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Conditional Defect in mRNA 3′ End Processing Caused by a Mutation in the Gene for Poly(A) Polymerase

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Maturation of most eukaryotic mRNA 3′ ends requires endonucleolytic cleavage and polyadenylation of precursor mRNAs. To further understand the mechanism and function of mRNA 3′ end processing, we identified a temperature-sensitive mutant of *Saccharomyces cerevisiae* defective for polyadenylation. Genetic analysis showed that the polyadenylation defect and the temperature sensitivity for growth result from a single mutation. Biochemical analysis of extracts from this mutant shows that the polyadenylation defect occurs at a step following normal site-specific cleavage of a pre-mRNA at its polyadenylation site. Molecular cloning and characterization of the wild-type allele of the mutated gene revealed that it (*PAP1*) encodes a previously characterized poly(A) polymerase with unknown RNA substrate specificity. Analysis of mRNA levels and structure in vivo indicate that shift of growing, mutant cells to the nonpermissive temperature results in the production of poly(A)-deficient mRNAs which appear to end at their normal cleavage sites. Interestingly, measurement of the rate of protein synthesis after the temperature shift shows that translation continues long after the apparent loss of polyadenylated mRNA. Our characterization of the *pap1-1* defect implicates this gene as essential for mRNA 3′ end formation in *S. cerevisiae*.

Polyadenylation of eukaryotic mRNAs requires specific endonucleolytic cleavage of a precursor mRNA followed by polymerization of adenosine residues to a tail length ranging from about 70 residues in the yeast *Saccharomyces cerevisiae* to nearly 300 residues in humans (4, 29, 46). In addition to an endonuclease and a poly(A) polymerase, this process appears to require several other enzymatic activities which enhance the specificity and the rate of each step (7, 8, 13, 14, 28, 41, 42). Recent progress on the purification and characterization of these enzymes has begun to shed light on the mechanism of polyadenylation as well as to underscore the complexity of the process itself (15, 26, 32, 40, 41, 45). Purification of poly(A) polymerases from calf thymus (32, 44), vaccinia virus (12), and *S. cerevisiae* (25) allowed the identification of the genes encoding these enzymes by reverse genetic techniques. However, only the bovine poly(A) polymerase, in conjunction with a specificity factor, demonstrates the substrate specificity required for cellular mRNA polyadenylation (32).

The development of a cell-free system for the accurate and efficient polyadenylation of *S. cerevisiae* mRNAs indicated the mechanistic similarities between mRNA 3′ end formation in lower and higher eukaryotes (4, 5). In the yeast system in vitro, polyadenylation follows site-specific cleavage by an endonuclease. Both steps demonstrate a high degree of specificity; mutated and metazoan mRNAs do not function correctly as cleavage substrates, and polyadenylation does not occur on uncleaved RNAs (1, 4, 6, 39). Fractionation of yeast polyadenylation extracts yields at least four factors, a poly(A) polymerase, a polyadenylation factor, and two cleavage factors, required to reconstitute the reaction (6). The RNA sequences specifying mRNA 3′ processing in yeast cells appear to be different from those in mammalian cells (4, 20, 21). While mammalian cells clearly require the highly conserved sequence AAUAAA (11, 47), no universally accepted signal for mRNA polyadenylation has emerged from recent studies on mRNA 3′ end formation (22, 36, 38). This finding implies that similarities that may exist between the enzymes required for polyadenylation in the two systems may belie important differences in their biological specificity.

The availability of the yeast system has opened the way for the application of powerful genetic techniques to the study of the mechanism and function of polyadenylation. As one step in this type of approach, we applied a polyadenylation assay in vitro to screen a collection of temperature-sensitive mutants for defects in mRNA 3′ end processing. We report here the identification and characterization of a temperature-sensitive mutant with a defect in the yeast poly(A) polymerase.

MATERIALS AND METHODS

Yeast strains and genetic techniques. General yeast genetic techniques were carried out as described by Rose et al. (35). The experiments reported here were carried out in the following strains: A364A (*MATa ade1 ade2 ura1 his7 lys2 tyr1 gal1*), EJ101 (*MATα his pep4-3 prb1 prc1*), S288C (*MATα gal2*), T481 (*MAT ade2-101 lys2-801 ura3-52*), T581 (*MATα ade2-101 his3-208 ura3-52*), UR3148-1B (*MATa ade1/ade2 lys2 gal1? ura3-52 pap1-1*). The collection of temperature-sensitive mutants derived from mutagenesis of A364A (18) was generously provided by Mike Culbertson.

The ts148 isolate (the 148th isolate tested) was successively outcrossed to S288C, T581, and T481 to finally generate UR3148-1B, which was used in the experiments in this study.

Revertants of *pap1-1* were isolated by spreading 10^8 cells on each of 10 YEPD plates and incubating each at 37°C. After 10 days, 15 temperature-resistant colonies were picked and were characterized further.

