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Transcription termination downstream of the *Saccharomyces cerevisiae FPB1* poly(A) site does not depend on efficient 3′ end processing

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ABSTRACT

Efficient transcription termination downstream of poly(A) sites has been shown to correlate with the strength of an upstream polyadenylation signal and the presence of a polymerase pause site. To further investigate the mechanism linking termination with 3′-end processing, we analyzed the *cis*-acting elements that contribute to these events in the *Saccharomyces cerevisiae FBP1* gene. *FBP1* has a complex polyadenylation signal, and at least three efficiency elements must be present for efficient processing. However, not all combinations of these elements are equally effective. This gene also shows a novel organization of sequence elements. A strong positioning element is located upstream, rather than downstream, of the efficiency elements, and functions to select the cleavage site in vitro and in vivo. Transcription run-on analysis indicated that termination occurs within 61 nt past the poly(A) site. Deletion of two UAUAUA-type efficiency elements greatly reduces polyadenylation in vivo and in vitro, but transcription termination is still efficient, implying that *FBP1* termination signals may be distinct from those for polyadenylation. Alternatively, assembly of a partial, but nonfunctional, polyadenylation complex on the nascent transcript may be sufficient to cause termination.

Keywords: efficiency element; polyadenylation signal; positioning element; RNA polymerase pausing; run-on analysis; transcriptional interference; yeast

INTRODUCTION

Creation of the 3′ end is an essential step in mRNA synthesis. In eukaryotes, the mRNA must be separated from the DNA template so that it can be transported to the cytoplasm and used as a substrate for translation, and the RNA polymerase II (pol II) must be released to initiate new transcription. The mature mRNA is generated from a precursor RNA that extends between 100 and 4,000 nt beyond the mature 3′ end. This processing involves endonucleolytic cleavage at a specific site coupled to polymerization of a poly(A) tail onto the upstream cleavage fragment (Manley & Takagaki, 1996; Wahle & Keller, 1996). This tail plays a key role in the subsequent life of the mRNA because it functions in mRNA turnover (Beelman & Parker, 1995) and in facilitating translation (Sachs et al., 1997).

The desire to understand the molecular mechanism of mRNA 3′ end formation has provoked an extensive search for the mRNA sequences and the *trans*-acting factors involved in this event (Manley & Takagaki, 1996; Wahle & Keller, 1996). The yeast *Saccharomyces cerevisiae* has been extremely useful in studies of the biochemistry and genetics of this process and, overall, the 3′-end processing reaction resembles that of mammalian cells. The sequence motifs in the pre-mRNAs that dictate the reaction are different, but similarities can be found in many of the proteins required for polyadenylation (Manley & Takagaki, 1996). In *S. cerevisiae*, three elements working in concert are sufficient to specify a poly(A) site (for review, Guo & Sherman,

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1996). There is a PyA_n motif at the cleavage site and two upstream elements—a TA-rich element, consisting most often of alternating pyrimidines and purines or a TTTTTAT motif, and, downstream of this sequence, an A-rich element. Deletion of the TA-rich element decreases the efficiency of processing. Recognition of the A-rich motif appears to be closely coupled to cleavage at the nearest downstream PyA_n site. Mutation of the A-rich element shifts processing to other sites without affecting efficiency unless there are no redundant signals on the transcript that can substitute for its function. For this reason, it has been referred to as a positioning element, whereas the TA-rich motif is called an efficiency element. However, some yeast polyadenylation signals are complex, and have multiple efficiency elements that cumulatively create a strong signal (Egli et al., 1995; Guo et al., 1995).

