyces cerevisiae* poly(A) polymerase, Pap1. Uba2 is a homolog of ubiquitin-activating (E1) enzymes and Ufd1 is a protein whose function is probably also linked to the ubiquitin-mediated protein degradation pathway. These two proteins interact with Pap1 and with each other, but not with eight other target proteins which were tested in the two-hybrid system. The last 115 amino acids of Uba2, which contains an 82-amino acid region not present in previously characterized E1 enzymes, is sufficient for the interaction with Pap1. Both Uba2 and Ufd1 can be co-immunoprecipitated from extracts with Pap1, confirming in vitro the interaction identified by two-hybrid analysis. Depletion of Uba2 from cells produces extracts which polyadenylate precursor RNA with increased efficiency compared to extracts from nondepleted cells, while depletion of Ufd1 yields extracts which are defective in processing. These two proteins are not components of polyadenylation factors, and instead may have a role in regulating poly(A) polymerase activity.

Key words *Saccharomyces cerevisiae* mRNA 3′-end processing · Poly(A) polymerase · Ubiquitin pathway

Communicated by C. P. Hollenberg

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Introduction

Polyadenylation is an essential step in the synthesis of eukaryotic mRNA, and this modification contributes to the cytoplasmic stability of mRNA and increases the efficiency of translation (Sachs and Wahle 1993). In addition, defects in polyadenylation can lead to inefficient export of mRNA from the nucleus (Huang and Carmichael 1996; Long et al. 1995). During the polyadenylation reaction, precursor RNA is cleaved at the poly(A) site, and a poly(A) tail is added to the new 3′ end (Wahle and Keller 1996). With an in vitro processing system using yeast whole cell extracts (Butler and Platt 1988), it has been possible to identify four different factors which are required for the polyadenylation of yeast precursor mRNA (Chen and Moore 1992). Cleavage factors (CF) I and II are required for the cleavage step, while poly(A) polymerase (Pap1), CF I and PF I are needed for the poly(A) addition step. Several genes encoding proteins which participate in yeast mRNA 3′-end processing have been identified (Manley and Takagaki 1996). The gene for Pap1 was the first one cloned (Lingner et al. 1991; Patel and Butler 1992), followed by the identification of Rna14 and Rna15 as components of CF I (Minvielle-Sebastia et al. 1994), Fip1 (Preker et al. 1995) and Brr5/Ysh1 (Chanfreau et al. 1996; Jenny et al. 1996) as subunits of the PF I factor, and Cft1 as part of CF II (Stumpf and Domdey 1996). The CF I factor can be separated into two functional components, CF IA and CF IB (Kessler et al. 1996). CF IA contains four polypeptides, two of which are Rna14p and Rna15p, while CF IB is a single 73 kDa protein. In addition, the Ref2 protein stimulates cleavage at otherwise poorly utilized poly(A) sites (Russnak et al. 1995).

Purified yeast Pap1 is nonspecific with regard to RNA substrate, but in the presence of CF I and PF I, it is only active on cleaved mRNA precursors. Pap1 is also capable of synthesizing tails up to 1000 nucleotides long, but cellular factors cause it to terminate poly(A) syn-

thesis at much shorter lengths. So far, only the Fip1 protein has been shown to interact directly with Pap1. In order to identify additional factors which confer specificity on poly(A) polymerase or otherwise regulate its activity, we used the *PAP1* coding sequence as a bait in the yeast two-hybrid system (Chien et al. 1991; Gyuris et al. 1993). Two proteins which interact specifically with Pap1 were identified. While characterization of the genes encoding these proteins was in progress, these genes, *UBA2* and *UFD1,* were also cloned by different approaches in the laboratory of A. Varshavsky (Dohmen et al. 1995; Johnson et al. 1995), as possible components of the ubiquitin-mediated protein degradation pathway. We show that these two proteins, Uba2 and Ufd1, interact with Pap1 in vitro. Depletion of either of these two proteins from cells by using a repressible promoter to drive their expression produces extracts which are altered in their in vitro polyadenylation activity.

Materials and methods

Strains, media and DNAs

Growth and manipulation of yeast strains was done according to standard procedures (Kaiser et al. 1994). The yeast strains used in these experiments are listed in Table 1 and the plasmids in Table 2. All experiments involving the two-hybrid system were performed in the strain EGY40. *S. cerevisiae* cultures were grown at 30° C in rich (YP) or synthetic (S) media containing either 2% dextrose (YPD or SD media) or 2% galactose (YPG or SG media). The libraries of partial *Sau*3AI digests of yeast genomic sequences in the pGAD vectors are described in Chien et al. (1991). The pSH18-34 reporter plasmid (a high-copy-number *URA3* plasmid containing eight LexA operators upstream of the gene *GAL1-lacZ*) was a gift of S. Hanes and R. Brent. Plasmid pBTM116 was obtained from P. Bartel and S. Fields (State University of New York, Stony Brook). pLexA-Fus3 and pLexA-Cdc2, which contain the entire yeast Fus3 and human Cdc2 proteins, respectively, are described in Gyuris et al. (1993), and LexA-bicoid, which encodes residues 2-160 of the *Drosophila bicoid* gene product, in Zervos et al. (1993). LexA-lamin, with the complete coding sequence of human lamin C in frame with LexA was obtained from S. Fields (Bartel et al. 1993). LexA-Ras and LexA-Rb were a gift of S. Reddy and B. Cochran (Tufts University, Boston). Plasmids pHG52 (pBTM116 containing the sequence encoding amino acids 83-566 of yeast topoisomerase I fused in frame to LexA) and pCTC59 (pBTM116 containing the coding sequence for amino acids 742-1358 of yeast Sir4 fused in frame with LexA) were sent to us by R. Sternglanz (State University of New York, Stony Brook). pGAD-DP1, which contains a fusion with an E2F homolog, was obtained from Amy Yee (Tufts University), and pGAD-FIP1 from W. Keller (Preker et al. 1995). Coding sequences of the interacting genes were placed under the control of the *GAL1* promoter in the yeast plasmid pBM272T, a derivative of pBM272, received from M. Johnston (Washington University, St. Louis, Mo.). β-Galactosidase assays were performed as described previously using *o*-nitrophenyl-β-D-galactoside (ONPG) as substrate (Kaiser et al. 1994) in multiples for at least three independent transformants, and activity is expressed in arbitrary units using the formula $100 \times OD_{420}/[OD_{600} \times$ volume assayed (ml) $\times$ time of reaction (min)].

