MOLECULAR AND CELLULAR BIOLOGY, Dec. 1995, p. 6979–6986 Vol. 15, No. 12
0270-7306/95/$04.00+0

Effects of Mutations in the *Saccharomyces cerevisiae RNA14*, *RNA15*, and *PAP1* Genes on Polyadenylation In Vivo

ELISABETH MANDART† AND ROY PARKER*

Department of Molecular and Cellular Biology and Howard Hughes Medical Institute, University of Arizona, Tucson, Arizona 85721

Received 19 July 1995/Returned for modification 29 August 1995/Accepted 15 September 1995

The *RNA14* and *RNA15* gene products have been implicated in a variety of cellular processes. Mutations in these genes lead to faster decay of some mRNAs and yield extracts that are deficient in cleavage and polyadenylation in vitro. These results suggest that the *RNA14* and *RNA15* gene products may be involved in both adenylation and deadenylation in vivo. To explore the roles of these gene products in vivo, we examined the site of adenylation and the rate of deadenylation for individual mRNAs in *rna14* and *rna15* mutant strains. We observed that the rates of deadenylation are not affected by lesions in either the *RNA14* or the *RNA15* gene. This result suggests that the proteins encoded by these genes are not involved in regulation of the deadenylation rate. In contrast, we observed that the site of adenylation for the *ACT1* transcript can be altered in these mutants. Interestingly, we also observed that mutation of the poly(A) polymerase gene altered the site of *ACT1* polyadenylation. These observations suggest that the RNA14, RNA15, and PAP1 proteins are involved in poly(A) site choice. This alteration in poly(A) site choice in the *rna14* mutant can be corrected by the *ssm4* suppressor, indicating that this suppression acts at the level of polyadenylation and not by slowing mRNA degradation.

Most eukaryotic mRNAs have a polyadenylate sequence at the 3′ end which is added in a posttranscriptional process. This poly(A) tail is important for the proper stability of transcripts (for a review, see reference 9), for efficient initiation of translation (11, 17, 32), and for efficient nuclear-cytoplasmic transport (25, 34). In addition, the site at which the poly(A) tail is added can be regulated, thereby leading to the production of alternative gene products from a single transcriptional unit.

Examination of the process of polyadenylation in mammalian cell extracts has led to substantial progress in understanding the mechanism by which the poly(A) tail is added (for reviews, see references 18, 38, and 40). Addition of a poly(A) tail is initiated by assembly of a complex of proteins on the sequences that specify a polyadenylation site. This complex cleaves the pre-mRNA and then adds a short poly(A) tail. The addition of a nuclear poly(A) binding protein, PABII, then alters the adenylation reaction to allow rapid elongation of the short poly(A) tail to its full length (3). In *Saccharomyces cerevisiae*, the use of an in vitro system has also shown that mRNA 3′ end maturation is similar in that the precursor mRNA is also cleaved at a specific site to which the poly(A) tail is added (5). However, there are clearly differences in the sequences that specify a polyadenylation site in *S. cerevisiae* and mammals (for reviews, see references 18, 38, and 40).

To understand the process of 3′ end formation, the proteins involved in cleavage and polyadenylation need to be identified and their functions need to be determined. In mammalian cells, fractionation of extracts has identified several factors required for adenylation, in addition to the poly(A) polymerase and PABII. These include a factor that specifies the site of cleavage, termed CPSF (cleavage and polyadenylation specificity factor), and a factor that stimulates cleavage, termed CstF (cleavage stimulation factor).

* Corresponding author. Phone: (520) 621-9347. Fax: (520) 621-4524.

† Permanent address: Centre National de la Recherche Scientifique, Institut de Génétique Moléculaire, 34033 Montpellier, France.

A number of groups have begun to utilize genetic approaches to identify the gene products required for cleavage and polyadenylation in *S. cerevisiae*. Such approaches have led to the identification of the yeast *REF2* gene, which encodes a protein that stimulates the cleavage reaction (37), and the yeast *FIP1* gene, which encodes a protein that directly interacts with the poly(A) polymerase (35). Three other yeast gene products known to be involved in adenylation are encoded by the *RNA14*, *RNA15*, and *PAP1* genes. The *PAP1* gene encodes the poly(A) polymerase (21, 22). Mutations in the *RNA14* and *RNA15* genes yield cell extracts that are deficient in the polyadenylation of pre-mRNAs in vitro, suggesting that the products of these genes are required for addition of the poly(A) tail in the nucleus (26). Interestingly, the products of the *RNA14* and *RNA15* genes have also been hypothesized to affect deadenylation of mRNAs in the cytoplasm for two reasons. First, *rna14* and *rna15* mutants show a loss of total cellular poly(A) and an increase in the rate of decay of the stable *ACT1* mRNA (27), which could be explained by an increased rate of cytoplasmic deadenylation due to the *rna14* and *rna15* mutations. Second, the *RNA14* gene product is found to be localized to both the nucleus and the cytoplasm (4), suggesting that it has a cytoplasmic function. To determine the functions of these proteins, we examined the sites of adenylation and the rate of deadenylation for individual mRNAs in *rna14*, *rna15*, and *pap1* mutant strains. Our results suggest that the RNA14 and RNA15 proteins are not involved in deadenylation rate regulation but are required, along with the poly(A) polymerase, for proper recognition of poly(A) sites.

MATERIALS AND METHODS

Yeast strains and medium. The *S. cerevisiae* strains used for analysis were the wild-type strain W303-1B (*MAT*α *ura3-1 trp1-1 ade2-1 leu2-3,112 his3-11,15*) (R. Rothstein, Columbia University, New York, N.Y.) and the isogenic *rna15-2* mutant strain. The *rna14-3*, *rna14-3 ssm4*Δ, and *ssm4*Δ mutant strains were derived from strain W303 (24). The *pap1-1* mutant strain (*MAT***a** *ura3-52 lys2-201 ade2 ade1 pap1-1*) was a kind gift of S. Butler (36). The yeast strains were transformed by the modified lithium acetate technique (13), and plasmids were maintained by growth in selective medium.

For experiments examining the endogenous *ACT1* mRNA, cells were grown at 24°C to the mid-log phase in 2% glucose and shifted to 35°C in the same medium, and aliquots were removed at different times. For transcriptional pulse-chase experiments, cells were grown at 24°C to the mid-log phase in medium containing 2% raffinose and 2% sucrose, shifted to 35°C for 20 min, harvested, and resuspended in medium containing 2% galactose to induce transcription. After 8 or 10 min (depending on the experiment), the cells were harvested and resuspended in medium containing 2% glucose (thereby repressing transcription) and cell aliquots were removed at various times.

mRNA analysis. Total RNA was extracted as described previously (6). RNase H and polyacrylamide Northern (RNA) blot assays were done with 10 to 20 μg of total RNA as previously described (31). Northern blots were analyzed on a Betascope (Betagen, Waltham, Mass.) or a PhosphorImager 445SI (Molecular Dynamics). Poly(A) tail length and approximate location were calculated by the marker size program. The amounts of the different transcripts were calculated with the manual quantification program and were standardized between lanes by using the transcript scR1 (10) as a control (6).