Transcription termination downstream of a poly(A) site is thought to be important in reducing nonproductive transcription and in avoiding transcriptional interference at downstream promoters (Eggermont & Proudfoot, 1993 and references therein) and chromosomal elements such as centromeres (Hegemann & Fleig, 1993) and origins of replication (Chen et al., 1996). In higher eukaryotes, the efficiency of transcription termination correlates with the strength of the polyadenylation signal and the presence of a termination site or region located downstream of the poly(A) site (Proudfoot, 1989; Edwalds-Gilbert et al., 1993; Ashfield et al., 1994). In some cases, this termination site has been shown to pause the elongating polymerase (Enriquez-Harris et al., 1991; Eggermont & Proudfoot, 1993). In *S. cerevisiae*, termination and polyadenylation also appear to be coupled. This conclusion is based on studies showing that mutations in polyadenylation signals cause transcriptional read-through into centromeres or autonomously replicating sequences (ARSs), resulting in plasmid instability (Snyder et al., 1988; Russo & Sherman, 1989; Russo, 1995), or read-through past a splice site, allowing synthesis of reporter mRNAs (Hyman et al., 1991; Irniger et al., 1991; Hyman & Moore, 1993). Pause sites may also be involved in transcription termination in yeast. Unpolyadenylated transcripts ending 50–100 nt beyond the poly(A) site can be detected after in vitro transcription of the *GAL7* and *ADH2* poly(A) sites in yeast extracts (Hyman & Moore, 1993). A region between 25 and 100 nt downstream of the *CYC1* poly(A) site is necessary for termination in vivo (Russo & Sherman, 1989), whereas only 15 nt beyond the *CYC1* poly(A) site is sufficient for polyadenylation in vitro (Sadhale & Platt, 1992), suggesting a specific role for the farther sequence in termination. A pause site has been demonstrated recently in the *ura4* gene of the yeast *Schizosaccharomyces pombe*. This study used a transcription run-on (TRO) analysis in combination with an assay that showed insertion of the pause site between two competing polyadenylation signals increased utilization of the upstream site in vivo (Birse et al., 1997). As for mammalian cells, efficient termination required both the pause site and a functional polyadenylation signal.

In this paper, we address the question of the sequences involved in the 3′-end formation of the *S. cerevisiae FBP1* gene. Our results indicate that it is a complex polyadenylation signal, and that at least three possible efficiency elements are necessary for efficient processing. The *FBP1* gene also shows a novel organization of sequence elements, with a positioning element located upstream of the efficiency elements. Surprisingly, transcription termination downstream of the *FBP1* poly(A) site can be uncoupled from 3′ end processing, and continues to occur efficiently upon removal of two of the efficiency elements.

RESULTS

A 397-bp fragment of the *S. cerevisiae FBP1* gene is sufficient for in vivo 3′-end formation and functions bidirectionally

To identify the elements required for 3′-end formation in the *S. cerevisiae FBP1* gene, we started with a fragment containing 3′ flanking sequence between positions 1054 and 1445 (with 1 as the translation start) of the *FBP1* gene. This 397-bp region (Fig. 1) includes the 3′ noncoding region of the *FBP1* gene, and the 5′ flank and part of the coding region of *YLR376c*, an ORF of unknown function that begins 195 bp after the *FBP1* translation stop codon. This fragment contains several TATATA-like sequences that could be efficiency elements—TATATGT, an almost perfect $(TA)_{14}$ sequence, and TATGTA. It also includes an A_{11} run that matches the positioning element consensus, but is in an unusual position, i.e., upstream of the potential efficiency elements.

To determine the function of the 397-nt *FBP1* fragment in 3′-end formation, we used a poly(A) signal competition assay, in which this fragment was inserted in either orientation into the pME729 plasmid (Irniger et al., 1992), between the 5′ part of the *ACT1* gene and the *ADH1* polyadenylation signal (Fig. 2A). In this assay, the strength of the insert in specifying 3′-end formation influences how many transcripts are polyadenylated at the downstream *ADH1* site. Plasmids were introduced into the yeast strain SEY2101, and total RNA isolated from the resulting strains was analyzed by northern blots (Fig. 2A). The probe used for hybridization was derived from a fragment of the *ACT1* gene, and detects transcripts from the plasmid as well as from the chromosomal copy of *ACT1*. The 1.4-kb *ACT1* mRNA thus served as an internal control for the amount of RNA applied to the gel. Transcription from the original vector, pME729, gives a unique band of 0.6 kb, corresponding to an RNA extending from the actin pro-