Isolation of interacting genes using the two-hybrid system

The yeast strain EGY40, containing pLexA-PAP and the reporter plasmid pSH18-34, was transformed with libraries of genomic DNA fragments in the three alternate-frame pGAD expression vectors (Chien et al. 1991), using the lithium acetate method (Kaiser et al. 1994), and transformants were selected on minimal medium with histidine at 30° C. Colonies (1–1.5 mm diameter) were replica-plated onto plates containing X-Gal. Blue colonies were identified, purified by restreaking, and retested for blue color. Plasmid DNA from positive colonies was used to transform the *E. coli* strain C600 (*supE44 hsdR thi-1 thr 1 leuB6 lacY1 tonA21 hflA150* [*chr::*Tn*10* (Tet^r)]). Amp^R Leu^+ transformants were selected.

DNAs were sorted into classes based on their restriction map patterns, and at least one representative from each class was partially sequenced by the dideoxy method using the US Biochemicals sequanase kit. Full-length DNA clones for the interacting genes were obtained by colony hybridization from a yeast library made in a centromeric *LEU2*-containing, pBR322-derived plasmid (ATCC #77162). The coding sequence was compared to sequences in databases using the FASTA (Pearson and Lipman 1988) and BLAST (Altschul et al. 1990) programs.

Gene disruptions and regulated expression of *UBA2* and *UFD1*

For gene disruptions (Rothstein 1991), the fragments containing the coding sequence were cloned in pUC-derived vectors, and the *HIS3* gene obtained from the pUC18-HIS3 plasmid by *Hin-*

Table 1 *S.cerevisiae* strains used in this study

Strain	Genotype	Source
EGY40	*MATα*, *ura3-52*, *his3*, *trp1*, *leu2*	R. Brent
W303	*MAT***a**/*MATα*, *leu2-3*/*leu2-3*, *can1-100*/*can1-100*, *ura3-1*/*ura3-1*, *ade2-1*/*ade2-1*, *his3-11,15*/*his3-11,15*, *trp1-1*/*trp1-1*	R. Rothstein
W303-1A	*MAT***a** *leu2-3*, *can1-100*, *ura3-1*, *ade2-1*, *his3-11,15*, *trp1-1*	R. Rothstein
MD1	*leu2-3*, *can1-100*, *ura3-1*, *ade2-1*, *his3-11,15*, *trp1-1*, Δ*UBA2-1784::HIS*. Haploid, mating type undetermined	This study
MD2	*MAT***a**/*MATα*, *leu2-3*/*leu2-3*, *can1-100*/*can1-100*, *ura3-1*/*ura3-1*, *ade2-1*/*ade2-1*, *his3-11,15*/*his3-11,15*, *trp1-1*/*trp1-1*, *UBA2*/Δ*UBA2(285-795)::HIS3*	This study
MD3	*MAT***a**/*MATα*, *leu2-3*/*leu2-3*, *can1-100*/*can1-100*, *ura3-1*/*ura3-1*, *ade2-1*/*ade2-1*, *his3-11,15*/*his3-11,15*, *trp1-1*/*trp1-1*, *UFD1*/Δ*UFD1(898)::HIS3*	This study
MD4	*leu2-3*, *can1-100*, *ura3-1*, *ade2-1*, *his3-11,15*, *trp1-1*, Δ*UBA2(285-795)::HIS3*, pBM272T-FL227. Haploid, mating type unknown	This study
MD5	*leu2-3*, *can1-100*, *ura3-1*, *ade 2-1*, *his3-11,15*, *trp1-1*, Δ*UFD1(898)::HIS3* pBM272T-FL237. Haploid, mating type undetermined	This study
MD6	*leu2-3*, *can1-100*, *ura3-1*, *ade2-1*, *his3-11,15*, *trp1-1*, Δ*UFD1(898)::HIS3*, p237–13. Haploid, mating type unknown	This study

Table 2 Plasmid constructions

Plasmid	Construction
pLexA-PAP	The entire *PAP1* ORF cloned in frame with the LexA DNA-binding domain at the *Bam*HI and *Pst*I sites of pBTM116, a *TRP1* yeast 2-micron plasmid
p237-13	A *Sau*3AI genomic fragment containing the full-length *UFD1* gene cloned in the *Bam*HI cloning site of p366, a *LEU2* yeast CEN-ARS based plasmid
pGAD-PIP3(523-1086)	The *UFD1* coding sequence from positions 523-1086 cloned in frame with the *GAL4* activating domain in pGAD.2F
pGAD-PIP2(1566-1911)	The *UBA2* coding sequence from positions 1566-1911 cloned in frame with the *GAL4* activating domain in pGAD.2F
pLexA-UBA2	The entire *UBA2* ORF cloned in frame with the LexA DNA-binding domain at the *Bam*HI site of pBTM116
pGAD-UFD1	The entire *UFD1* coding sequence cloned in frame with the *GAL4* activating domain in pGAD.1F
pBM272T	The *ADH1* 3′-end (a *Sma*I-*Nae*T fragment from pBTM116) cloned downstream of the *GAL1* promoter of pBM272, a *URA3* yeast CEN-ARS based plasmid
pBM272T-FL227	The FLAG epitope was introduced at the N-terminus of the *UBA2* ORF by PCR and the product cloned into the *Bam*HI site of pBM272T
pBM272T-FL237	The FLAG epitope was introduced at the N-terminus of the *UFD1* ORF by PCR and the product cloned into the *Bam*HI site of pBM272T
pGEX-2TK-227	GST fusion to the complete coding sequence of *UBA2* in pGEX-2TK
PGEX-2TK-237	GST fusion to the complete coding sequence of *UFD1* in pGEX-2TK

*c*II + *Sma* I digestion was introduced at a convenient location inside the insert. In the case of *PIP1,* the disruption was made close to the 5′ end of the gene by insertion of the *HIS3* gene at position 835. A disruption of *UBA2* was made by replacing the fragment from *Sty*I (285)–*Nhe*I (795) by the *HIS3* gene. For the partial disruption of *UBA2,* the *HIS3* gene was introduced in the *Cla*I site at position 1784. For the *UFD1* gene disruption, the *Xcm*I site at position 898 of this gene was used. The constructs were digested with the appropriate restriction enzymes in order to produce a fragment with which to transform the diploid yeast strain W303, and His^+ transformants were selected. Disruptions were verified by Southern analysis of chromosomal DNA digests, and yeast sporulation and tetrad analyses performed (Kaiser et al. 1994).

To regulate the expression of the *UBA2* and *UFD1* genes, we placed the complete coding sequence of these genes under the control of the *GAL1* promoter. These plasmids (pBM272T-FL227D and pBM272T-FL237D) were used to transform the diploid strains MD2 and MD3. Tetrad dissections were performed to identify His^+ Ura^+ spores carrying the disruption and the covering plasmid, and yielded strains MD4 and MD5.