The oligonucleotide used with RNase H to cleave the PGK1 mRNA was oRP70 (5′-CGGATAAGAAAGCAACACCTGG-3′), which is complementary to nucleotides 9 to 30 5′ of the *PGK1* stop codon. Oligonucleotide oRP121 (5′-AATTCCCCCCCCCCCCCCCCCA-3′), which is complementary to a 42-nucleotide sequence inserted 3′ of the *PGK1* stop codon (8), was used as a probe for this transcript. Oligonucleotide oRP30 (5′-GAAGATGGAGCCAAAGC-3′), which is complementary to nucleotides 160 to 180 5′ of the *ACT1* stop codon, was used to direct RNase H cleavage of the ACT1 mRNA, while oRP166 (5′-CATACGCGCACAAAAGCAGAG-3′), which is complementary to nucleotides 1 to 21 3′ of the *ACT1* stop codon, was used as a probe to detect the ACT1 or PGK1-ACT1 mRNA. Oligonucleotide oRP179 (5′-GTGACACCGTACT TCTTAGCG-3′), which is complementary to nucleotides 6 to 26 5′ of the *Bgl*II site of the *PGK1* gene, was also used with RNase H to cleave the PGK1-ACT1 chimeric mRNA.

Plasmids. The plasmid expressing the *PGK1* gene under control of the *GAL1* promoter has been previously described (pRP469 [8]). The plasmid expressing the hybrid *PGK1-ACT1* gene was constructed by replacing the 374-nucleotide *Hin*dIII-*Bgl*II fragment corresponding to the 3′ end of the *PGK1* gene from pRP469 with the 525-nucleotide *Bgl*II-*Pvu*II fragment of pRP253 (14), which contains the 3′ portion of the *ACT1* coding region and the transcriptional terminator. Partial deletion of the *ACT1* gene in pRP253 created a *Bgl*II site in place of the *Xho*II site in the *ACT1* coding sequence (14). In this *PGK1-ACT1* fusion, the reading frame was maintained at the junction.

RESULTS

Analysis of the *PGK1* mRNA deadenylation rate in *rna14* and *rna15* mutant strains. On the basis of loss of the total poly(A)$^+$ RNA and faster *ACT1* mRNA decay in mutant cells, it has been proposed that the *RNA14* and *RNA15* gene products might play a role in mRNA deadenylation (27). To examine this possibility, we measured the deadenylation rate of the *PGK1* mRNA in *rna14* and *rna15* temperature-sensitive mutants at the restrictive temperature. To limit our analysis to transcripts synthesized at the restrictive temperature, we utilized a *PGK1* gene under control of the *GAL1* upstream activation sequence to induce new transcription by addition of galactose following a shift to the restrictive temperature. Subsequently, transcription was inhibited by addition of glucose, thereby allowing measurement of deadenylation rates. Such an approach, termed a transcriptional pulse-chase, has been used to measure the rates of deadenylation of yeast transcripts (1, 8, 29, 30).

To perform this experiment, the wild-type strain and thermosensitive *rna14-3* and *rna15-2* mutant strains were transformed with plasmid pRP469, which contains the *PGK1* gene under control of the inducible *GAL1* promoter (8). The cells were first grown at 24°C in medium containing raffinose and then shifted to the restrictive temperature in the same medium and incubated for 20 min. Transcription of the *PGK1* gene was induced with galactose for 8 min, and then glucose was added to repress transcription (see Materials and Methods). Aliquots of cells were harvested at different times, and the poly(A) tail lengths on the transcripts were analyzed on acrylamide Northern gels. To avoid detection of the endogenous *PGK1* transcript, which would contain a mixture of transcripts made before and after the shift to the restrictive temperature, the blot was probed with an oligonucleotide specific to the *PGK1* transcript under *GAL1* control (see Fig. 1 and Materials and Methods).

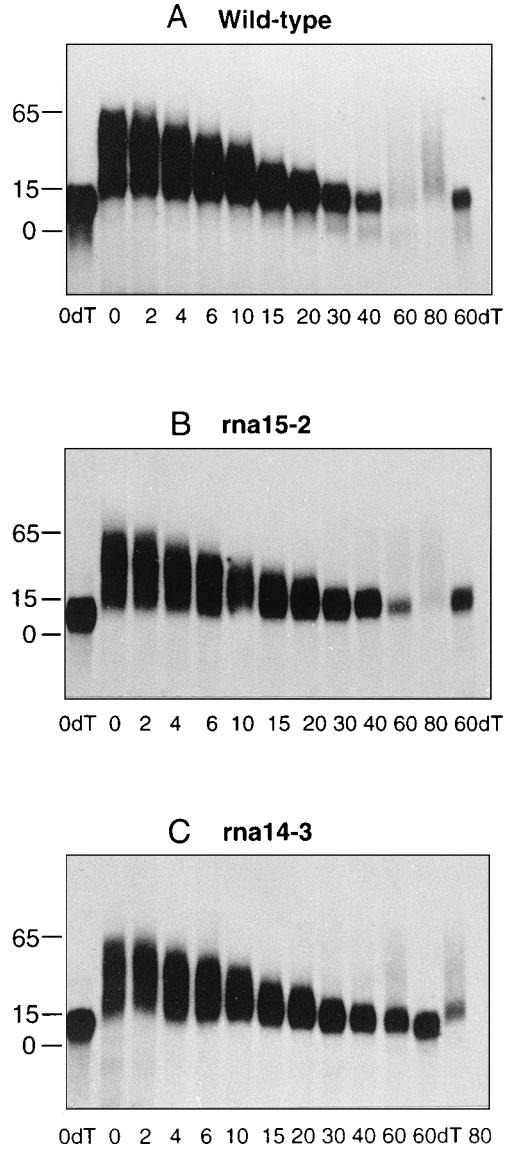


FIG. 1. Deadenylation of the *PGK1* mRNA in *rna14* and *rna15* mutant strains. Shown is a polyacrylamide Northern blot of a pool of *PGK1* transcripts produced at the restrictive temperature during 8 min of induction with galactose 20 min after a shift to 35°C. Samples were taken at various times after transcriptional repression by glucose, as indicated. Total RNA from each sample was treated with RNase H in the presence of oligonucleotide oRP70. Lanes 0dT and 60dT correspond to RNAs treated with RNase H in the presence of oligonucleotide oRP70 and oligo(dT) to remove the poly(A) tail. The probe was end-labeled oligonucleotide oRP121, which is complementary to a 42-nucleotide sequence inserted 3′ of the stop codon. This insertion has no effect on *PGK1* mRNA stability (8). At the left are the approximate numbers of adenylate residues based on comparison to size markers and a sample treated with RNase H and oligo(dT). (A) The W303 control strain. (B) The *rna15-2* mutant strain. (C) The *rna14-3* mutant strain. For each strain, the smear that can be seen at late time points probably corresponds to transcripts having long poly(A) tails, since the same RNAs treated with oligo(dT) and RNase H do not have this smear but present a more intense signal corresponding to transcripts without a poly(A) tail. This suggests that some low-level transcription can take place late, even though the strains are in glucose.

The key result in this experiment is that the deadenylation profile of the *PGK1* transcripts in the *rna15-2* or *rna14-3* mu-

tant strain (Fig. 1B and C) was very similar to that in the W303 control strain (Fig. 1A). In all strains at the time of transcription repression (0 min), the new transcripts had a distribution of poly(A) tails that ranged from a few nucleotides to ~65 adenylate residues. In these experiments, poly(A) tail lengths were based on comparison to a sample in which the poly(A) tails had been removed by treatment with oligo(dT) and RNase H (0dT lanes) and to molecular weight markers (not shown). The poly(A) tails were more heterogeneous than those observed in similar transcriptional induction experiments done at 24°C (8), presumably because of the more heterogeneous behavior of a population of transcripts synthesized at 35°C. By measuring the longest poly(A) tail lengths at each time point, we estimated that in the wild-type and the *rna15-2* and *rna14-3* mutant strains, the maximum deadenylation rate of the *PGK1* transcript was approximately three adenylate residues per min. This rate is similar to what has previously been described in other wild-type strains (8).