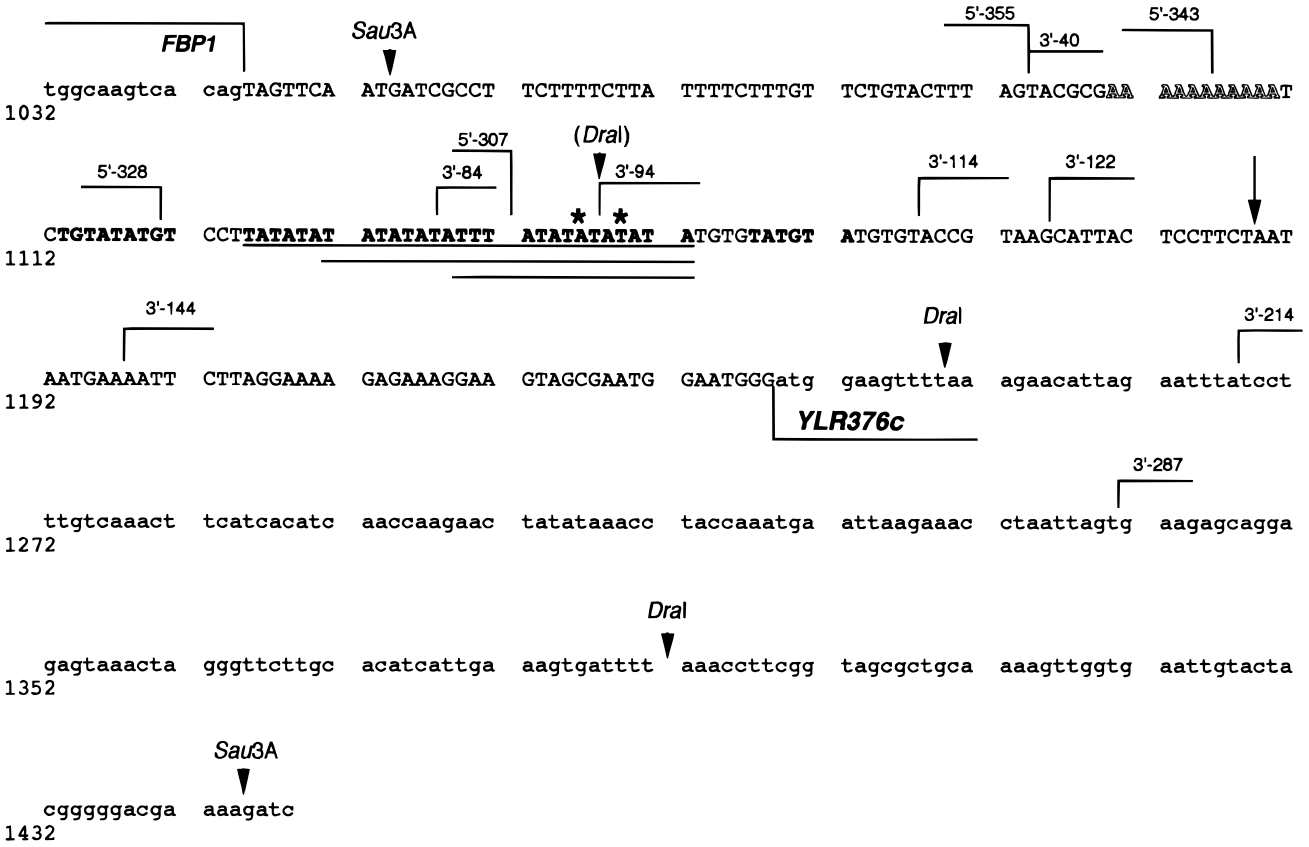


FIGURE 1. Sequence of the *S. cerevisiae FBP1* gene between positions 1032 and 1448 (considering 1 as the translation start). The efficiency element matches are in bold-type. One of these matches is the almost perfect $(TA)_{14}$ repeat located between positions 1125 and 1152. The region of this repeat deleted in the D3 deletion is underlined, the sequence deleted in the D2 deletion is underlined twice, and the sequence deleted in the D1 deletion is underlined three times. The 5′ deletion and the 3′ deletion end points are indicated with the length of *FBP1* sequence conserved in each case. The arrow marks the major poly(A) site. Arrowheads indicate the relevant restriction sites. Lowercase letters indicate the coding regions of *FBP1* and *YLR376c*. Asterisks correspond to the nucleotides that have been changed to their complementary ones in the DM deletion. In this case, a new *Dra* I restriction site appears, which is indicated in parenthesis. The A_{11} tract is shadowed.

moter to the *ADH1* poly(A) site (Irniger et al., 1992). When the *FBP1* fragment is in the forward orientation (pVPA397), an abundant 0.4-kb truncated (T) transcript and a minor 1.0-kb readthrough (R) species are detected. The size of the major band corresponds to the use of a polyadenylation site at the beginning of the *FBP1* fragment, whereas the minor band has the size expected if the *ADH1* poly(A) site is used. More than 98% of the transcripts end at the *FBP1* poly(A) site, indicating that this fragment contains a very efficient polyadenylation signal. This fragment also functions well in the reverse orientation (pVPA397R), with 89% of the mRNAs using a site located approximately at the end of the *FBP1* insert. Northern blot analysis of poly(A)$^+$ RNA purified on oligo(dT) columns gave identical results to the ones obtained with total RNA in both efficiency and location of the poly(A) sites (data not shown). Therefore, total RNA was used for subsequent experiments.

We used the RT-PCR technique to map the poly(A) sites at the nucleotide level in plasmids pVPA397 and pVPA397R, as well as in pRS2083, which contains the entire *FBP1* gene under control of its own promoter. Sequencing of the RT-PCR products revealed that the major poly(A) site in the wild-type gene is most often after a T at position 1188 (Fig. 3). In some cases, a C at 1187, a T at 1191, or a G at 1195 is used instead. Thus, the wild-type sites are concentrated in a small 9-nt region between 1187 and 1195. No major differences were seen between the sites used in the 397-nt fragment and the whole gene, confirming that the *FBP1* poly(A) site was being recognized appropriately in the competition assay construct. In the reverse orientation, the poly(A) site was found at five different positions, four inside the *FBP1* fragment and one outside. This analysis indicated that the sequence contained in the 397-nt fragment was sufficient for accurate and efficient polyadenylation.