In vitro activity assays

Cell extracts for in vitro polyadenylation assays were prepared from the haploid strains W303-1A, MD1, MD4, MD5, and MD6. One-liter cultures were grown to an OD of 1 in SD media and harvested, or grown to an OD of 0.5 in SG media, harvested, resuspended in 1-l of either SG or SD medium and grown for an additional 5 h. Cells were harvested and extracts prepared by the protocol previously described by Chen and Moore (1992), using the modifications indicated below. Cells were collected and resuspended in 60 ml of buffer A (1 M sorbitol, 50 mM TRIS-HCl pH 7.8, 10 mM $MgCl_2$, 30 mM DTT), agitated at 80 rpm at 30° for 60 min, chilled for 15 min on ice, collected and resuspended in 4–4.5 ml of buffer B (10 mM HEPES-KOH pH 7, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1 mM PMSF, 2 mM pepstatin A, 0.6 mM leupeptin), to which 7 ml of glass beads (0.5 mm diameter) were then added. The mix was vortexed for four 30-s pulses with a 1-min cooling period on ice between pulses. The KCl concentration was adjusted to 0.2 M, and the tubes were rotated for 60 min at 4°. The solution was spun for 30 min at 15 000 × *g*, the supernatant removed, and cleared by ultracentrifugation at 50 000 rpm in an SW50.1 rotor (Beckman) for 1 h. Ammonium sulphate was added to the supernatant to give 40% saturation. After 30 min of rotation, the precipitate was collected by centrifugation at 12 000 × *g* for 30 min at 4°. The pellet was resuspended in 100 ml of buffer C (20 mM HEPES-KOH pH 7.9, 0.2 mM EDTA, 0.5 mM DTT, 20% glycerol, 1 mM PMSF, 2 mM pepstatin, 0.6 mM leupeptin) and dialyzed for 2 h (with one change after 1 h) against 1-l buffer C at 4°. The extract was frozen in liquid nitrogen and stored at −70°. The protein concentration of each extract was determined using the BioRad protein assay kit.

The extracts were tested for in vitro cleavage and polyadenylation activity using the protocol previously described (Chen and Moore 1992). The substrate was a ^{32}P-labeled RNA generated by in vitro transcription of *Ava*I-digested pGAL7-1 DNA (Chen and Moore 1992). This plasmid contains a fragment of *GAL7* encompassing the poly(A) site, and the precursor generated from its transcription allows the detection of cleavage and polyadenylation activity. Reactions were performed using equal amounts of protein. RNAs purified from the reactions were run on 8 M urea/5% polyacrylamide gels to resolve the bands corresponding to precursor and $poly(A)^+$ product. The percentage of total RNA which was polyadenylated was calculated using phosphorimager analysis to determine the amounts of precursor and polyadenylated RNA in each lane.

To assay the topoisomerase I activity, processing extract at different dilutions was mixed with 1 µg of supercoiled pBR322 plasmid in a 10-µl reaction containing 100 µg/ml bovine serum albumin, 1 mM DTT, 0.2 mM PMSF, 10 mM HEPES-KOH, pH 7.5, 150 mM KCl, 10 mM magnesium acetate, 10% glycerol, and 0.5 mM EDTA (Zhelkovsky and Moore 1994) and incubated for 15 min at 30° C. The reactions were stopped by the addition of 2 µl 5xSDS sample buffer (10% SDS, 10 mM DTT, 50% glycerol, 0.01% bromophenol blue), electrophoresed in a 1% agarose gel in 89 mM TRIS-borate, 2 mM EDTA, pH 8.0, and DNAs visualized by ethidium bromide staining.

Antibodies, immunoblotting and immunoprecipitations

Antibodies specific for Uba2 and Ufd1 were generated using GST-fusion proteins as antigens. The complete coding sequence of the *UBA2* or *UFD1* gene was introduced into the polylinker region of the pGEX-2TK vector (Pharmacia) to overexpress the proteins as GST fusion in *E. coli*. The GST fusion proteins were purified according to the Pharmacia guidelines, and rabbit polyclonal sera produced (Cocalico Biologicals Reamstorm, Pa.).

To purify polyclonal sera, affinity columns were prepared by first rehydrating 1.3 g of activated CH Sepharose-4B (Pharmacia) in 50 ml of 1 mM HCl at 4° C for 60 min, followed by three washes with 50 ml of 1 mM HCl, to give a final matrix volume of 4 ml. Some 6 mg of each GST fusion protein was dialyzed against

Coupling Buffer (0.5 M NaCl, 100 mM $NaHCO_3$ pH 8.0) for 2 h with one change of buffer. The protein solution was then brought to a volume of 40 ml with Coupling Buffer and covalently bound to the column matrix according to the manufacturer's instructions. Then 10 ml of polyclonal antiserum raised against either GST-Uba2 or GST-Ufd1 was diluted to 100 ml with 10 mM TRIS-HCl, pH 7.5. A GST affinity column was recursively perfused with serum at 30 ml per h, for 10 h to remove antibodies specific for the GST tag. The flowthrough fraction was applied to either a GST-Uba2 or GST-Ufd1 affinity column and recirculated for 10 h. Columns were washed with 20 bed volumes of 10 mM TRIS-HCl, pH 7.5 and then with 20 bed volumes of 0.5 M NaCl, 10 mM TRIS-HCl, pH 7.5, and antibodies eluted with 15 bed volumes 100 mM triethylamine pH 11.5. Eluent was collected in 2-ml fractions into tubes containing 100 mM Tris-HCl, pH 8.0, and peak fractions pooled and dialyzed against 100 volumes of phosphate-buffered saline (PBS), and stored at −20° C.

For immunoblotting, samples were run on 10% polyacrylamide/SDS gels and electrotransferred to Immobilon-P membranes (Millipore). The blots were blocked and processed for immunodetection of antigens as described by Kessler et al. (1995). Protein bands were detected using alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse antibodies (Promega) and the alkaline phosphatase substrates 5-bromo-4-chloro-3-indolyl phosphate and *p*-nitrotetrazolium blue (Promega), or with peroxidase-coupled anti-rabbit or anti-mouse antibodies and the ECL Detection Kit (Amersham).