Preparation of polyadenylation extracts. To facilitate the screening of the large number of isolates in the Hartwell

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collection (18), a rapid, small-scale method for preparing yeast whole-cell extracts was derived from the protocol of Lin et al. (24). Cells were grown with shaking in YEPD medium (35) at 25°C to an A_{600} of between 2 and 6. Cells were harvested by centrifugation at 1,000 × *g* for 5 min, and the pellet was resuspended in 0.15 ml of buffer S (50 mM Tris-HCl [pH 7.8], 1 M sorbitol, 10 mM $MgCl_2$, 30 mM dithiothreitol [DTT]) and transferred to a sterile 1.5-ml microcentrifuge tube. The cell suspension was incubated at 25°C for 15 min, and the cells were harvested by centrifugation at 1,000 × *g* for 5 min. Cells were converted to spheroplasts by resuspension in 0.15 ml of buffer S containing zymolyase (100T; ICN) at 0.13 mg/ml and then incubated at 25°C for 40 min with gentle shaking. The spheroplasts were collected by centrifugation (4°C) at 1,000 × *g* for 5 min and resuspended in 0.1 ml of buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES]-KOH [pH 7.0], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT; 4°C) and disrupted with a microcentrifuge tube sample pestle (Marsh Biomedical, Rochester, N.Y.). The suspension was supplemented with 0.01 ml of 2 M KCl and incubated at 4°C for 30 min with frequent mixing. The cellular debris was removed by centrifugation (4°C) at 10,000 × *g* for 30 min, and the collected supernatant was assayed immediately or frozen on dry ice and stored at −70°C. The activity of these preparations was stable for at least 12 months and was not significantly diminished by thawing and refreezing.

Large-scale, ammonium sulfate-fractionated polyadenylation extracts were prepared and assayed as previously described (4).

Polyadenylation in vitro. Processing reactions were carried out as previously described, using synthetic radiolabeled *CYC1* pre-mRNA as a substrate (4), except that the small-scale extracts described above were substituted for more purified extract at a concentration of 20% (vol/vol).

Temperature shift and isolation of total RNA from yeast cells. Cells were grown in YEPD to a concentration of 2 × 10^6 to 4 × 10^6 cells per ml at 25°C, at which time the culture was split and half was incubated with shaking at 35°C. At various times, approximately 4 × 10^8 cells were pelleted by centrifugation at 1,000 × *g* for 5 min, washed with 1 ml of water, and pelleted by centrifugation; the cell pellet was frozen on dry ice and stored at −20°C.

Total RNA was isolated from pellets containing approximately 4 × 10^8 cells. Pellets were resuspended in 0.4 ml of RIB buffer (0.2 M Tris-HCl [pH 7.5], 0.5 M NaCl, 1% sodium dodecyl sulfate [SDS]) plus 2 μl each of diethylpyrocarbonate and 2-mercaptoethanol. To each tube was added 400 ml of acid-washed glass beads and 0.4 ml of PCI-RIB (phenol-chloroform-isoamyl alcohol [48:1:1] saturated with RIB buffer). Cells were disrupted by mixing on a Vortex mixer at maximum speed for 90 s and then cooled on ice for 30 s. The mixing-cooling cycle was repeated three times, after which the mixture was centrifuged at 10,000 × *g* for 2 min. The supernatant was removed to a sterile 1.5-ml microcentrifuge tube containing 0.4 ml of PCI-RIB and 2 μl of diethylpyrocarbonate, the aqueous and organic phases were mixed thoroughly, and the mixture was centrifuged for 3 min at 10,000 × *g*. The aqueous phase was removed and extracted one more time with 0.4 ml of PCI-RIB and centrifuged as before; the nucleic acids were precipitated from the aqueous phase by the addition of 0.8 ml of 95% ethanol (−20°C) and incubated at −20°C for 1 h. The precipitated nucleic acids were collected by centrifugation at 10,000 × *g* for 10 min. The pellet was washed with 0.2 ml of 70% ethanol, dried, and resuspended in 0.1 ml of sterile water.

Fractionation of RNA on oligo(dT)-cellulose. RNAs were fractionated on oligo(dT)-cellulose (Collaborative Research type III) as described by Herrick et al. (19). RNA (50 μg or less) in 25 μl of water was mixed with an equal volume of 2× BB (40 mM HEPES-KOH [pH 7.5], 1 M NaCl, 2 mM EDTA, 0.2% SDS) and incubated at 65°C for 15 min. This mixture was added to 50 μl of oligo(dT)-cellulose equilibrated in 1× BB, and the mixture was incubated at room temperature for 15 min with frequent mixing. The mixture was centrifuged at 10,000 × *g* for 5 min; the supernatant was removed and saved. The resin was washed with 0.1 ml of 1× BB and centrifuged for 5 min at 10,000 × *g*, and the supernatant was combined with that from the previous step; this unbound RNA was precipitated by the addition of 2 volumes of ethanol. The resin was washed twice as described above with LSBB (20 mM HEPES-KOH [pH 7.5], 0.1 M NaCl, 1 mM EDTA, 0.1% SDS), and the supernatants were discarded. The bound poly(A)$^+$ RNA was eluted from the resin by mixing with 0.2 ml of sterile water followed by centrifugation at 10,000 × *g* for 5 min. The supernatant was removed to a sterile 1.5-ml microcentrifuge tube containing 10 μg of *Escherichia coli* tRNA and 6.7 μl of 3 M potassium acetate (pH 5), and 0.4 ml of ethanol (−20°C) was added. After incubation at −20°C for at least 2 h, the precipitated RNAs were collected by centrifugation at 10,000 × *g* for 10 min. RNA pellets were washed with 70% ethanol, dried, and resuspended in 20 μl of sterile water before further use. A quantitative assessment of this technique, using products of cleavage and polyadenylation of synthetic, radiolabeled *CYC1* pre-mRNA in vitro, indicated that the unbound fraction contains greater than 90% of the unpolyadenylated substrate, while the bound fraction contains greater than 90% of the poly(A)$^+$ product [average poly(A) tail length of 60 residues].