Two important conclusions were drawn from these experiments. First, the *rna14-3* and *rna15-2* mutations do not affect the deadenylation rate of the *PGK1* mRNA (see Discussion). Second, we observed no difference between the wild-type and mutants strains in the poly(A) tail length of the *PGK1* transcript. In vitro, the RNA14 and RNA15 proteins are required for 3′ end processing (26). However, our results indicated that in vivo, the temperature-sensitive mutations still allow adenylation of the *PGK1* mRNA, although we cannot determine if there are minor changes in the efficiency of cleavage at this adenylation site. To determine if the *RNA14* and *RNA15* gene products do affect the adenylation of specific mRNAs in vivo, we analyzed the adenylation of a second mRNA.

The *rna14* and *rna15* mutations alter the relative levels of different 3′ ends of the *ACT1* mRNA. We chose to examine the adenylation of *ACT1* transcript in the *rna14* and *rna15* mutants for two reasons. First, it has been reported that *ACT1* mRNA stability is modified in the *rna14-3* and *rna15-2* mutant strains (24, 27), suggesting that the metabolism of this transcript is somehow altered in these mutants. In addition, on the basis of in vitro experiments, this gene has a set of relatively poor polyadenylation sites and therefore might be more sensitive to a decrease in the efficiency of cleavage and polyadenylation than is the *PGK1* mRNA (15).

To determine the effects of the *rna14* and *rna15* mutations on the *ACT1* mRNA, we examined the distribution of 3′ ends of the endogenous *ACT1* transcript at various times after a shift to the restrictive temperature. To resolve the multiple 3′ ends of the *ACT1* transcript, the mRNA was analyzed on an acrylamide Northern gel after cleavage with RNase H and an oligonucleotide near the 3′ end (see Materials and Methods and Fig. 2A). In the wild-type strain (Fig. 2B), the *ACT1* mRNA has five 3′ ends, labeled 1 to 5 for discussion purposes (Fig. 2A and B). There was a reasonable correlation between the 3′ ends determined in our experiment and the multiple 3′ ends previously described for the *ACT1* mRNA (15). These multiple *ACT1* 3′ ends are most easily observed in the lane in which the heterogeneous poly(A) tails have been removed with RNase H and oligo(dT). In wild-type cells, the 5′-most polyadenylation site (site 1) was clearly the major 3′ end throughout the time course of the experiment and corresponds to approximately 60% of the total *ACT1* 3′ ends present (Table 1). Furthermore, it is important to note that the levels of the minor transcripts (corresponding to 6, 11, and 25% of the total *ACT1* transcripts) did not significantly change with time and therefore were not greatly affected by the temperature shift (Table 1; compare lanes 0dT and 70dT in Fig. 2).

In an *rna15-2* mutant strain (Fig. 2C), examination of the 3′ ends of the *ACT1* mRNA after a shift to the restrictive temperature revealed several differences. At the permissive temperature (lane 0dT), the same five polyadenylated transcripts seen in wild-type cells were present in the *rna15* mutant strain at roughly the same ratios (Table 1). However, following a shift to 35°C, the poly(A) tails shortened and the transcript levels decreased for 3′ ends 1, 2, and 3. This change is consistent with inhibition of polyadenylation at these sites and decay of the pre-existing cytoplasmic mRNA. Surprisingly, the most distal 3′ end (transcript 5) increased in both relative and absolute levels throughout the time course of the experiment to reach 50% of the total *ACT1* 3′ ends present after 70 min at 35°C. This alteration is easily seen by comparing the 0- and 70-min time point samples treated with RNase H and oligo(dT) and is quantified in Table 1.

Similar results were also seen with the *rna14-3* mutant strain (Fig. 2D and Table 1). The relative levels and polyadenylation status of the different ends are similar to those of wild-type cells at the permissive temperature. However, following a shift to the restrictive temperature, the poly(A) tail length of the four shorter transcripts decreased while the longest transcript (from site 5) remained at significant levels with a distribution of poly(A) tail lengths. Moreover, while its amount was constant during the time course, its relative level increased from 10 to 50% (Table 1). These results suggest that the *rna14-3* and *rna15-2* mutations lead to an alteration in the specificity of the site of polyadenylation (see below).

The *rna14* and *rna15* lesions lead to preferential use of the most distal *ACT1* polyadenylation site. The above results indicated that at the restrictive temperature, the *rna14* and *rna15* mutations led to the production of different levels of the multiple 3′ ends of *ACT1* mRNA. There are two possible explanations for this phenotype. First, in the mutant strains, the shorter transcripts might be degraded faster than in the wild-type strain, thereby leading to a change in the relative levels of each 3′ end. Alternatively, the *rna14* and *rna15* lesions might lead to a change in the actual efficiencies with which different polyadenylation sites are recognized. To distinguish between these possibilities, we utilized the *GAL1* promoter to induce the transcription of mRNAs with the *ACT1* 3′ end at the restrictive temperature and then examined the ratio of the newly made 3′ ends. If the *rna14* and *rna15* lesions alter the relative decay rates, then we would expect first to see the same relative levels of each 3′ end in the wild-type and mutant strains after a brief pulse of transcription, followed by different rates of mRNA decay for the shorter transcripts. Alternatively, if the *rna14* and *rna15* lesions alter the choice of polyadenylation site, then we would expect to see a different distribution of 3′ ends immediately after the pulse of transcription.

Since overexpression of *ACT1* is toxic for the cell (12), we constructed a chimeric gene in which we replaced the *PGK1* 3′ end with sequences from the *ACT1* 3′ end sufficient to allow production of all five *ACT1* 3′ ends (see Materials and Methods). By cleaving the RNA prior to gel electrophoresis with RNase H and an oligonucleotide directed against the *PGK1* sequences and then probing the blot with an oligonucleotide specific for the *ACT1* sequences, we were able to detect specifically mRNAs arising from the hybrid gene without detecting the endogenous *PGK1* or *ACT1* mRNA. A centromeric plasmid bearing this chimeric gene was introduced into the wild-type and *rna14-3* and *rna15-2* mutant strains, and the *ACT1* transcripts produced after 20 min at the restrictive temperature were determined in a transcriptional pulse-chase experiment (see Materials and Methods).

The analysis of the *PGK1-ACT1* chimeric mRNA in wild-type cells is shown in Fig. 3A. At the 0-min time point, when