For immunoprecipitations, 1-l cultures of the MD4 and MD5 strains were grown in SG media to an OD_{600} of 1, harvested, washed once with PBS and resuspended in 2 ml of lysis buffer containing 30 mM HEPES pH 7.4, 100 mM potassium acetate, 2 mM magnesium acetate, 0.4 M sorbitol, 1% Triton X-100,10 mM N-ethylmalemide (NEM), 1 mM PMSF, 0.3 mM Tosylphenylalanine chloromethyl ketone (TPCK), and 0.14 mM Tosyllysine chloromethyl ketone (TLCK). Cells were lysed by vortexing for 2 min with 2 g of 0.5-mm glass beads, and insoluble cell debris pelleted by centrifugation at 14 000 × *g* for 20 min at 4° C. Cell extract (1.5 mg) was incubated with 5 µg of recombinant Pap1 (Kessler et al. 1995) and the indicated antibodies in a total volume of 1 ml lysis buffer for 2 h on ice, followed by the addition of 50 µl of Protein A-Sepharose beads (Gibco BRL) and further incubation on a rotator for 2 h at 4° C. The beads were then collected and washed four times with the lysis buffer. Proteins were released from the beads by boiling in 30 µl of protein sample buffer plus 10 mM DTT for 3 min, followed by electrophoresis on a 10% acrylamide/SDS gel and immunoblotting as described above. In this case, the wash solutions for the blot included 0.05% sodium deoxycholate. LexA antiserum was obtained from R.Brent, Pap1 monoclonal antibodies from M. Kessler (Kessler et al. 1995), and FLAG M5 antibodies from Eastman Kodak.

Results

Identification of Pap1-interacting proteins by the two-hybrid system

In order to identify factors implicated in yeast polyadenylation, we have used the two-hybrid method to detect protein-protein interactions (Chien et al. 1991; Gyuris et al. 1993). In this screen, expression in yeast of a LacZ reporter gene under control of the LexA operator sequence depends on recruitment of the Gal4 activator domain to the promoter via the interaction of two different hybrid proteins. For this purpose, we created a fusion protein consisting of the entire Pap1 protein joined to the C-terminus of LexA. The LexA-Pap1 fusion was detectable in immunoblots using LexA-(Golemis and Brent 1992) or Pap1-specific (Kessler et al. 1995) antibodies (data not shown), and alone, failed to activate the reporter gene (Table 3). The reporter strain with LexA-Pap1 fusion was then transformed with three different plasmid libraries bearing yeast genomic fragments fused to sequences encoding the SV40 T-antigen nuclear localization signal followed by the Gal4 activation domain.

Approximately 500 000 transformants from each library were screened for β-galactosidase expression. Thirty-four plasmids activated the reporter gene in a manner dependent on cotransformation with pLexA-Pap1. For further study, we selected the twelve plasmids which gave the strongest activation. By partial sequencing of the inserts in these plasmids, we determined that these represented three different open reading frames (ORFs) fused in frame to the Gal4 activating domain. In plasmids containing the first ORF, the shortest common region included 621 nucleotides before the stop codon. We called this gene *PIP1*, for *P*ap1 *I*nteracting *P*rotein. In the second set (*PIP2*), 345 nucleotides were shared before the stop codon, and in the third one (*PIP3*), there were 564 nucleotides before the stop codon. PCR was used to subclone the partial sequence of the three ORFs in frame with the Gal4 activation domain. In all three cases, this sequence was sufficient for activation of the reporter gene, indicating that the interaction does not require additional sequences in the library insert.

To determine the specificity of the interaction between these proteins and Pap1, we tested their ability to interact with other LexA fusion proteins. These test proteins have different functions inside the cell, from cell architecture to regulation of gene expression, and included *Drosophila* Bicoid, human lamin C, human Cdc2, yeast Fus3, human Ras, human Rb, yeast topoisomerase 1, and yeast Sir4. In the case of Pip1, a strong interaction with lamin C, topoisomerase I and Sir4 (data not shown) suggested a less specific role for Pip1 in the yeast cell, and we did not pursue analysis of the gene's function.

Table 3 Interaction of Pip2 (Uba2) and Pip3 (Ufd1) carboxyl domains with various LexA fusion proteins

Fusion Protein	GAD[a]	GAD-PIP2[a] (1566-1911)	Gad-PIP3[a] (523-1086)
LexA	1.1 ± 0.2	1.5 ± 0.2	1.3 ± 0.2
LexA-Pap1	1.3 ± 0.2	16.3 ± 2.0	24.0 ± 3.1
LexA-Bicoid	1.3 ± 0.2	1.5 ± 0.2	2.0 ± 0.3
LexA-Cdc2	0.9 ± 0.1	0.6 ± 0.1	1.2 ± 0.2
LexA-Fus	1.5 ± 0.2	2.0 ± 0.3	1.9 ± 0.3
LexA-Lamin	1.6 ± 0.3	1.9 ± 0.3	1.1 ± 0.2
LexA-Ras	0.5 ± 0.1	0.7 ± 0.1	0.9 ± 0.2
LexA-Rb	0.9 ± 0.2	0.9 ± 0.2	1.1 ± 0.2
LexA-Sir4	1.5 ± 0.3	1.7 ± 0.3	1.8 ± 0.3
LexA-Top1	1.3 ± 0.3	1.7 ± 0.3	1.3 ± 0.2

[a] Interaction was assayed as the relative β-galactosidase activity of EGY40 strains carrying the reporter plasmid and the indicated fusion constructs
GAD, Gal4 activating domain

For Pip2 and Pip3, the interaction with Pap1 was clearly very specific, with no significant interaction being detected between these proteins and any of the controls (Table 3). This suggests that the Pip2 and Pip3 proteins may be involved in some aspect of Pap1 activity in the yeast cell.

Cloning of the PIP genes

A low-copy-number CEN plasmid library was screened to obtain the complete coding sequence of the three interacting genes. The *PIP1* gene encodes a 105-kDa protein of unknown function, which has previously been designated as gene YER032w, on Chromosome V, nucleotides 63006-65783, in the GenBank database. Disruption of the gene indicated that it was not essential. In the case of *PIP2*, the ORF frame is 1911 nucleotides long, giving a protein of 636 amino acids and a predicted molecular mass of about 71 kDa. Concurrently with this study, this gene was also isolated by Dohmen et al. (1995) as a homolog of E1 ubiquitin-activating enzymes, and given the designation *UBA2* by this group. For *PIP3*, the potential ORF has 1086 nucleotides, coding for a protein of 361 amino acids with a molecular mass of approximately 40 kDa. In the same time period, this gene was cloned by Johnson et al. (1995) as a gene involved in the ubiquitin-mediated protein degradation pathway, and named *UFD1*. For the remainder of this paper, we will refer to *PIP2* and *PIP3* as *UBA2* and *UFD1*, respectively.