RNA hybridization (Northern blot) analysis. Total RNA samples (10 to 30 μg) were suspended in 50 μl of MFF (1× MOPS [0.2 M morpholinopropanesulfonic acid {pH 7.0}, 50 mM sodium acetate, 5 mM EDTA], 6.5% formaldehyde, 50% deionized formamide) and incubated at 65°C for 15 min. The denatured RNA was mixed with 10 μl of 1 mM EDTA (pH 8)–0.25% bromophenol blue–0.25% xylene cyanol–50% glycerol and loaded onto a 1.2% agarose–1× MOPS–2.2 M formaldehyde gel; the RNAs were separated by electrophoresis at 5 V/cm until the xylene cyanol dye had migrated 14 cm from the origin. The gel was rinsed for 20 min in 250 ml of 10× SSC (1× SSC is 150 mM NaCl plus 15 mM trisodium citrate [pH 7.0]), and the RNA was transferred to a GeneScreen Plus (Dupont) membrane by capillary action (27). After transfer, the membrane was washed in 10× SSC for 15 min and the gel was dried under vacuum at 80°C for 2 h. The membrane was prehybridized at 60°C for 8 h in 4 ml of 25 mM $KHPO_4$ (pH 7.4) per ml–5× SSC–5× Denhardt's solution (27)–50 μg of sonicated, denatured salmon sperm DNA per ml–50% deionized formamide. Hybridization was carried out under the same conditions for 16 h with the addition of the antisense RNA probe specific to the RNA transcript to be detected (5 × 10^5 cpm/ml). The membrane was then washed twice for 15 min in 250 ml of 1× SSC–0.1% SDS and twice in 0.25× SSC–0.1% SDS. These washes were carried out at 25°C when nonspecific hybridization to rRNA was to be visualized and at 60°C when this nonspecific hybridization was to be removed. Hybrids were visualized by radioautography with Kodak X-Omat AR5 film for 6 to 24 h at −70°C.

Antisense RNA probes were prepared by in vitro tran-

scription of linearized plasmids, using SP6 or T7 RNA polymerase (Bethesda Research Laboratories) in 50-μl reaction volumes. For T7 RNA polymerase, reaction mixtures contained 40 mM Tris-HCl (pH 8.0), 25 mM NaCl, 8 mM $MgCl_2$, 2 mM spermidine, 5 mM DTT, 0.4 mM ATP, 0.4 mM CTP, 0.4 mM UTP, 0.02 mM GTP, 40 U of RNasin, 1 μg of plasmid DNA, 40 μCi of 5'-[α-P^{32}]GTP (410 Ci/mmol; Amersham), and 50 U of T7 RNA polymerase. For SP6 RNA polymerase, reaction mixtures contained 40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 1 mM DTT, 0.4 mM ATP, 0.4 mM CTP, 0.4 mM UTP, 0.02 mM GTP, 40 U of RNasin, 1 μg of plasmid DNA, 40 μCi of 5'-[α-^{32}P]GTP, and 15 U of SP6 RNA polymerase. RNA synthesis in each case was carried out for 60 min at 37°C and terminated by ethanol precipitation. The RNA probes were separated from unreacted substrate by chromatography on Sephadex G-50 columns (1.0 ml) in water. The probe for *ACT1* is complementary to positions +42 to +196 of *ACT1*, where position +1 corresponds to the start site of transcription. The probe for *HTB2* is complementary to positions −160 to approximately +300 of *HTB2*, where position +1 corresponds to the first nucleotide following the stop codon of *HTB2*.

Determination of poly(A) content. Poly(A) content was determined as described by Sachs and Davis (37). Total RNA samples (2 to 20 μg for *PAP1* [23 and 35°C] and *pap1-1* [23°C] or 20 to 60 μg for *pap1-1* [35°C]) were incubated for 15 min at 65°C in 60 μl of buffer A (300 mM NaCl, 20 mM Tris-HCl [pH 8.0], 20 mM EDTA, 0.3 mg of *E. coli* tRNA per ml) plus 833 nCi of poly[5-^{3}H]uridylic acid per ml (72 Ci/mmol; Amersham) and then incubated at 37°C for 30 min. The mixture was mixed with 1 ml of buffer A (4°C) plus RNase A (20 μg/ml) and incubated on ice for 30 min. Hybrids were precipitated by the addition of trichloroacetic acid (TCA) to 5% and incubation on ice for 1 h. The precipitates were collected by filtration through Whatman GF/C filters and then washed with 5 ml of 5% TCA and 1 ml of 95% ethanol. The filters were dried, and the hybrids were quantitated by liquid scintillation counting. All assays were carried out with three different amounts of each RNA to ensure that the labeled poly(U) was in excess.