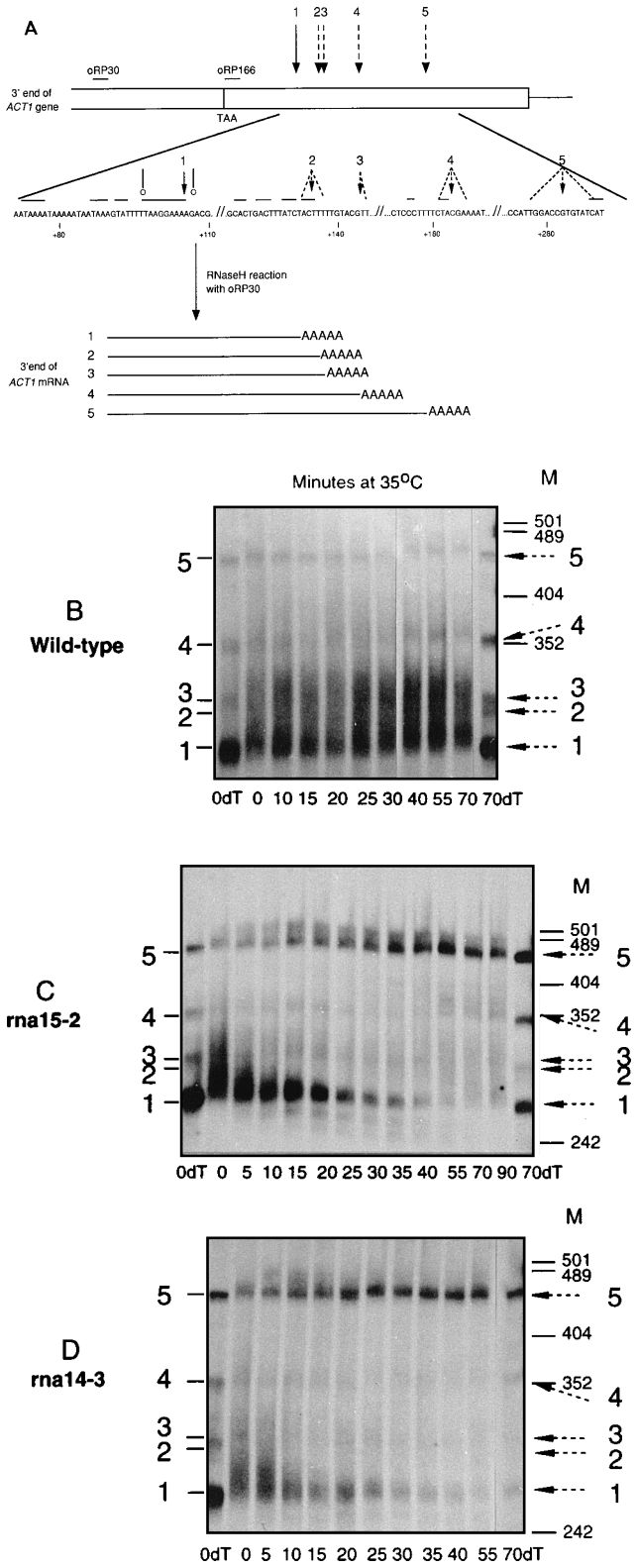


FIG. 2. Examination of the *ACT1* mRNA 3′ ends after a shift to 35°C in the wild-type strain and *rna14* and *rna15* mutant strains. (A) The schematic shows the 3′ end structure of the *ACT1* gene. The oligonucleotides utilized for RNase H cleavage (oRP30) and for probing (oRP166) are also shown. The solid arrow indicates the major polyadenylation site, and the dashed arrows show the other minor sites observed in this study. The 3′ ends of transcripts were determined (as described in Materials and Methods) from two experiments, and the average lengths are indicated by the arrows on the sequence: the limitations of our gel analysis are represented by the dashed lines. The determination of the most distal 3′ end is less precise than that of the others because of the lower resolution of high-molecular-weight species in the gel. On the sequence, the localization of the cleavage sites is compared with that of the 3′ end described in the literature: the open circles indicate the cleavage sites mentioned in reference 12, and the overlined sequences represent the range of 3′ ends described in reference 15. The number of each kind of transcript (1 to 5) corresponds to the poly(A) site utilized. (B) Polyacrylamide Northern blot of *ACT1* transcripts produced at the restrictive temperature in the wild-type strain at steady state. Samples were harvested at the different times indicated after a shift to 35°C. Total RNA from each sample was treated with RNase H in the presence of oligonucleotide oRP30. The samples taken at 0 and 70 min after the shift were also treated in the presence of oligo(dT) to remove the poly(A) tail (lanes 0dT and 70dT). The probe used was end-labeled oligonucleotide oRP166. The numbers 1 to 5 correspond to the transcript numbers in Fig. 3A, and the other numbers are molecular sizes (m) in nucleotides. Polyacrylamide Northern blots of *ACT1* transcripts produced in the *rna15-2* mutant strain (C) and in the *rna14-3* mutant strain (D) are also shown.

TABLE 1. Quantification of the different 3′ ends of the *ACT1* mRNA after a shift to 35°C[a]

Time point (min)	Site(s)	% of total *ACT1* mRNA 3′ ends in:			
		Wild type	*rna15-2* mutant	*rna14-3* mutant	*rna14-3 ssm4*Δ mutant
0	1	57.6	62.0	57.0	66.6
	2, 3	24.9	20	19	13.1
	4	6.2	7.5	11.9	8.6
	5	11.2	9.5	12	11.6
70	1	61.4	26.3	24.8	38.3
	2, 3	26.1	9.8	14.7	12.3
	4	7.9	14.2	9.6	16.0
	5	4.5	49.5	50.8	33.2

[a] The quantification and site numbers of actin 3′ ends correspond to the experiment described in Fig. 3. The number of counts was obtained with a Betascope. Similar results were obtained in other experiments.

transcription was repressed, transcripts mapping to all five 3′ ends were present. The major 3′ end of the chimeric mRNA (57% of the total 3′ ends) produced in the pulse of transcription corresponds to the major 3′ end of the *ACT1* transcript seen under steady-state conditions (site 1) and is the site most proximal to the stop codon (compare with Fig. 2B). In contrast, the longest transcript (site 5) is barely detectable (~9% of the total 3′ ends) in the sample in which the poly(A) tail has been removed (lane 0dT). By comparison, in the *rna15-2* mutant strain (Fig. 3B) and in the *rna14-3* mutant strain (Fig. 3C), transcripts with all of the different 3′ ends are also present but the most 3′ site (site 5) is the most abundant (~40% in both strains), even immediately after the brief pulse of transcription (Fig. 3, lanes 0dT, and Table 2). This result was also seen in an experiment (Fig. 4) in which we ran the RNA from an early time point in each transcriptional pulse-chase on the same gel after cleavage with RNase H and oligo(dT). This observation indicated that most of the newly synthesized transcripts in the *rna14* and *rna15* mutant strains were produced by utilizing a poly(A) site different from that used in wild-type cells.

The above-described experiment also demonstrated that the *rna14* and *rna15* lesions do not alter the deadenylation rate or decay rate of any of the transcripts. All of the transcripts were synthesized with a poly(A) tail, which was shortened at similar rates on all of the transcripts to an oligo(A) tail (at approximately 40 min for all of the different 3′ ends), at which point the levels of the transcript body began to decrease. These results are consistent with a general pathway of mRNA decay in *S. cerevisiae* in which deadenylation of the transcript leads to