Disruptions of the *UBA2* and *UFD1* genes showed that these two genes were essential, in agreement with previous reports (Dohmen et al. 1995; Johnson et al. 1995). In the case of *UBA2*, we also made a partial disruption close to the 3′end of the gene, to eliminate the region necessary for the interaction with Pap1, as defined by the minimal *UBA2* sequence which was active in the two-hybrid screen. This disruption was tolerated, but the haploid strain (MD1) grew significantly more slowly than the wild-type W303-1A strain, with a division time double that of the wild type. Microscopic examination of the MD1 cells indicated that they were larger and more elongated than the wild-type cells.

Uba2 and Ufd1 interact with Pap1 in vitro

It is important to confirm two-hybrid results by demonstrating that a complex of the two relevant proteins forms in vitro. We were not able to find evidence of interaction of Pap1 and Uba2 or Ufd1 by co-immunoprecipitation from cell extracts, probably because of the low abundance of these proteins in the extracts. However, we were able to show a specific interaction of Pap1 and Uba2 and Ufd1 from these extracts if the concentration of Pap1 was increased 25-fold in the extracts by addition of recombinant Pap1. For these experiments, we used extracts from strains expressing FLAG-tagged versions of either Uba2 or Ufd1 (strains MD4 and MD5, respectively). The yeast Pap1 can be efficiently immunoprecipitated by monoclonal antibodies against epitopes in either the amino or carboxyl termini of Pap1 (Kessler et al. 1995), as shown in the immunoblots of Fig.1A (lanes 1, 2, 6, and 7), which were probed with a mixture of the two Pap1 antibodies and developed with an alkaline phosphatase color reaction. The same immunoblots were also probed with rabbit polyclonal antibodies against either Uba2 or Ufd1 and immunoreactive proteins visualized with a luminescent detection reagent (Fig.1B). This analysis showed that a fraction of the Uba2 in the extract was associated with Pap1

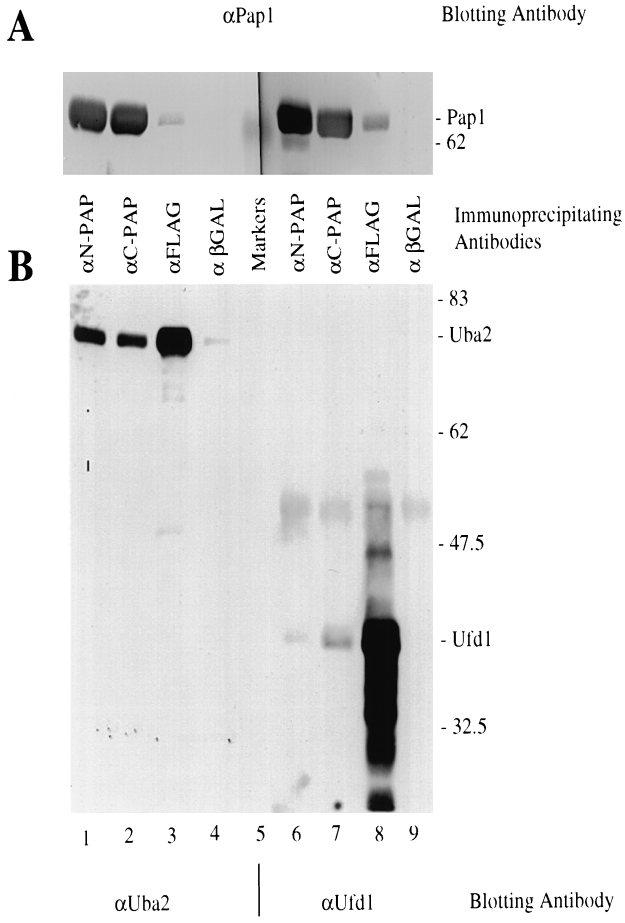

Fig. 1A, B Protein complex formation detected by immunoprecipitation. Some 1.5 mg of extract from cells expressing either FLAG-tagged Uba2 (lanes 1–4) or Ufd1 (lanes 6–9) was mixed with 5 μg of pure yeast rPap1 and the mixture immunoprecipitated with the indicated antibodies. Lanes 1 and 6, antibody against the N-terminal epitope of Papl (αN-PAP); lanes 2 and 7, antibody against the C-terminal epitope of Pap1 (αC-PAP); lanes 3 and 8, antibody against the FLAG epitope (αFLAG); lanes 4 and 9, antibody against *β*-galactosidase (α*β*GAL); lane 5, prestained protein markers, whose sizes are indicated on the right. **A** The blot was probed with Pap1 antibodies and immunoreactive proteins visualized by alkaline phosphatase color staining. **B** Lanes 1–4 were probed with antibodies against Uba2 and lanes 6–9 with antibodies against Ufd1, and immunoreactive proteins were detected by horseradish peroxidase-dependent luminescence

(Fig. 1B, lanes 1 and 2). Interestingly, Ufd1 could only be detected by co-immunoprecipitation with Pap1 antibody if the antibody specific for the C-terminal region of Pap1 was used (Fig. 1B, lanes 6 and 7), suggesting that Ufd1 might be interacting with Pap1 in such a way as to block binding of the N-terminal specific antibody. In a reciprocal experiment, aliquots of the same extracts were immunoprecipitated with the anti-FLAG antibody and blots probed for the presence of Pap1, Uba2, or Ufd1. In this case, Pap1 also co-immunoprecipitated with either protein (Fig. 1A, B, lanes 3 for Uba2 and lanes 8 for Ufd1). No Pap1, Uba2 or Ufd1 can be detected if a control antibody against β-galactosidase is used (Fig. 1A, B, lanes 4 and 9). These results indicate a specific, though not particularly robust interaction between Pap1 and Uba2 or Ufd1.

Analysis of the role of Uba2 and Ufd1 in poly(A) polymerase function

Immunoblot analysis of factors necessary for reconstitution of the in vitro polyadenylation reaction (Kessler et al. 1996) indicated that Uba2 and Ufd1 did not co-fractionate with the CF I, CF II, PF I, or PAP activities (data not shown) and suggests that these proteins are not components of the actual processing factors.