Measurement of protein synthetic rates in vivo. Cells were grown in sulfate-depleted medium (33) supplemented with glucose (2% [wt/vol]) to a density of 1×10^6 to 2×10^6 cells per ml at 23°C; 3×10^7 cells were concentrated by centrifugation at 1,000 × *g* for 5 min and resuspended at a density of 9×10^7 cells per ml in 40 mM KH_2PO_4 (pH 6.0)–2% (wt/vol) glucose at the appropriate temperature. The cells were labeled by the addition of L-[^{35}S]methionine (1,000 Ci/mmol; Amersham) to 200 μCi/ml. Samples (10 μl) were withdrawn at 0, 1, 5, 10, and 20 min after addition and spotted onto Whatman GF/C filters, which were immediately suspended in 5% (wt/vol) TCA (20 ml per filter). The filters were heated at 100°C for 10 min in 5% TCA, washed in 10 ml of 5% TCA per filter, washed with 95% ethanol, and dried, and the bound radioactivity was determined by liquid scintillation counting. Rates were calculated by linear regression analysis of four or five time points.

RESULTS

Identification of a polyadenylation mutant. We developed a biochemical assay to directly screen a collection of temperature-sensitive mutants for defects in mRNA polyadenylation. The assay involves the preparation of crude extracts from cells grown at the permissive temperature followed by incubation of a synthetic, radiolabeled precursor mRNA in each at the permissive and nonpermissive temperatures. Resolution of the reaction products by polyacrylamide gel electrophoresis allows determination of the temperature dependence of polyadenylation in vitro. Figure 1 shows the temperature dependence of polyadenylation in a normal extract and the ts148 extract. Polyadenylation in the ts148 isolate shows two remarkable features: (i) at 23°C, the ts148 extract produces about 1/5 the amount of polyadenylated product observed in the normal control, and this falls to less than 1/10 at 30°C, while the normal control increases by half at the same temperature, and (ii) relatively large amounts of cleavage product result at 23 and 30°C in the mutant despite

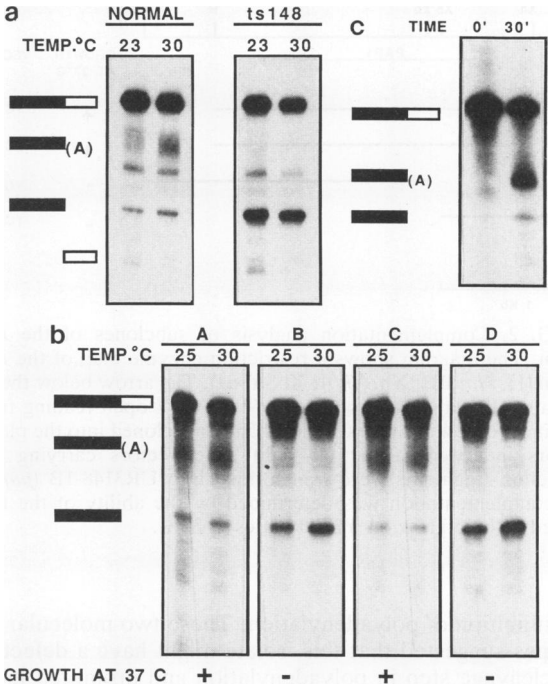

FIG. 1. (a) Temperature dependence of polyadenylation of the ts148 isolate in vitro. Synthetic radiolabeled *CYC1* pre-mRNA was incubated in miniextracts of a normal or ts148 extract for 30 min at 25 or 30°C, the reactions were stopped, and the products were analyzed as described in Materials and Methods. The rectangles to the left designate the positions of the substrate and products of the reaction. The filled rectangle represents the portion of the pre-mRNA 5' to the polyadenylation site, while the open rectangle represents the portion 3' to the site. The filled rectangle followed by (A) designates the position of the polyadenylated product. The identity of these products has been previously established (5). The RNA species migrating just below the polyadenylated RNA is generated during synthesis of the *CYC1* pre-mRNA, which was not gel purified in this case. Product levels mentioned in the text were determined by laser scanning densitometry (LKB Ultrascan XL) of autoradiographs. (b) Cosegregation of the biochemical and growth phenotypes of the *pap1-1* mutant. Synthetic radiolabeled *CYC1* pre-mRNA was incubated in the extracts from the meiotic progeny (A through D) of a single tetrad derived from a cross of a *pap1-1* strain (ts148) with a normal strain (S288C). The reactions were carried out and analyzed as described above. The growth phenotypes indicated at the bottom refer to the ability of the meiotic progeny to grow on YEPD plates at the indicated temperature; all grow at 25°C. (c) Coreversion of the biochemical and growth phenotypes associated with *pap1-1*. Polyadenylation was assayed as described above in an ammonium sulfate-fractionated extract (4) derived from a spontaneous, temperature-resistant revertant of *pap1-1*. Times are indicated in minutes.

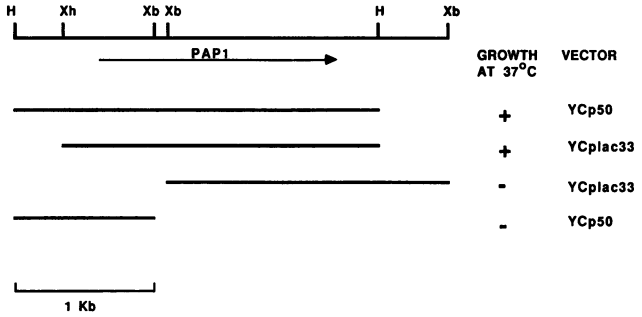

FIG. 2. Complementation analysis of subclones of the *PAP1* region. The diagram shows a restriction enzyme map of the *PAP1* region (H, *Hin*dIII; Xh, *Xho*I; Xb, *Xba*I). The arrow below the map indicates the length and position of the *PAP1* open reading frame. The lines represent the restriction fragments cloned into the plasmid vectors indicated at the far right. The vectors carrying these restriction fragments were transformed into UR3148-1B (*pap1-1*), and complementation was determined by the ability of the transformed cells to grow on YEPD plates at 37°C.