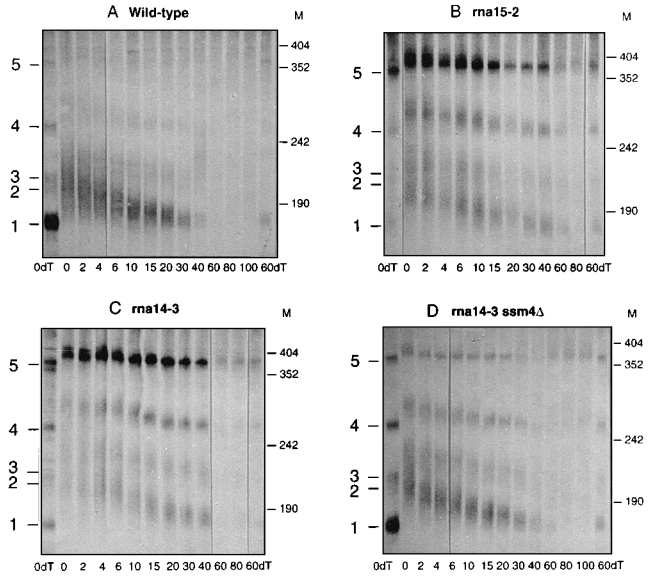


FIG. 3. Analysis of newly synthesized hybrid *PGK1-ACT1* mRNAs. Polyacrylamide Northern blot of a pool of hybrid *PGK1-ACT1* transcripts produced at the restrictive temperature during 10 min of induction with galactose after a shift for 20 min to 35°C. Samples were taken at the times indicated after transcriptional repression with glucose. The RNase H reaction was performed in the presence of *PGK1* oligonucleotide oRP179 and also with oligo(dT) for the time points 0 and 60 min (lanes 0dT and 60dT). The probe used was end-labeled oligonucleotide oRP166 or a labeled *ACT1* 3′ end DNA fragment. The numbers at the left correspond to the transcript numbers in Fig. 3, and those at the right are molecular sizes (m) in nucleotides. (A) Wild-type strain W303. (B) *rna15-2* mutant strain. (C) *rna14-3* mutant strain. The total RNA in lane 0dT was partially degraded, which explains the additional bands. (D) *rna14-3 ssm4*Δ mutant strain. Note that the time points in the transcriptional pulse experiments do not correspond to those at steady state: the 0-min lane (which is the time of repression of transcription) in the transcriptional pulse experiment corresponds to 30 min after the shift to the restrictive temperature (20 min prior to transcriptional induction plus 10 min of transcription). This time point corresponds to the 30-min lane in the experiments wherein the cultures were shifted to the restrictive temperature and the transcripts were analyzed from the time of the temperature shift (Fig. 2 and 5).

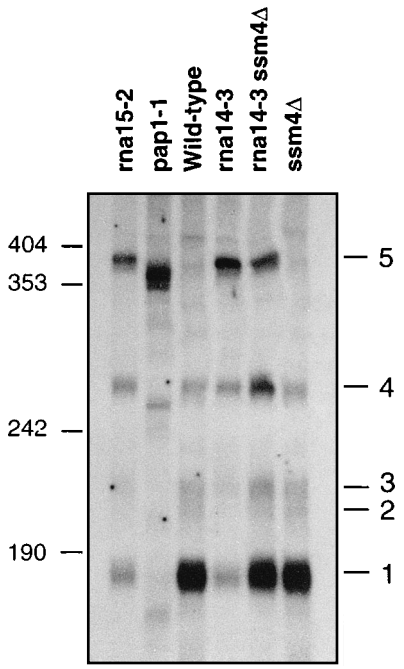


FIG. 4. Comparison of the *ACT1* poly(A) sites utilized in the different strains. Polyacrylamide Northern blot of the pool of hybrid *PGK1-ACT1* transcripts produced in the experiment described in Fig. 3. Samples taken in the different strains at 4 min after transcriptional repression were treated with RNase H in the presence of *PGK1* oligonucleotide oRP179 and oligo(dT) to remove the poly(A) tail. The numbers at the right correspond to the transcript numbers represented in Fig. 3, and the numbers at the left are size markers (in nucleotides).

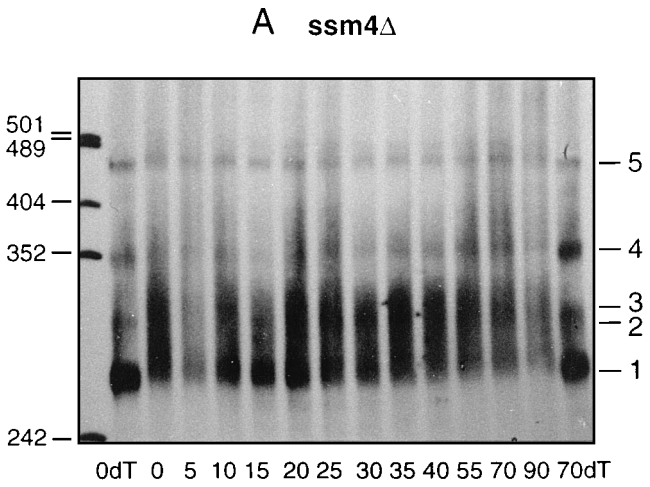
degradation of the transcript body (2, 8, 9). We interpret this set of results to indicate that the difference in the 3′ end distribution of the *ACT1* transcripts between the wild-type strain and the *rna14* and *rna15* mutant strains is due to a change in the choice of poly(A) site and not to a difference in mRNA stability (see Discussion).

The *ssm4* suppressor corrects the polyadenylation site alteration seen in the *rna14-3* mutant strain. The *SSM4* gene is a nonessential gene that was isolated as an extragenic suppressor of *rna14* thermosensitive mutations. Mutations or deletions in the *SSM4* gene suppress the thermosensitivity of the *rna14-3* allele but do not suppress either deletion of the *RNA14* gene or mutations in the *RNA15* gene (24). To determine how the *ssm4* suppressor might function, we asked if, in the presence of the *ssm4* suppressor, the alteration in the poly(A) site choice for the *ACT1* mRNA seen in the *rna14-3* strain was corrected.

We first analyzed the distribution of 3′ ends on the endogenous *ACT1* mRNA at various times after a shift to 35°C in an *ssm4*Δ mutant strain and an *rna14-3 ssm4*Δ mutant strain. In the *ssm4*Δ strain, the distribution of *ACT1* 3′ ends is identical to the wild-type profile (compare Fig. 5A with Fig. 2B). This is not surprising, since the *ssm4*Δ mutation has no effect on viability at any temperature (24). This observation indicated that, by itself, the deletion of *SSM4* did not alter poly(A) site choice on the *ACT1* mRNA. In the *rna14-3 ssm4*Δ mutant strain, as in the *rna14* mutant strain (Fig. 2B), we first observed a change in poly(A) site choice and a decrease in poly(A) tail length for the shorter transcripts following a shift to the restrictive temperature. However, unlike the *rna14* mutant strain, following ~30 min at 35°C, we observed a smear for each 3′ end which corresponds to newly synthesized adenylated mRNAs. Comparison of the transcripts present after 70 min at the restrictive temperature (lane 70dT) with the transcripts present at the permissive temperature (lane 0dT) demonstrated that the *ssm4* suppressor partially corrects the alteration of the *ACT1* 3′ ends seen in the *rna14* mutant (Table 1). In principle, this could be because the *ssm4* suppressor acts by enhancing the recognition of the major poly(A) site or by altering the relative stability of the different 3′ ends.

To distinguish between these alternatives, we utilized the *PGK1-ACT1* chimera under *GAL1* control to examine the 3′ ends of newly synthesized transcripts in the *ssm4* suppressor strains. If the correction by *ssm4*Δ does not affect poly(A) site choice, we expect most of the newly synthesized transcripts in the *rna14-3 ssm4*Δ strain to end at the most 3′ poly(A) site, as in the *rna14-3* mutant strain. Alternatively, if the loss of SSM4 function allows recovery of the choice of poly(A) sites, we expect that many of the new transcripts will be synthesized by using the most proximal poly(A) site, as in the wild-type strain.