To address the question of what role the Ufd1 and Uba2 proteins might have in poly(A) polymerase function or regulation, we used the M4 and M5 strains, in which the only source of the proteins was the plasmid-borne, galactose-inducible gene. By shifting cells containing these plasmids to growth on glucose, the expression of the gene is repressed and the effects on processing activity of extracts can be examined. These haploid strains had the same doubling time as wild-type cells, demonstrating that the disruptions were covered by the *GAL1* plasmids. Northern blot analyses showed that the expression of the *UBA2* and *UFD1* genes was regulated by glucose in these cells, and no mRNA was detectable 1 h after the shift from galactose to this carbon source (data not shown). Immunoblots of cell extracts showed that the expression of each protein was not significantly increased when the *GAL1* promoter was used instead of the natural promoter (Fig. 2, lanes 1, 2, 4 and 5), and suggests that the cells maintain a tight regulation of the levels of these two proteins. After a 5-h shift to glucose-containing media, the level of each protein is greatly diminished (Fig. 2, lanes 2, 3, 5 and 6). In comparison, the level of Pap1 in the extracts did not change when cells were incubated with glucose instead of galactose (Fig. 2, lanes 7–10).

We tested the polyadenylation activity of extracts from cells grown continuously in galactose or from cells depleted of Ufd1 or Uba2 by a 5-h incubation in glucose-containing media. The cells continued to grow and doubled in density during the 5-h shift. The assay for polyadenylation uses radioactive precursor containing the *GAL7* poly(A) site (Fig. 3A, lane 1). Upon incubation with extract and ATP, this 295-nucleotide RNA is cleaved at the poly(A) site, and rapidly extended by the addition of 50–90 adenosines (Chen and Moore 1992). The 161-nucleotide upstream cleavage product does not accumulate because of this rapid polyadenylation, and the 134-nucleotide downstream product is quickly degraded, such that neither cleavage product is detected under the conditions used in this study. RNA purified from such reactions can be resolved into these different species by electrophoresis on urea/acrylamide gels, followed by autoradiography (Fig. 3). Depletion of Ufd1 resulted in extracts with only one-third of the polyadenylation activity of extracts from cells grown in galactose (Fig. 3, lanes 3 and 4). Two additional sets of extracts showed a similar processing defect in extracts from cells lacking Ufd1 (Fig. 3, lanes 5–8). In contrast to Ufd1 depletion, the percentage of polyadenylated RNA derived from extracts depleted of Uba2 was greater than that from extracts made from cells grown in galactose

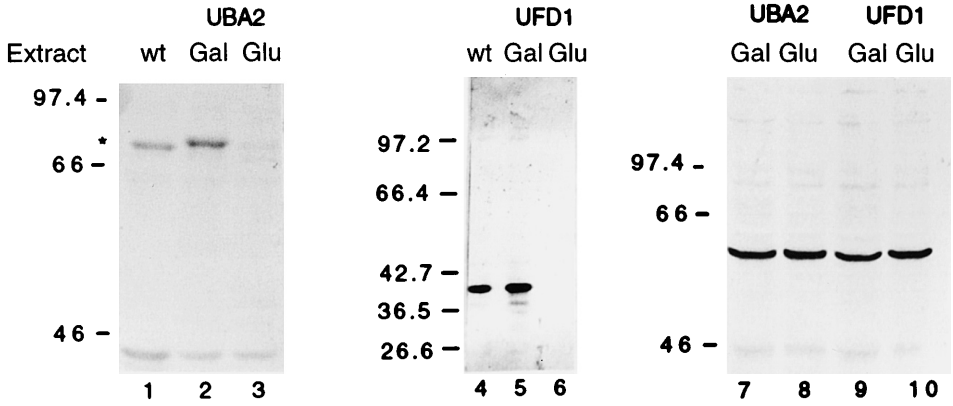

Fig. 2 Immunoblot analysis of yeast extracts using anti-Uba2 (lanes 1–3), anti-Ufd1 (lanes 4–6), or anti-Pap1 (lanes 7–10) antibodies. Lanes 1 and 4 contain extracts from the yeast W303-1A strain; lanes 2 and 7, extract from the M4 strain grown in galactose-containing media; lanes 3 and 8, extract from the same strain grown with galactose, then shifted for 5 h to glucose-containing medium; lanes 5 and 9, extract from the M5 strain grown with galactose; lanes 6 and 10, extract from this strain grown with galactose, then shifted for 5 h to glucose-containing medium. Equal amounts of protein were loaded in each lane. The positions of marker proteins are indicated

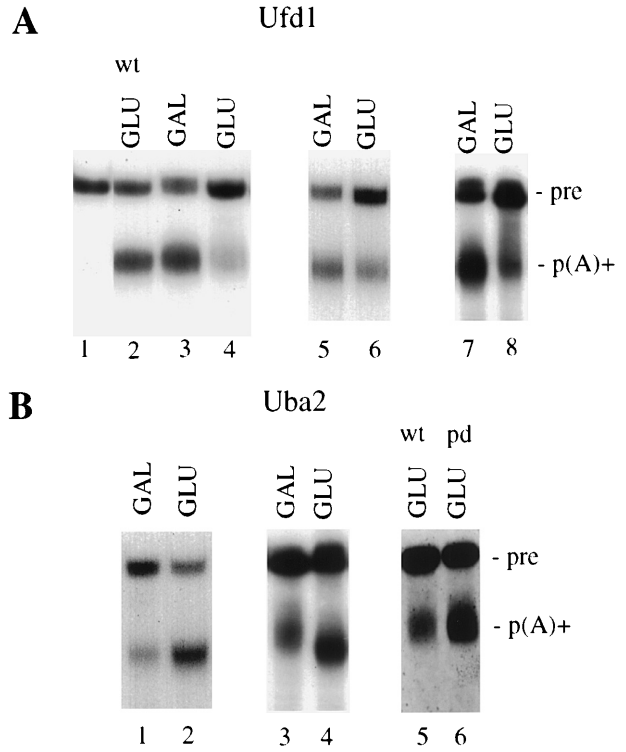

Fig. 3A, B In vitro polyadenylation assays. **A** In vitro assays using extracts from the MD5 strain grown continuously in galactose-containing medium (lanes 3, 5, and 7) or shifted for 5 h to glucose-containing medium (lanes 4, 6, and 8). Reactions were carried out as described in Materials and methods, the RNAs were purified, and resolved on a 5% acrylamide-8M urea gel, and visualized by autoradiography. Three independent preparations of extracts are shown. Lane 1 contains untreated precursor; lane 2 shows products from a reaction using extract from strain MD6, which contains the *UFD1* disruption covered by a plasmid bearing a genomic fragment containing the *UFD1* gene. The positions of precursor RNA (pre) and polyadenylated product [p(A)+] are indicated on the right. **B** In vitro assays using two separate extract preparations from the MD4 strain grown continuously in galactose-containing medium (lanes 1 and 3) or shifted for 5 h to glucose-containing medium (lanes 2 and 4); extract from wild-type W303-1A cells grown in glucose (lane 5); and extract from the MD1 strain containing the partial disruption of *UBA2* (lane 6). RNAs were analyzed as described in **A**

medium (Fig. 3B, lanes 1 and 2). Approximately 1.7 times more RNA had been processed in the extracts from glucose-grown cells, and a duplicate set of extracts showed the same increase in processing efficiency (Fig. 3B, lanes 3 and 4). Extracts were also prepared from the haploid strain MD1 carrying the partial *UBA2* disruption which was not lethal for cell growth. These extracts also showed an increase in polyadenylation activity when compared to wild type extracts (Fig. 3B, lanes 5 and 6). Extracts prepared from cells grown with either glucose or galactose as the carbon source show no difference in the in vitro processing of *GAL7* precursor (Sadhale and Platt 1992), suggesting that the changes in processing efficiencies described above are a consequence of loss of Ufd1 or Uba2.