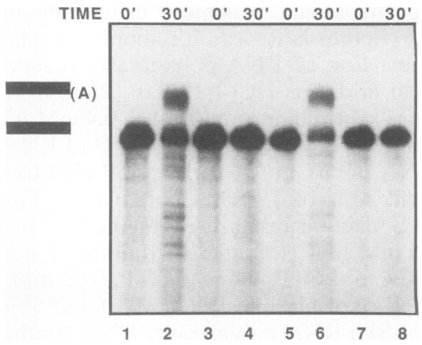

FIG. 3. Polyadenylation of precleaved *CYC1* RNA in extracts derived from a *PAP1* (EJ101) or a *pap1-1* (UR3148-1B) strain. Synthetic radiolabeled *CYC1* pre-RNA was cleaved at its polyadenylation site in ammonium sulfate-fractionated extracts of either a *PAP1* (lanes 1 to 4) or *pap1-1* (lanes 5 to 8) strain, and the cleavage product was purified as previously described (5). The purified, precleaved RNA was incubated in a *PAP1* (lanes 1, 2, 5, and 6) or *pap1-1* (lanes 2, 4, 7, and 8) extract at 30°C for the time (in minutes) indicated and analyzed as described in Materials and Methods.

the inhibition of polyadenylation. These two molecular phenotypes suggested that this isolate might have a defect in a postcleavage step in polyadenylation and prompted its further characterization.

The collection of temperature-sensitive mutants from which the ts148 isolate originated arose from chemical mutagenesis and contains many isolates with multiple mutations (18, 48). Further study of this mutation thus required outcrossing the original mutation into a normal genetic background. Analysis of tetrads from three successive outcrosses always showed 2:2 segregation of the temperature-sensitive growth phenotype after every cross (19 complete tetrads). More importantly, biochemical analysis of five complete tetrads from different outcrosses showed that the molecular and growth phenotypes always cosegregate as exemplified in Fig. 1b.

Phenotypic cosegregation cannot always distinguish between two phenotypes arising from a single mutation and two phenotypes arising from two closely linked mutations. Therefore, we isolated spontaneous, temperature-resistant revertants of the ts148 mutation and characterized these genetically and biochemically. A cross between the revertant UR3148-1CR1 and a normal strain yielded a diploid which upon sporulation produced meiotic progeny that were all temperature resistant (nine complete tetrads). This finding indicates that the loss of the original temperature-sensitive phenotype results from intragenic reversion rather than suppression. More importantly, the revertant also shows a normal polyadenylation phenotype (Fig. 1c). These results indicate that the temperature sensitivity and the polyadenylation defect result from a mutation in a single gene.

The conditional mutation occurs in the *PAP1* gene encoding poly(A) polymerase. We cloned the normal allele of the ts148 gene by complementation of the temperature sensitivity with a plasmid vector library (34). Screening of 14,000 transformants revealed 10 which, when reintroduced into the mutant background, allowed growth at 37°C. All 10 plasmids contained inserts with overlapping restriction maps. Deletion analysis of one of these clones showed that complementation of the temperature-sensitive growth phenotype requires DNA sequence contained on a 2,281-bp *Xho*I-*Hin*dIII restriction fragment (Fig. 2). DNA sequence analysis of this restriction fragment revealed a single, complete 1,707-bp open reading frame which corresponds to the *PAP1* gene encoding poly(A) polymerase (25).

The *pap1-1* mutation causes a postcleavage defect in polyadenylation. Endonucleolytic cleavage of pre-mRNAs at their polyadenylation sites in human systems shows an apparent requirement for poly(A) polymerase in vitro (7, 8, 14, 42, 43). We decided, therefore, to test whether the *pap1-1* mutation (ts148) might cause the production of an abnormal RNA 3′ end unrecognizable in the polyadenylation step or whether the mutation causes a defect in the polyadenylation step itself. The former possibility predicts (i) that a cleavage product from a *pap1-1* extract should not function as a polyadenylation substrate in a normal *PAP1* extract and (ii) that a cleavage product from a normal extract should function as a substrate for polyadenylation in a *pap1-1* extract. Figure 3 shows that a normal (*PAP1*) extract polyadenylates cleavage products previously isolated from either a *PAP1* or *pap1-1* extract. In contrast, polyadenylation occurs on neither substrate in a *pap1-1* extract. The fact that a normally cleaved RNA fails as a substrate in a *pap1-1* extract favors a model in which a defect occurs in a step following the correct cleavage of the pre-mRNA.

Thermal inactivation of the *pap1-1* gene product results in the shutoff of polyadenylation in vivo. If the *pap1-1* mutation affects polyadenylation, then shifting growing *pap1-1* cells to the nonpermissive temperature (35°C), which causes the cells to stop growing (Fig. 4A), should result in a decrease in the poly(A) content of the cells. Measurement of cellular poly(A) content by its ability to protect poly[5-^{3}H]uridylic acid from RNase hydrolysis shows that, indeed, the amount of poly(A) decreases about 80% within 30 min after the shift, and by 60 min less than 5% of the original poly(A) remains (Fig. 4B). Shift of normal cells to 35°C has no such effect on poly(A) content. These results give only a relative quantitation of poly(A) content. Since we do not know the lower limit of detection of this assay for the amounts or length of poly(A), these results may underestimate the real amount of cellular poly(A). Nevertheless, these results clearly show that the *pap1-1* mutation causes a temperature-dependent defect affecting the amount of poly(A) in vivo and, together with the results in vitro described above, indicate that the mutation disrupts polyadenylation in vivo.