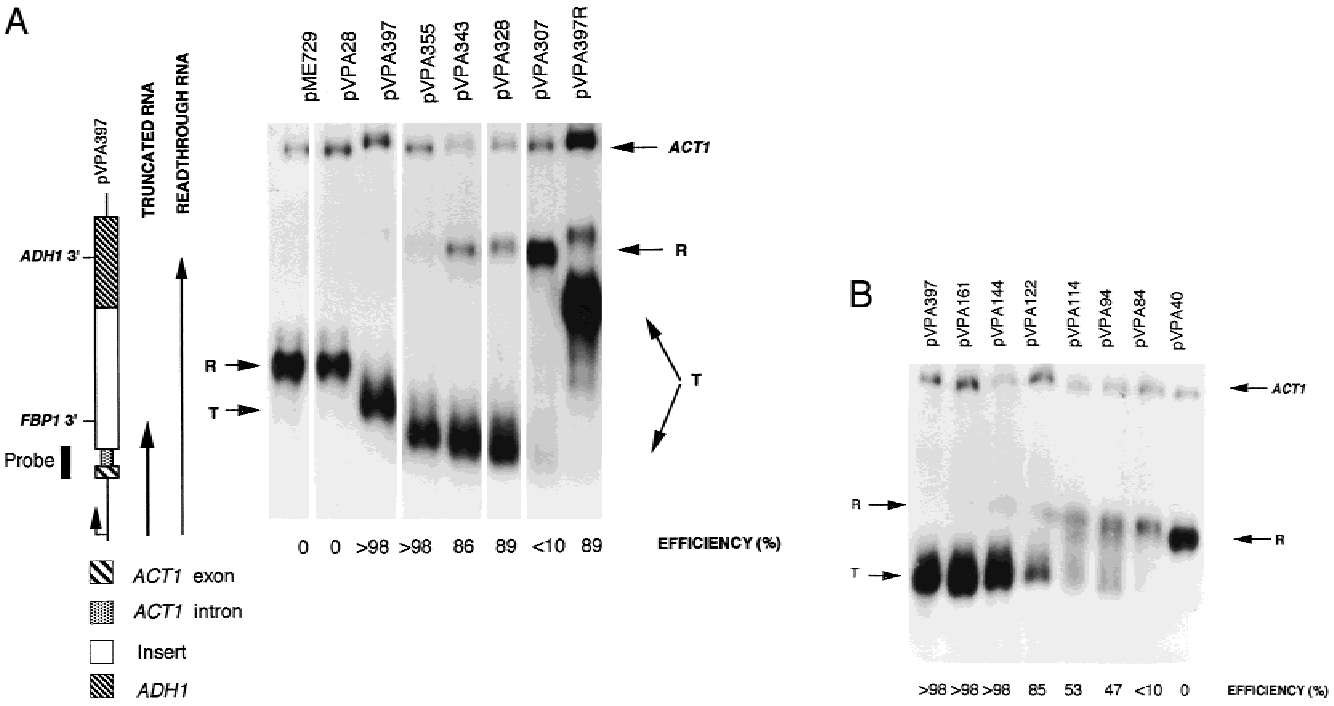


FIGURE 2. Poly(A) signal competition assays. **A:** Analysis of in vivo polyadenylation of pVPA plasmids carrying deletions of the 397-bp fragment from the 5′ end (see Materials and Methods for a complete description of these plasmids). Total RNA was isolated from yeast cells carrying the plasmid indicated above each lane (R stands for reverse orientation). Samples were analyzed by a 2% agarose-formaldehyde gel. The blot was hybridized with probe complementary to *ACT1* sequence, as shown in the diagram on the left. The polyadenylation efficiency (expressed as the percentage of truncated transcripts, T/T+R) is shown at the bottom of each lane, and was determined as an average from three different experiments, with the standard deviation error being lower than 3% in all cases. *ACT1*, R, and T stand for the chromosomal *ACT1*, readthrough, and truncated mRNAs, respectively. pME729 is the original plasmid without insert and pVPA28 has only the $(TA)_{14}$ inverted repeat. **B:** Analysis of in vivo polyadenylation of pVPA plasmids carrying deletions of the 397 fragment from the 3′ end (experimental conditions and symbols as in A). **C:** Analysis of the effect of the D1, D2, and D3 deletions on the processing efficiency of the 397 and 144 fragments. Conditions and symbols are as described for panel A. In this case, we also indicate at the bottom another measure of the efficiency, which is the percentage of truncated mRNA relative to the wild-type fragment and normalized with regard to the *ACT1* mRNA.