The analysis of the chimeric *PGK1-ACT1* mRNA produced at 35°C in the *ssm4*Δ mutant strain showed the same pattern as in the wild-type strain (data not shown and Fig. 4), confirming that *ssm4*Δ by itself does not affect the poly(A) site choice of *ACT1* mRNA. In the *rna14-3 ssm4*Δ double mutant (Fig. 3D and 4), the major 3′ end (47.7% of the total *ACT1* 3′ ends; Table 2) produced during a brief pulse of transcription corresponds to the major site seen in wild-type cells (Fig. 3A and 4)



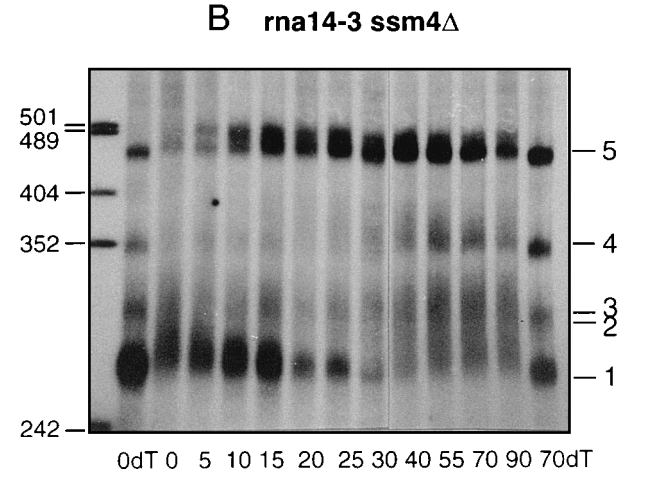


FIG. 5. Analysis of *ACT1* mRNA in strains with the *ssm4* suppressor. Polyacrylamide Northern blot of endogenous *ACT1* transcripts produced at 24°C and following a shift to 35°C in the *ssm4*Δ mutant strain (A) and the *rna14-3 ssm4*Δ mutant strain (B). The numbers on the left are molecular sizes in nucleotides.

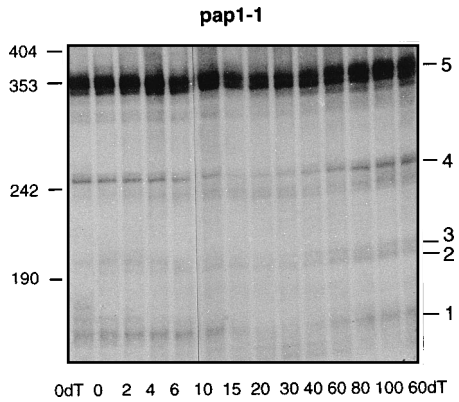


FIG. 6. Analysis of *PGK1-ACT1* mRNAs produced in the *pap1-1* mutant. Shown is a polyacrylamide Northern blot of a pool of hybrid *PGK1-ACT1* transcripts produced at the restrictive temperature under the conditions described in the legend to Fig. 3. Numbers 1 to 5 correspond to the transcript numbers in Fig. 2A. The numbers on the left are molecular sizes in nucleotides.

and not to the predominant site utilized in *rna14-3* mutant strains (Fig. 3C and 4). This observation indicated that *ssm4*Δ suppressor restored the recognition of the major poly(A) site. Although the pattern of 3′ ends observed is almost identical to that obtained in the wild-type W303 strain (Fig. 3A), the largest transcript is still more abundant in the double mutant strain (15.7% versus 8.9% in the wild-type strain). This indicated that the suppression of 3′ end alteration by *ssm4*Δ is not complete (Table 2 and Fig. 4).

TABLE 2. Quantification of the different *ACT1* 3′ ends on newly synthesized transcripts at the restrictive temperature[a]

Site(s)	% of total *ACT1* 3′ ends in:					
	Wild type	*rna15-2* mutant	*rna14-3* mutant	*rna14-3 ssm4*Δ mutant	*ssm4*Δ mutant	*pap1-1* mutant
1	57.0	23.2	20.1	47.7	57.2	17.2
2, 3	22.6	21.2	20.7	19.2	20.2	12.2
4	11.3	18.5	18.5	17.2	11.6	15.6
5	8.9	37.0	40.6	15.7	10.5	54.9

[a] Quantification of the different actin 3′ ends at the time of transcriptional repression (0-min time point) in a transcriptional pulse-chase experiment. Each datum point corresponds to the mean of two values. The number of counts was obtained with a PhosphorImager. The site numbers are the same as those in Fig. 3A.

Mutations in the poly(A) polymerase gene also alter poly(A) site recognition. In mammalian cells, the poly(A) polymerase has been reported to be part of the multicomponent assembly that specifies the cleavage and polyadenylation sites. In addition, loss of poly(A) polymerase decreases the efficiency with which poly(A) sites are recognized, suggesting that the poly(A) polymerase contributes to the choice of the cleavage site (3, 19). In *S. cerevisiae*, it has been suggested, on the basis of in vitro experiments, that the yeast poly(A) polymerase is not required for assembly of the cleavage complex and thereby does not influence the choice of the polyadenylation site (7). However, since it has been reported that RNA14p, RNA15p, and PAP1p belong to the same complex (20, 26), we examined if mutations in the *PAP1* gene also alter poly(A) site recognition.

To do this experiment, we transformed a temperature-sensitive poly(A) polymerase mutant (*pap1-1*) with a plasmid bearing the *PGK1-ACT1* chimeric construction. We then examined the distribution of the *ACT1* 3′ ends produced from the chimeric mRNA in a 10-min pulse of transcription after a shift for 20 min to the restrictive temperature. As shown in Fig. 6, all of the 3′ ends are utilized in the *pap1-1* mutant. Consistent with the notion that the poly(A) polymerase is required for addition of the poly(A) tail, the transcripts produced are not adenylated [compare 0-min results with and without oligo(dT) and RNase H treatment]. Surprisingly, the most abundant 3′ end (54.9% of total *ACT1* 3′ ends) corresponded to the most 3′-distal poly(A) site, which was also preferentially used in the *rna14* and *rna15* mutants. This result suggests that defects in the poly(A) polymerase can affect the specificity of polyadenylation. Interestingly, all of the transcripts produced at the restrictive temperature in the *pap1-1* mutant are slightly shorter than those produced in the other strains (Fig. 4). This observation implies that lesions in the poly(A) polymerase may alter the precise site of cleavage or polyadenylation, after a general area for adenylation has been specified.

One surprising result is that unadenylated *PGK1-ACT1* transcripts produced in a *pap1-1* mutant are more stable than in the wild-type and *rna14* and *rna15* mutant strains (compare Fig.

3A, B, C, and D with Fig. 6). This observation was unexpected because, for several yeast mRNAs, deadenylation leads to decapping following by a rapid 5′ to 3′ degradation of the body transcript (9, 29, 30). Thus, it was expected that unadenylated mRNA would be more rapidly degraded than the corresponding adenylated mRNA. Although we do not understand the basis for this effect, one possible explanation is that the PAP1 protein is involved in a later step in mRNA biogenesis, such as mRNA transport or maturation.

DISCUSSION

Mutations in the *RNA14* and *RNA15* genes do not affect the deadenylation rate. Two observations indicated that the rates of mRNA deadenylation were not affected by the *rna14-3* or *rna15-2* mutations. First, the deadenylation rate for the *PGK1* mRNA was the same in the wild-type and the *rna14* and *rna15* mutant strains (Fig. 1). In addition, the deadenylation rates of the ACT1 3′ ends, analyzed in the context of a chimeric *PGK1-ACT1* transcript, were also the same in the wild-type and the *rna14* and *rna15* mutant strains (Fig. 3A, B, and C). We interpret these results to indicate that the *RNA14* and *RNA15* gene products are not involved in deadenylation rate regulation. This implies that the shortening of the total poly(A) population observed at the restrictive temperature in the *rna14* and *rna15* mutant strains (24, 27) is due solely to a decrease in the synthesis of new polyadenylated transcripts.