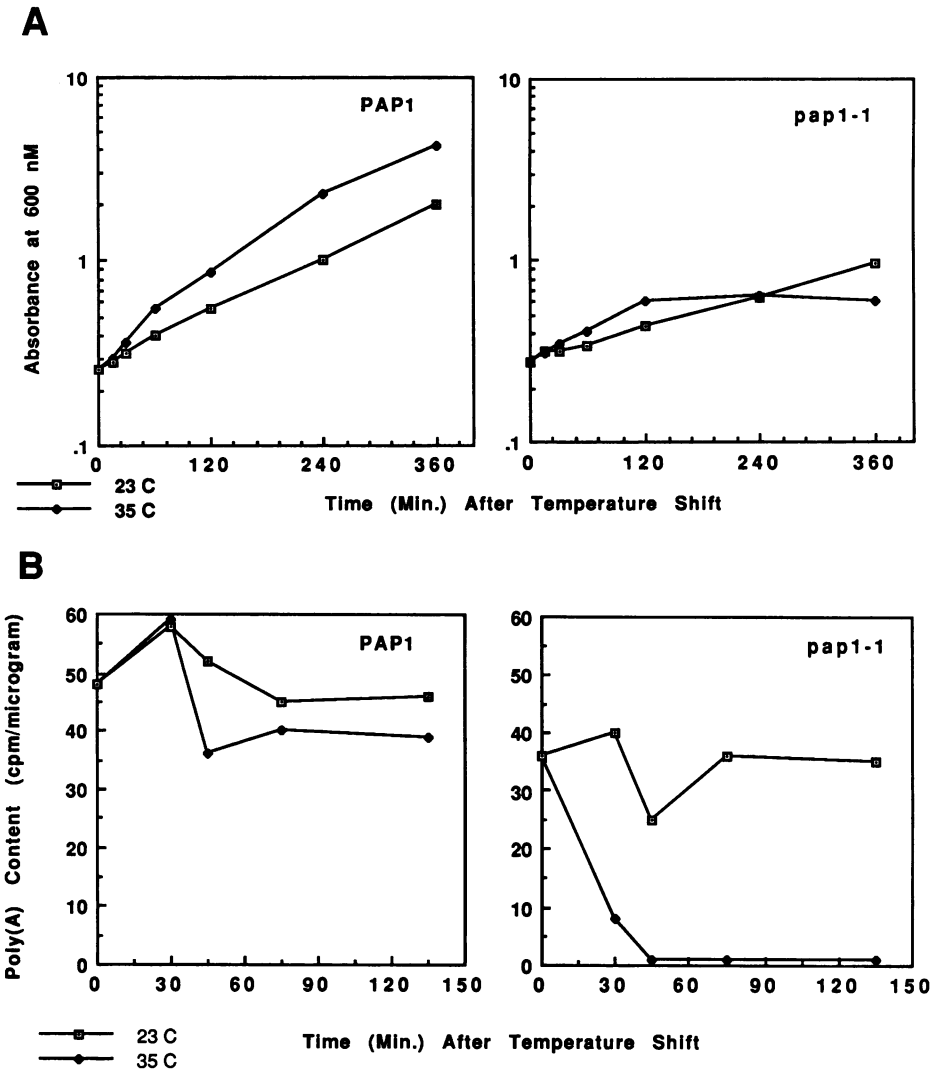

FIG. 4. (A) Growth of *PAP1* (A364A) and *pap1-1* (UR3148-1B) cells in YEPD as a function of time at 23 or 35°C. Cell growth is expressed as an increase in A_{600} in a Hitachi U-1100 spectrophotometer. In this instrument, an A_{600} of 0.5 corresponds to 5×10^6 cells per ml. (B) Poly(A) content of *PAP1* and *pap1-1* strains as a function of incubation time at 23 or 35°C. Poly(A) content is expressed as the amount of poly[5-^{3}H]uridylic acid in counts per minute protected from RNase A digestion with 1 μg of total RNA. For cells grown at 23°C, this value is 48 cpm/μg for *PAP1* and 36 cpm/μg for *pap1-1*.