Deletions of the 397-nt *FBP1* fragment delineate a short region required for in vivo processing

To determine more precisely the sequences required for 3′-end formation in the *FBP1* gene, we created a nested series of deletions in the 397-nt fragment from both ends (Fig. 1) and cloned them into pME729. RNAs were again analyzed by northern blots (Fig. 2A for 5′ deletions and Fig. 2B for 3′ deletions) and poly(A) sites mapped by RT-PCR (Fig. 3). Four deletions from the 5′ end were tested. A construct in which the first 42 nt (5′-355) were removed showed wild-type polyadenylation efficiency. A deletion of an additional 12 nt (5′-343) shortened the A_{11} tract to five A's, but still yielded almost wild-type levels of truncated RNAs and a wild-type pattern of poly(A) site utilization. The third deletion (5′-328) eliminated the entire A_{11} tract and the TATATGT motif. This truncation also gave a high efficiency of polyadenylation, but exhibited much greater variability in the positions of the poly(A) sites. Six different sites were used, with two of them, at positions 1204 and 1219, not occurring naturally. Moreover, the major poly(A) site was no longer used preferentially in the 5′-328 mutant. This result suggests that some element in the region between positions 1105 and 1120, possibly the AAAAA or the TATATGT sequences, plays a role in the precise selection of the poly(A) site. The

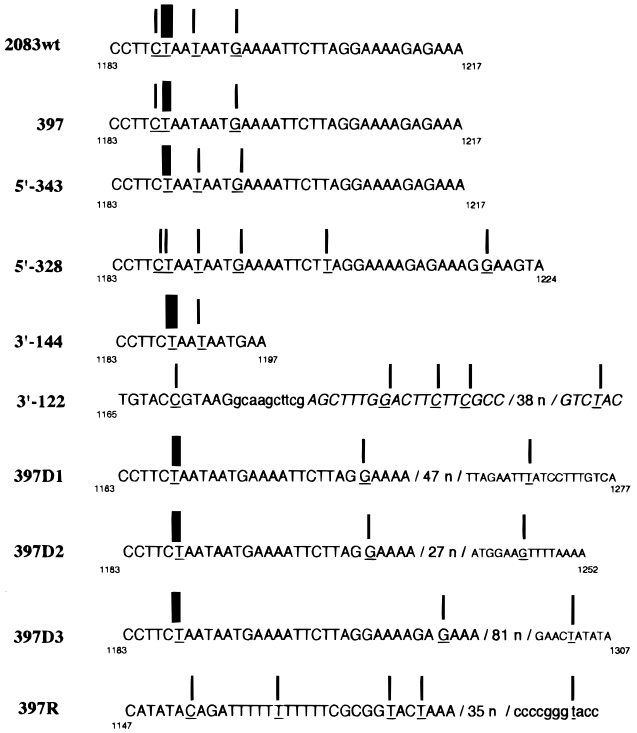


FIGURE 3. Poly(A) sites. Underlined letters indicate the last nucleotide before the poly(A) tail of several cDNAs obtained by RT-PCR from mRNAs of the whole gene (named 2083) or several pVPA plasmids. Thickness of the lines above the letters is proportional to the number of clones in which this site is used. The coding region of *YLR376c* is in small capital letters, the polylinker sequences is in small letters, and the *ADH1* sequence is in italics.

fourth deletion, which lacks the first seven TA pairs of the $(TA)_{14}$ sequence, causes a dramatic decrease in polyadenylation efficiency to less than 10% of transcripts being truncated. We have not been able to accurately map the poly(A) sites for this construct due to the very low amounts of transcript.

The 3′ deletions resulted in a more gradual reduction in polyadenylation efficiency (Fig. 2B). The longest fragments tested (up to 3′-144) show wild-type levels of processing and choice of poly(A) site. In the 3′-122 deletion, the wild-type poly(A) sites and sequences immediately surrounding these sites were eliminated, and the polyadenylation ability declines slightly to 85%. Poly(A) sites were now scattered over a wide region (Fig. 3), consistent with removal of the wild-type poly(A) sites. With the next deletion (3′-114), the proportion of truncated species is reduced to 53%, suggesting that the sequence in these eight nucleotides contributes to efficiency. Interestingly, removal of the putative efficiency element TATGTA in deletion 3′-94 did not result in a further drop in efficiency from 3′-114 levels (47 versus 53%). On the other hand, deletion of 10 more bases (deletion 3′-84) causes a large decrease in the level of truncated transcripts, to less than 10%. In this mutation, only the first six TA pairs of the $(TA)_{14}$ region are conserved. Finally, the 3′-40 deletion, which does not contain any of the TA repeats, the TATATG motif, or the A_{11} tract, produced only readthrough mRNA. Taken together, this deletion analysis shows that a short region of 77 nt, extending from positions 1121 to 1197 and defined by the 5′-328 and 3′-144 deletions, is adequate for efficient polyadenylation in the *FBP1* sequence. However, an additional 15 nt of upstream sequence is needed to maintain the accuracy of 3′-end processing.