Chromatographic fractions enriched in Ufd1 or Uba2 but lacking polyadenylation factors were added to Ufd1- or Uba2-depleted extracts in an effort to restore wild-type levels of processing, but these experiments were not successful (data not shown). The GST fusions used to prepare specific antibodies were not tested in this type of experiment because they were not active in attempts to demonstrate a direct interaction with Pap1 synthesized in vitro. These negative results are inconclusive since without further knowledge of the biochemical role of Ufd1 and Uba2, it is difficult to decide whether the various sources of the proteins are all active.

One indication of the consistency of the extract preparation was that the same amount of poly(A) polymerase was found in each extract (Fig. 2). As an independent assessment that other biochemical activities in the extracts were not changed, the ability of different extracts to relax supercoiled DNA was determined. This activity is due to the presence of topoisomerase I in the extracts. By two-hybrid analysis, neither Ufd1 or Uba2 interacted with the yeast topoisomerase I. Incubation of supercoiled plasmid DNA with extract results in the accumulation of partially relaxed circular DNAs (Fig. 4A, lanes 1 and 2). Serial dilutions of processing extracts prepared from cells carrying the galactose-regulated *UFD1* or *UBA2* gene showed that the extracts from cells grown in galactose- or in glucose-containing media had equivalent levels of topoisomerase I activity,

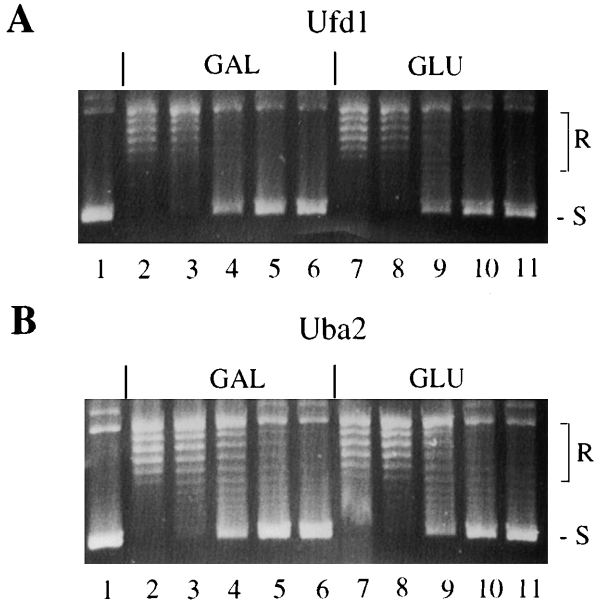

Fig. 4A, B Determination of the DNA relaxing capacity of extracts. Serial dilutions beginning with 3.5 μg of extract were incubated with supercoiled plasmid DNA as described in Materials and methods, and the products resolved on a 1% agarose gel. Lane 1, supercoiled DNA (S) without treatment; lanes 2 and 7, no dilution; lanes 3 and 8, 1/3.16 dilution; lanes 4 and 9, 1/10 dilution; lanes 5 and 10, 1/31.6 dilution; lanes 6 and 11, 1/100 dilution. **A** extracts from the MD5 strain were grown continuously in galactose-containing medium (lanes 2–6) or shifted for 5 h to glucose-containing medium (lanes 7–11). **B** Extracts from the MD4 strain were grown continuously in galactose-containing medium (lanes 2–6) or shifted for 5 h to glucose-containing medium (lanes 7–11)

Table 4 Interaction of Uba2 and Ufd1 as determined by β-galactosidase activity using two-hybrid analysis

LexA fusion	GAD fusion	β-galactosidase activity[a]
LexA-UBA2	GAD-UFD1	34.5 ± 6
LexA-UBA2	GAD-DP1	0
LexA-PAP1	GAD-UFD1	166 ± 6
LexA-PAP1	GAD-FIP1	234 ± 37
LexA-CDC2	GAD-UFD1	0

[a] Expressed as units/minute/number of cells

as judged by the amount of residual supercoiled DNA left in the reaction mixture as the dilution factor is increased (Fig. 4A for Ufd1 and Fig. 4B for Uba2).

Ufd1 and Uba2 interact with each other

The observation that Ufd1 and Uba2 interacted specifically with Pap1 and previous work suggesting that both proteins might be components of the ubiquitin-mediated protein degradation pathway raised the possibility that the two proteins might also interact with each other. To test this idea, a LexA-Uba2 fusion protein was created. This hybrid protein contains the entire *UBA2* coding sequence and can rescue the lethality of a *UBA2* gene disruption. A plasmid carrying this construct was introduced into the EGY40 reporter strain which had been previously transformed with a plasmid bearing the entire *UFD1* coding sequence fused to the *GAL4* activation domain. As measured by β-galactosidase activity, the interaction of Ufd1 and Uba2 was about one-fifth that of Ufd1 and Pap1 (Table 4). Interestingly, the interaction of Ufd1 and Pap1 was almost as strong (about two-thirds) as that of Pap1 and Fip1, a component of PF I which interacts directly with Pap1 (Preker et al. 1995).

Discussion

Using the two-hybrid system (Fields and Sternglanz 1994), we have isolated *UBA2* and *UFD1* as genes encoding proteins which interact specifically with the *S. cerevisiae* poly(A) polymerase (Pap1), an enzyme essential for the proper formation of mRNA 3′-ends. Both of these Pap1-interacting proteins have been linked to the ubiquitin-mediated protein degradation pathway (Dohmen et al. 1995; Johnson et al. 1995). This multistep process is involved in the turnover of many intracellular proteins, and is one of the ways used by the cell to control the levels of regulatory proteins such as cyclins and transcription factors (for review, see Ciechanover and Schwartz 1994; Hochstrasser 1995; Hochstrasser 1996). Uba2 has strong homology to ubiquitin-activating, or E1, enzymes, which activate ubiquitin by first adenylating it and then linking it to a conserved cysteine residue in E1. The activated ubiquitin is then transferred to a cysteine residue in one of several ubiquitin-conjugating (E2) enzymes. The E2 enzyme then transfers the ubiquitin to a target protein. This process sometimes requires a third factor, ubiquitin protein ligases (E3), which recognize the target protein and can also be an intermediate in the ubiquitin transfer. The further addition of a polyubiquitin chain leads to degradation in a 26S proteasomal complex.