Analysis of the amount and length of actin (*ACT1*) mRNA by Northern blotting (Fig. 5a and b) reveals that the amount of mRNA remains constant up to 15 min after the shift and then decreases about 50% by 30 min. *ACT1* mRNA remains at this level for up to 60 min and then shows a large decrease by 120 min, at which time the cells stop growing (Fig. 4A). We fractionated, by oligo(dT)-cellulose chromatography, total RNA samples isolated from normal and *pap1-1* cells after the shift to 35°C. Synthesis of poly(A) tails in vivo in yeast cells yields adenylate tracts of approximately 70 residues which upon deadenylation range in size from 1 to 60 residues (16, 17). Since RNAs with poly(A) tails shorter than about 20 residues do not bind to oligo(dT)-cellulose, some 40 to 50% of all yeast mRNAs do not bind to the resin (16, 19). Thus, oligo(dT)-cellulose chromatography concentrates poly(A)$^+$ RNA with long, newly synthesized poly(A) tails in the bound fraction. The results in Fig. 5c demonstrate this, as more than 90% of a synthetic, radiolabeled poly(A)$^+$ *CYC1* RNA [poly(A) tail length of 50 to 70 residues] binds oligo(dT)-cellulose, while some 50% of the cellular *ACT1* mRNA does not. Shift of the *pap1-1* cells to 35°C results in a rapid loss of *ACT1* mRNA from the pool of mRNAs with long poly(A) tails and an increase in the amount of mRNA in the poly(A)-deficient pool (Fig. 5c). These results, therefore, support a model in which thermal inactivation of the *pap1-1* gene product results in a shutoff of polyadenylation in vivo.

We also monitored by Northern analysis the amount and length of histone H2B2 (*HTB2*) mRNA. Yeast histone mRNAs, unlike their metazoan counterparts, carry poly(A) tails. *HTB2* mRNA has two cleavage and polyadenylation sites in vitro (4) as well as in vivo (Fig. 5a, 3). *HTB2* mRNA 3' end formation appears to change in response to the temperature shift in normal cells, as evidenced by the loss of the longer of the two mRNA species. The response in *pap1-1* cells shows two interesting features. First, the temperature shift causes loss of the longer species as in normal cells, and

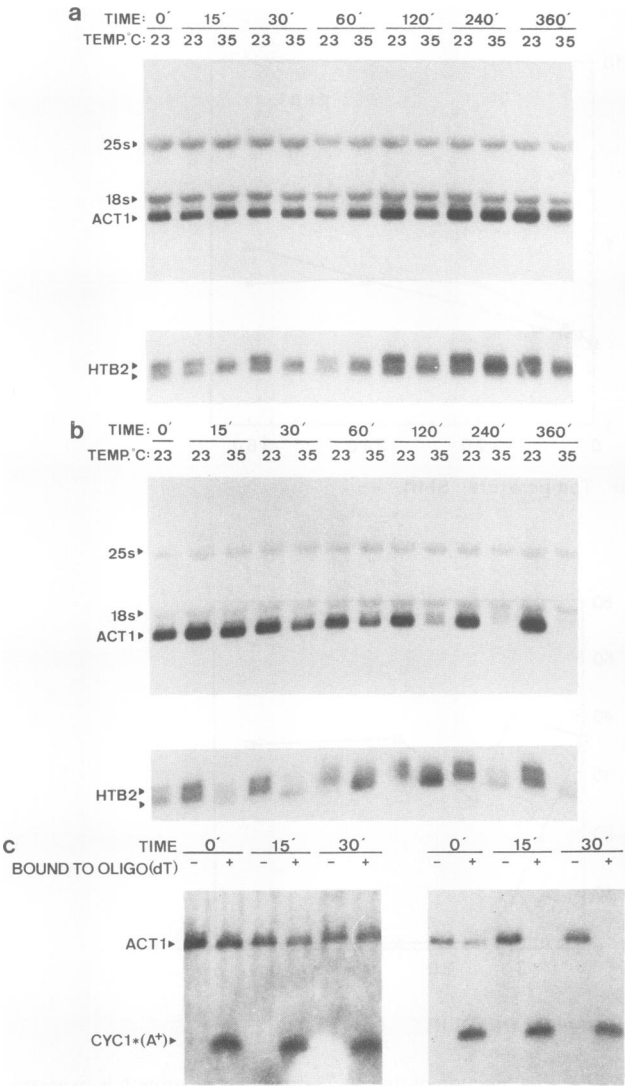

FIG. 5. (a and b) Northern blot analysis of *ACT1* and *HTB2* mRNAs from *PAP1* (A364A) (a) and *pap1-1* (UR3148-1B) (b) cells as a function of incubation time (minutes) at 23 or 35°C. The panels showing *ACT1* and *HTB2* were derived from the same blots, which were first hybridized with a probe for *ACT1* washed at low stringency to reveal rRNAs as well as *ACT1*. The blot was stripped and reprobed with a probe for *HTB2*. Product levels mentioned in the text were determined by laser scanning densitometry (LKB Ultrascan XL) of autoradiographs and were normalized to the amount of 18S rRNA. (c) Northern blot analysis of poly(A)$^+$ and poly(A)-deficient *ACT1* mRNA from *PAP1* and *pap1-1* cells incubated at 35°C for the amount of time indicated. CYC1* (A$^+$) indicates the position of a synthetic radiolabeled *CYC1* poly(A)$^+$ RNA having a poly(A) tail length of ca. 60 residues.

the remaining species decreases in length, consistent with the absence of its poly(A) tail. Second, after the temperature shift, we always observe an initial decrease in *HTB2* mRNA levels followed by a transient increase in the level of the shorter, apparently poly(A)$^-$ transcript. It remains unclear at this time whether this pattern reflects changes in *HTB2* mRNA stability, the rate of *HTB2* synthesis, or both.