In our experiments, we observed no alteration in the decay rates of the *PGK1* or *ACT1* mRNA in the *rna14* and *rna15* mutant strains compared with those in the wild-type strain. This observation was unexpected, since it has previously been reported that the *ACT1* mRNA decays more rapidly in these mutant strains (27). The difference in these results is likely to be due to the different methodologies used to measure the decay rate in each case. In our experiments, we utilized the glucose repression of the *GAL1* upstream activation sequence to determine the decay of each mRNA under similar conditions. In the previous experiments, the decay rate of the *ACT1* mRNA was assessed by determining the *ACT1* transcript levels remaining after a shift to the restrictive temperature. Since the *ACT1* transcript levels decreased more rapidly in the *rna14* and *rna15* mutant strains than in a temperature-sensitive RNA polymerase II (*rpb1-1*) mutant strain, it was suggested that the *rna14* and *rna15* mutations led to increased mRNA decay rates. However, the *rpb1-1* allele can show differences in how efficiently individual promoters are repressed after a shift to the restrictive temperature (33, 33a). A simple explanation for the *ACT1* mRNA level differences observed in the *rpb1-1* mutant and the *rna14* and *rna15* mutant strains is that transcription of *ACT1* mRNA is less repressed in the *rpb1-1* mutant than is *ACT1* mRNA polyadenylation in the *rna14* and *rna15* mutant strains.

The RNA14, RNA15, and PAP1 proteins can affect the choice of poly(A) sites. Several lines of evidence indicated that the RNA14 and RNA15 proteins can affect the choice of specific poly(A) sites. First, after a shift to the restrictive temperature the relative levels of the different 3′ ends of the *ACT1* transcript were altered in the mutant strains (Fig. 2B, C, and D). In addition, transcriptional pulse-chase experiments with the *PGK1-ACT1* chimeric transcript indicated that this difference was due to a change in the utilization of the different polyadenylation sites and not to a difference in mRNA decay rates (Fig. 3A, B, and C).

We also observed that a mutation in the poly(A) polymerase gene led to similar alterations in the use of the different *ACT1* polyadenylation sites (Fig. 6). These results were unexpected, since it has been suggested that in *S. cerevisiae* the poly(A) polymerase is not required for assembly of the cleavage complex and the actual cleavage reaction (7, 26). The simplest explanation for this effect is that in *S. cerevisiae*, as in mammals, the poly(A) polymerase is part of the assembled cleavage complex and the levels of PAP1p, or its conformation, can affect the efficiency with which the cleavage complex assembles. This hypothesis is consistent with the observation that immunodepletion of the poly(A) polymerase from yeast extracts inhibits cleavage and that yeast subcellular fractions essential for cleavage in vitro do appear to contain some poly(A) polymerase (26).

An interesting question is why mutations in either *RNA14*, *RNA15*, or *PAP1* lead to the same changes in *ACT1* 3′ end distribution. Since all of the mutations give qualitatively similar phenotypes, it is unlikely that these lesions create novel specificities in these gene products that promote the recognition of the distal polyadenylation site. A simple hypothesis is that any decrease in the functioning of the cleavage and polyadenylation machinery would lead to the use of the most distal site. One possible way this might arise is that the most distal site actually represents a transcriptional terminator and that in the absence of cleavage and adenylation, the RNA polymerase stops and this end is then nonspecifically polyadenylated. However, the sites of transcription termination that have been examined to date in *S. cerevisiae* in vivo are not adenylated (16). An alternative hypothesis is that a decrease in polyadenylation efficiency promotes the use of the most distal poly(A) site, either because this site is closer to the site of transcriptional termination (and thereby perhaps more efficiently recognized) or because this site is an efficient site for cleavage and adenylation but is normally not utilized to high levels because in the presence of efficient cleavage and adenylation, the 3′ end is generated, on average, before the RNA polymerase transcribes the most distal site.

Implications for the control of polyadenylation site choice in *S. cerevisiae* and mammals. Homologs of RNA14p and RNA15p have been identified in more complex eukaryotes. RNA14p shows 24.3% identity with a 77-kDa subunit of mammalian CstF, which is required for recognition of polyadenylation signals and for assembly of the polyadenylation complex. Similarly, the RNA binding domain of RNA15p is 42.5% identical to the RNA binding domain of the 64-kDa subunit of CstF (39). Interestingly, and analogous to our in vivo observations, changes in the levels of CstF activity in mammalian extracts have been shown to affect the choice of polyadenylation sites (23, 41). These observations imply that the regulation of poly(A) site choice occurs, at least in some cases, by modulation of the activity of the RNA14 and RNA15 proteins. In this light, it is interesting that mutations in a *Drosophila* homolog of RNA14p [the *Drosophila* su(f) protein] suppress the consequences of transposon insertions, perhaps by altering the site of polyadenylation (28).

Role of these gene products in the separable phases of cleavage and adenylation. In vitro experiments with yeast extracts have shown that the *RNA14* and *RNA15* gene products are involved in both cleavage and polyadenylation while the PAP1 protein is required only for polyadenylation (26). Our results show that, in vivo, all three of these gene products are implicated in polyadenylation site recognition and subsequent cleavage. We also observed that any 3′ end that is generated in the *rna14* and *rna15* mutants received a full-length poly(A) tail. This observation is slightly different from that of in vitro experiments with precleaved *CYC1* mRNA (26), in which extracts deficient in RNA14p and RNA15p did not efficiently adenylate a precleaved *CYC1* precursor. This suggests that

recognition of the poly(A) site by the RNA14 and RNA15 proteins is a preliminary step necessary for polyadenylation, even on precleaved substrates. We interpret these observations to suggest that the *rna14* and *rna15* mutations affect the assembly of the complex both in vivo and in vitro and that complex assembly is required both for cleavage and for efficient adenylation.

Role of the *SSM4* gene product. Mutations in the *ssm4* gene suppress the temperature sensitivity of the *rna14-3* allele but do not suppress the *rna15-2* mutation or an *rna14* deletion. The *SSM4* gene encodes a large protein with a calculated molecular mass of ~150 kDa and no homology to any known protein (24). Our results indicate that the absence of SSM4 function restores the use of the most proximal poly(A) site choice in the *rna14-3* mutant (Fig. 3D and 4). This result indicated that the suppression due to *ssm4*Δ occurs by the *ssm4*Δ mutation either directly or indirectly altering cleavage and polyadenylation.

ACKNOWLEDGMENTS

We thank S. Butler for the gift of the *pap1-1* mutant strain, F. Lacroute and J.-M. Rouillard for the *rna14* and *rna15* mutant strains used in this study and for discussions, and the members of R.P.'s laboratory for helpful comments on the manuscript.

This work was supported by the Howard Hughes Medical Institute and a grant to R.P. from the National Institutes of Health (GM 45443).