The TA repeat is necessary for efficient processing

The TATATA sequence has been reported to be one of the most effective efficiency elements (Irniger & Braus, 1994). The *FBP1* sequence contains an almost perfect 28-nt TA repeat, with only one TA pair replaced by TT (Fig. 1). This element could not direct processing by itself when subcloned into the pME729 vector (Fig. 2A, pVPA28). Nevertheless, the deletions 5′-307 or 3′-84, which remove either the first or second part of this region, cause a large reduction in processing, suggesting that the entire repeat might be important. This region in double-stranded DNA forms a stable cruciform structure in vitro (del Olmo & Pérez-Ortín, 1993), although not in yeast in vivo (Aranda et al., 1997), and is also predicted to form a stable RNA hairpin structure with a free energy of ~8.4 kcal/mol, according to the MFOLD program. This secondary structure would be destroyed in 5′-307 or 3′-84. To further explore what features of this region were important, we analyzed the effect of mutations and deletions in the TA repeat using the polyadenylation signal competition assay. We first constructed a double mutation in the 397-nt fragment (A to T at position 1146 and T to A at 1149, Fig. 1). This mutation maintained the proportion of A/T in the second half of the TA repeat, but destroyed the pattern of alternating pyrimidine/purines. However, analysis of RNAs from it showed no difference in processing efficiency compared to wild-type sequence (data not shown).

We then made deletions to reduce the TA content of this region without altering the surrounding sequence (Fig. 1). A deletion of 10 bp inside the $(TA)_{14}$ stretch (397D1) reduced this region to only nine TA pairs, but did not affect processing efficiency (Fig. 2C). It did have some effect on poly(A) site position, although the major wild-type site was still the predominant one (Fig. 3). The same result was observed with the 397D2 fragment, which conserved just three TA pairs. However, a complete deletion of the TA-rich sequence (397D3) caused a significant reduction in the amount of the truncated species, to 60% versus 98% for wild type (Fig. 2C). Again the wild-type poly(A) site is used predominantly. In derivatives of the 3′-144 fragment carrying the D2 and D3 deletions (Fig. 2C), we observed

efficiencies similar to that found for 397 derivatives, confirming that sequence beyond position 1197 was not involved in processing, even in the absence of the entire TA repeat.

For the polyadenylation competition experiments described previously, the polyadenylation efficiency of the upstream poly(A) site is calculated as the percentage of total transcripts (truncated plus readthrough species) originating from the actin promoter of the reporter plasmid. However, this definition assumes that all of the mRNA molecules that are not processed at the *FBP1* site reach the downstream *ADH1* site and are processed there. Inspection of the northern blot of RNAs derived from the construct with a complete deletion of the $(TA)_{14}$ tract (Fig. 2C) showed a reduction in the level of truncated species, but did not reveal a corresponding increase in readthrough transcripts. The most likely explanation for the lack of readthrough transcripts is that transcription is still terminating between the two poly(A) sites, and this possibility is explored in a later section. An alternative way to calculate the efficiency of polyadenylation at the *FBP1* site is to use the amount of actin mRNA in the sample as a standard and take the 3′-end forming efficiency of the 397 fragment to be the maximum possible, or 100%. By this determination, the relative efficiencies of the 397 D1, D2, and D3 constructs are 91, 61, and 16%, respectively (Fig. 2C, T relative levels), suggesting a much stronger effect of deletions in the TA repeat than calculated by comparison to the readthrough transcription. These results also indicate that the primary sequence of this region, rather than secondary structure, is the critical feature in its function as a polyadenylation signal.