Effect of thermal inactivation of the *pap1-1* gene product on protein synthesis. We decided to monitor the rates of total protein synthesis rather than that for specific species in order

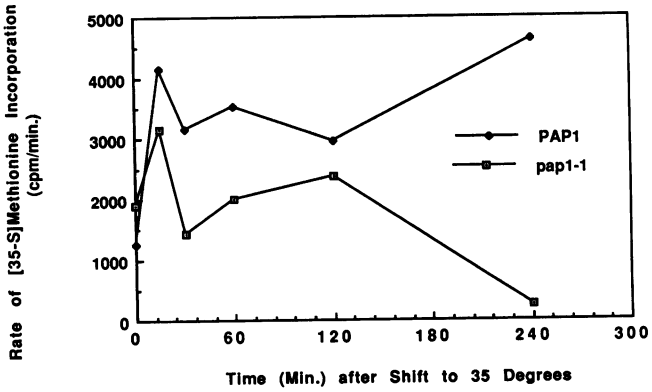

FIG. 6. Rate of [^{35}S]methionine incorporation into protein in *PAP1* (A364A) and *pap1-1* (UR3148-1B) cells as a function of time of incubation at 35°C. Each point represents the slope calculated from four or five time points taken between the time of addition of label to the culture and 20 min after.

to determine the generality of the effect of the *pap1-1* mutation on this process. Measurement of the rate of protein synthesis by pulse-labeling cells with [^{35}S]methionine after the shift to 35°C shows an increase in the rate of translation in *PAP1* cells (Fig. 6). Curiously, in *pap1-1* cells, the rate of protein synthesis remains relatively unchanged for at least 2 h after the temperature shift and then decreases to near zero by 4 h. This effect demonstrates that translation continues despite a significant decrease in the availability of poly(A)$^+$ mRNA in the cell.

DISCUSSION

The results reported here define the first conditional mutation known to directly affect the processing of mRNA 3′ ends. We identified this mutant by using a biochemical screen which allowed us to search for defects directly affecting the specific endonucleolytic cleavage and polyadenylation steps required in mRNA 3′ end formation. The mutation thus identified confers both a biochemical defect as well as temperature sensitivity for growth. The fact that these phenotypes result from the same mutation (Fig. 1) simplified the physiological characterization of the defect by temperature shift experiments and facilitated the cloning of the normal *PAP1* gene and its identification as the gene encoding poly(A) polymerase.

Two results in vitro suggest that the *PAP1* gene product acts at a step following correct cleavage of the pre-mRNA at its polyadenylation site. First, the *pap1-1* mutant accumulates large amounts of normal-length cleavage product and very little polyadenylated product (Fig. 1). The small amount of polyadenylated product produced in the mutant extracts probably indicates the production of an unstable protein, a result not atypical of temperature-sensitive proteins (2, 31). Second, the inability of the mutant extracts to polyadenylate a normal cleavage product despite its efficient polyadenylation in a normal extract (Fig. 3) suggests that the mutant extract cannot recognize a normal cleavage product as a substrate for polyadenylation.

Characterization of the *pap1-1* defect in vivo lends significant support to our conclusion that it acts to specifically polyadenylate mRNAs. Determination of the cellular poly(A) content shows that a shift of *pap1-1* cells to the nonpermissive temperature results in a complete loss of

measurable poly(A) by 60 min (Fig. 4). Characterization of *ACT1* and *HTB2* mRNAs under these conditions suggests that normal-length poly(A)-deficient products accumulate in the mutant (Fig. 5). Interestingly, the mRNA level patterns of these two messages show significant differences. *ACT1* mRNA begins to decrease by 30 min postshift and disappears after 2 h at the nonpermissive temperature. In contrast, *HTB2* mRNA levels decrease initially and then increase by 60 min postshift. We have observed both types of accumulation patterns among a relatively small set of mRNAs tested thus far in our laboratory (3). Messages with the steady-state pattern exemplified by *HTB2* may provide the transcripts which support ongoing translation in *pap1-1* cells at the nonpermissive temperature. In any case, the unexpected continuation of protein synthesis in the absence of detectable poly(A)$^+$ mRNA raises important questions about the requirement of poly(A) tails for mRNA function (23, 30). Further investigation of the effect of the *pap1-1* mutation on protein synthesis patterns may prove interesting in this regard.

The DNA sequence of the *PAP1* gene showed that it corresponds to the previously identified gene, *PAP1*, encoding a yeast poly(A) polymerase (25). A thorough biochemical characterization of the enzyme encoded by *PAP1* showed characteristics expected of the enzyme required to polyadenylate mRNAs, yet several features of the purified protein made its role in mRNA polyadenylation uncertain (26). First, like all pure poly(A) polymerases, the enzyme showed no apparent RNA substrate specificity; it polyadenylated nonsubstrate RNA primers and failed to preferentially polyadenylate a substrate ending at its natural polyadenylation site. Second, the purified enzyme catalyzes the synthesis of poly(A) tails of nonphysiological length (ca. 250 nucleotides). These features and the possibility that eukaryotes may contain multiple poly(A) polymerases (9, 10) suggested that this enzyme might not function in mRNA 3′ end formation. However, the experiments reported here show that a mutation (*pap1-1*) in *PAP1* causes a defect in its ability to specifically polyadenylate mRNAs in vitro and in vivo and thereby establishes this enzyme as essential for mRNA 3′ end formation in *S. cerevisiae*. The fact that the purified poly(A) polymerase does not demonstrate substrate specificity or synthesis of normal-length poly(A) tails suggests that *S. cerevisiae* requires other factors to confer these properties. The availability of a conditional defect in this activity should make possible the identification of such factors among the suppressors of the *pap1-1* mutation.

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