REFERENCES

1. **Beelman, C. A., and R. Parker.** 1994. Differential effects of translational inhibition in cis and in trans on the decay of the unstable yeast *MFA2* mRNA. J. Biol. Chem. **269:**9687–9692.
2. **Beelman, C. A., and R. Parker.** 1995. Degradation of mRNA in eukaryotes. Cell **81:**179–183.
3. **Bienroth, S., W. Keller, and E. Wahle.** 1993. Assembly of a processive messenger RNA polyadenylation complex. EMBO J. **12:**585–594.
4. **Bonneaud, N., L. Minvielle-Sebastia, C. Cullin, and F. Lacroute.** 1994. Cellular localization of RNA14p and RNA15p, two proteins involved in mRNA stability. J. Cell Sci. **107:**913–921.
5. **Butler, J. S., and T. Platt.** 1988. RNA processing generates the mature 3′ end of yeast *CYC1* messenger RNA *in vitro*. Science **242:**1270–1274.
6. **Caponigro, G., D. Muhlrad, and R. Parker.** 1993. A small segment of the *MATα1* transcript promotes mRNA decay in *Saccharomyces cerevisiae*: a stimulatory role for rare codons. Mol. Cell. Biol. **13:**5141–5148.
7. **Chen, J., and C. Moore.** 1992. Separation of factors required for cleavage and polyadenylation of yeast pre-mRNA. Mol. Cell. Biol. **12:**3470–3481.
8. **Decker, C. J., and R. Parker.** 1993. A turnover pathway for both stable and unstable mRNAs in yeast: evidence for a requirement for deadenylation. Genes Dev. **7:**1632–1643.
9. **Decker, C. J., and R. Parker.** 1994. Mechanisms of mRNA degradation in eukaryotes. Trends Biochem. Sci. **19:**336–340.
10. **Felici, F., G. Cesareni, and J. M. X. Hughes.** 1989. The most abundant small cytoplasmic RNA of *Saccharomyces cerevisiae* has an important function required for normal cell growth. Mol. Cell. Biol. **9:**3260–3268.
11. **Gallie, D. R.** 1991. The cap and poly(A) tail function synergistically to regulate mRNA translational efficiency. Genes Dev. **5:**2108–2116.
12. **Gallwitz, D., F. Perrin, and R. Seidel.** 1981. The actin gene in *Saccharomyces cerevisiae*: 5′ and 3′ end mapping, flanking and putative regulatory sequences. Nucleic Acids Res. **9:**6339–6350.
13. **Gietz, D., A. St. Jean, R. A. Woods, and R. H. Schiestl.** 1992. Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. **20:**1425.
14. **Heaton, B., C. Decker, D. Muhlrad, J. Donahue, A. Jacobson, and R. Parker.** 1992. Analysis of chimeric mRNAs identifies two regions within the *STE3* mRNA which promote rapid mRNA decay. Nucleic Acids Res. **20:**5365–5373.
15. **Heidmann, S., B. Obermaier, K. Vogel, and H. Domdey.** 1992. Identification of pre-mRNA polyadenylation sites in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **12:**4215–4229.
16. **Hyman, L. E., and C. L. Moore.** 1993. Termination and pausing of RNA polymerase II downstream of yeast polyadenylation sites. Mol. Cell. Biol. **13:**5159–5167.
17. **Jackson, R. J., and N. Standart.** 1990. Does the poly(A) tail and 3′ untranslated region control mRNA translation? Cell **62:**15–24.
18. **Keller, W.** 1995. No end yet to messenger RNA 3′ processing! Cell **81:**829–832.
19. **Keller, W., S. Bienroth, K. M. Lang, and G. Christofori.** 1991. Cleavage and polyadenylation factor (CPF) specifically interacts with the pre-mRNA 3′ processing signal AAUAAA. EMBO J. **10:**4241–4249.
20. **Kessler, M. M., A. M. Zhelkovsky, A. Skvorak, and C. L. Moore.** 1995. Monoclonal antibodies to yeast poly(A) polymerase (PAP) provide evidence for association of PAP with cleavage factor 1. Biochemistry **34:**1750–1759.
21. **Lingner, J., J. Kellermann, and W. Keller.** 1991. Cloning and expression of the essential gene for poly(A) polymerase from *S. cerevisiae*. Nature (London) **354:**496–498.
22. **Lingner, J., I. Radkte, E. Wahle, and W. Keller.** 1991. Purification and characterization of poly(A) polymerase from *Saccharomyces cerevisiae*. J. Biol. Chem. **266:**8741–8746.
23. **MacDonald, C. C., J. Wilusz, and T. Shenk.** 1994. The 64-kilodalton subunit of the CstF polyadenylation factor binds to pre-mRNAs downstream of the cleavage site and influences cleavage site location. Mol. Cell. Biol. **14:**6647–6654.
24. **Mandart, E., M.-E. Dufour, and F. Lacroute.** 1994. Inactivation of *SSM4*, a new *Saccharomyces cerevisiae* gene, suppresses mRNA instability due to *rna14* mutations. Mol. Gen. Genet. **245:**323–333.
25. **Maquat, L. E.** 1991. Nuclear mRNA export. Curr. Opin. Cell Biol. **3:**1004–1012.
26. **Minvielle-Sebastia, L., P. J. Preker, and W. Keller.** 1994. RNA14 and RNA15 proteins as components of a yeast pre-mRNA 3′-end processing factor. Science **266:**1702–1705.
27. **Minvielle-Sebastia, L., B. Winsor, N. Bonneaud, and F. Lacroute.** 1991. Mutations in the yeast *RNA14* and *RNA15* genes result in an abnormal mRNA decay rate; sequence analysis reveals an RNA-binding domain in the RNA15 protein. Mol. Cell. Biol. **11:**3075–3087.
28. **Mitchelson, A., M. Simonelig, C. Williams, and K. O'Hare.** 1993. Homology with *Saccharomyces cerevisiae* RNA14 suggests that phenotypic suppression in *Drosophila melanogaster* by *suppressor of forked* occurs at the level of RNA stability. Genes Dev. **7:**241–249.
29. **Muhlrad, D., C. J. Decker, and R. Parker.** 1994. Deadenylation of the unstable mRNA encoded by the yeast *MFA2* gene leads to decapping followed by 5′ to 3′ digestion of the transcript. Genes Dev. **8:**855–866.
30. **Muhlrad, D., C. J. Decker, and R. Parker.** 1995. Turnover mechanisms of the stable yeast PGK1 mRNA. Mol. Cell. Biol. **15:**2145–2156.
31. **Muhlrad, D., and R. Parker.** 1992. Mutations affecting stability and deadenylation of the yeast *MFA2* transcript. Genes Dev. **6:**2100–2111.
32. **Munroe, D., and A. Jacobson.** 1990. mRNA poly(A) tail, a 3′ enhancer of translational initiation. Mol. Cell. Biol. **10:**3441–3455.
33. **Nonet, M., C. Scafe, J. Sexton, and R. Young.** 1987. Eucaryotic RNA polymerase conditional mutant that rapidly ceases mRNA synthesis. Mol. Cell. Biol. **7:**1602–1611.

33a. **Parker, R.** Unpublished data.

34. **Piñol-Roma, S., and G. Dreyfuss.** 1992. Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm. Nature (London) **355:**730–732.
35. **Precker, P. J., J. Lingner, L. Minvielle-Sebastia, and W. Keller.** 1995. The *FIP1* gene encodes a component of a yeast pre-mRNA polyadenylation factor that directly interacts with poly(A) polymerase. Cell **81:**379–389.
36. **Proweller, A., and S. Butler.** 1994. Efficient translation of poly(A) deficient mRNA in *Saccharomyces cerevisiae*. Genes Dev. **8:**2629–2640.
37. **Russack, R., K. W. Nehrke, and T. Platt.** 1995. *REF2* encodes an RNA-binding protein directly involved in yeast mRNA 3′-end formation. Mol. Cell. Biol. **15:**1689–1697.
38. **Sachs, A., and E. Wahle.** 1993. Poly(A) tail metabolism and function in eukaryotes. J. Biol. Chem. **268:**22955–22958.
39. **Takagaki, Y., and J. L. Manley.** 1994. A polyadenylation factor subunit is the human homologue of the *Drosophila suppressor of forked* protein. Nature (London) **372:**471–474.
40. **Wahle, E., and W. Keller.** 1992. The biochemistry of 3′-end cleavage and polyadenylation of messenger RNA precursors. Annu. Rev. Biochem. **61:**419–440.
41. **Weiss, E. A., G. M. Gilmartin, and J. R. Nevins.** 1991. Poly(A) site efficiency reflects the stability of complex formation involving the downstream element. EMBO J. **10:**215–219.