Uba2 has a cysteine equivalent to the one which forms the ubiquitin-thioester bond in Uba1, a yeast protein with E1 enzymatic properties (McGrath et al. 1991). While this cysteine is essential for Uba2 function in vivo, a thioester linkage of Uba2 with ubiquitin could not be demonstrated under conditions which readily yield this adduct with Uba1 (Dohmen et al. 1995). This result implies that the function of Uba2 in the cell is not strictly analogous to that of an E1 enzyme, and led to the postulate that Uba2 might act more like an E2 enzyme, or even form a thioester conjugate with a protein other than ubiquitin. Uba2 is found in the nucleus (Dohmen et al. 1995), while Uba1 is primarily cytoplasmic (McGrath et al. 1991). The *UFD1* gene was previously cloned by analysis of mutations which stabilized a ubiquitin-β-galactosidase fusion in yeast cells (Johnson et al. 1995). The missense allele of *UFD1* isolated in this screen did not prevent the post-translation multi-ubiquitination of the β-galactosidase fusion, suggesting that Ufd1 functions at a later step in the degradation pathway.

Because Uba2 and Ufd1 do not cofractionate with factors needed to reconstitute the in vitro polyadenylation reaction, it is unlikely that they are actual components of the polyadenylation machinery. However, the very specific interaction with Pap1 in the two-hybrid analysis in vivo, the in vitro co-immunoprecipitation with Pap1, and the fact that depletion of cells of these proteins affects the efficiency of in vitro polyadenylation suggests that Uba2 and Ufd1 may have a role in regulating Pap1 activity, and that ubiquitination may be involved. Any model invoking Pap1 regulation must accommodate the observations that Pap1 levels did not change when either *UBA2* or *UFD1* expression was shut off, that a new species of Pap1 was not detected, and that removal of Uba2 or Ufd1 has opposite effects on polyadenylation efficiency. Mutation of genes involved in ubiquitination results in marked accumulation of previously unstable proteins (Ciechanover and Schwartz 1994; Hochstrasser 1995). The fact that Pap1 did not accumulate upon Uba2 or Ufd1 depletion suggests that any Uba2/Ufd1-mediated regulation does not occur via effects on Pap1 stability.

Uba2 could directly regulate Pap1 activity via a modification, perhaps adenylation or ubiquitination, which is labile and therefore not detected in our analysis. Alternatively, the function of Uba2 or Ufd1 could be to modify a factor associated with Pap1, and this would explain why there was no change in the level of Pap1 or its mobility. This factor could be a regulatory factor or one of the constitutive polyadenylation factors which recruit Pap1

to cleaved mRNA precursor (Wahle and Keller 1996). In further support of these possibilities, evidence is accumulating that ubiquitination may have a role other than direct targeting of proteins to the proteasome (Hochstrasser 1996). For example, ubiquitination, but not proteolysis, of the IκB kinase, or a regulatory subunit, is necessary for kinase activity (Chen et al. 1996).

Post-translational modifications have not been reported for the yeast poly(A) polymerase (Kessler et al. 1995; Lingner et al. 1991). However, the hyperphosphorylation of vertebrate poly(A) polymerase (Ballantyne et al. 1995; Thuresson et al. 1994) has been shown to down-regulate its activity and this inhibition probably contributes to the general repression of mRNA synthesis during mitosis (Colgan et al. 1996). The yeast enzyme does not have the serine/threonine-rich region found at the carboxyl end of the vertebrate enzyme, which is the likely site of phosphorylation. An alternate mechanism such as ubiquitination may be used to regulate Pap1 activity as yeast cells enter the M-phase of the cell cycle. The two-hybrid analysis indicates that Ufd1 has an affinity for Pap1 which is similar to that of Fip1, a component of a factor which helps to recruit Pap1 to cleaved mRNA precursor. The co-immunoprecipation data suggests that Ufd1 is interacting with the N-terminus of Pap1, which contains a domain essential for specific polyadenylation (Zhelkovsky et al. 1995). Interaction with specific factors increases the processivity of poly(A) polymerase in physiological conditions (Wahle and Keller 1996), and a plausible means of inhibiting poly(A) polymerase activity would be to disrupt its association with these factors via post-translational modification of Pap1 or interaction with a regulatory protein.

If Uba2 is involved in down-regulating Pap1 activity, removal of Uba2 would result in an increase in polyadenylation efficiency. Ufd1 may somehow be necessary to remove the modified Pap1 from the cellular pool of usable polyadenylation components, or to simply remove a modification made by Uba2. With either scenario, in the absence of Ufd1, an inactive Pap1 would accumulate and possibly compete with active Pap1 in the assembly of polyadenylation complexes, leading to a decrease in polyadenylation efficiency.

Further experiments should reveal more about the function and mode of action of Uba2 and Ufd1 with regard to the regulation of mRNA polyadenylation. The two-hybrid interaction of Uba2 and Ufd1 supports the hypothesis that they function in the same pathway, and it will be interesting to see if a pathway involving Uba2 and Ufd1 has a broader role in cell function. Since Uba2 is localized to the nucleus (Dohmen et al. 1995), other targets of its action are likely to be nuclear proteins.

Acknowledgements We appreciate the generosity of P. Bartel, R. Brent, B. Cochran, D. Dawson, S. Fields, R. Finley, J. Gyuris, S. Hanes, P. Hieter, M. Johnston, S. Reddy and R. Rothstein in providing the gifts of plasmids and strains necessary for these experiments. We would also like to thank E. Birney for searching for RNA recognition motifs in our sequences. We acknowledge B. Dagarag, S. Helmling, L. Hyman, M. Kessler, S. Schneider, A. Skvorak, Jing Zhao, and A. Zhelkovsky for their helpful comments and technical assistance and F. Estruch and J. E. Perez for critically reading the manuscript. We especially thank J. Dohmen and A. Varshavsky for sharing results prior to publication. This work was supported by grant #NP909 from the American Cancer Society to C. L. Moore.

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Note added in proof

A recent report by Russnak et al. has identified Pip1 as Fir1. Fir1 interacts with Ref2, a protein which stimulates the processing of weak poly(A) sites.

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