The lack of an inverse correlation of readthrough and truncated transcripts is most obvious for the D3 deletion shown in Figure 2C, and this mutation was used to investigate other possible reasons for the decrease in truncated species. Southern analysis indicated that the copy number of the plasmids was not affected by the deletion (data not shown). Furthermore, if the D3 deletion is introduced into the *FBP1* gene carried on a single copy plasmid, the amount of *FBP1* mRNA derived from this construct is 2.7 times lower than that produced from the equivalent wild-type construct (data not shown). This reduction again suggests that the absence of the TA repeat is affecting the mRNA levels. The mutant mRNA is perfectly active because this plasmid can complement a disruption in the chromosomal *FBP1* gene in the RG1-5d strain. However, the levels of fructose-1,6-bisphosphatase activity are 3.3-fold less in the strain carrying the D3 deletion compared to the wild-type gene (41 versus 136 mU/mg of protein), in agreement with the decrease in the mRNA levels. According to this data, translation of the D3 mRNA is normal, suggesting that an appropriate length of poly(A) tail has been added.

TRO analysis (see below) shows that the level of RNA polymerases transcribing the region of *FBP1* upstream of the poly(A) site is similar in both wild-type and D3 constructs. Therefore, a decrease in transcription rate is not responsible for the lower amounts of D3 mRNA. This reduction could be explained by an effect of the $(TA)_{14}$ element on mRNA stability. To check this possibility, the *FBP1* 397WT and 397D3 fragments were cloned downstream of the galactose inducible promoter in the pHZ18Δ2SMA plasmid (Hyman et al., 1991). This plasmid is a high copy *Escherichia coli/S. cerevisiae* shuttle vector that contains a fusion gene under the control of the *GAL* upstream activator sequence and driven by the *CYC1* promoter. The ribosomal protein (rp)51 untranslated region and an AUG codon, followed by the 5′ splice site and a shortened intron and 3′ splice site, are fused in frame to the *lacZ* gene. We subcloned the fragments in the forward orientation inside the rp51 intron and transformed the W303-1a strain with the resulting plasmids. A similar decrease in the amount of processed transcripts was observed for the D3 fragment in comparison to WT, as was described for these same fragments in the pVPA plasmids (data not shown), indicating that the defect in accumulation of processed RNAs was independent of the surrounding in which the fragment was placed. With strains carrying the pHZ18D2SMA constructs, it is possible to induce transcription through the inserts with galactose and to repress it by shifting to a glucose-containing medium, and thus to compare the stability of the RNAs carrying (WT) or not (D3) the TATATA sequence. The RNA decay can be monitored by analyzing RNA from aliquots of cells taken at different times following the shift to glucose. This experiment gave a half life of 6.3 ± 1 min for the wild-type sequence and 6.0 ± 1 min for the D3 deletion (data not shown). Thus, the $(TA)_{14}$ deletion causes a reduction in levels of mRNA containing the *FBP1* sequence without affecting the stability of the mature poly(A)$^+$ mRNA.

In vitro experiments confirm that the *FBP1* TA repeat is involved in pre-mRNA processing

The data described above suggest that the TA repeat is important for optimal processing efficiency in vivo. We also tested the activity of wild-type and mutant constructs in an in vitro processing system in which radioactive precursor is incubated with yeast whole-cell extract in the presence of ATP (Butler & Platt, 1988). In this system, functional precursor is cleaved at the poly(A) site and the upstream fragment becomes polyadenylated. Products from the reaction are then purified, resolved on a denaturing urea/acrylamide gel, and detected by autoradiography. Because the 3′-144 fragment was processed at wild-type levels in vivo, we used RNA containing this sequence as substrate for the in vitro reactions. When incubated with extract, 62%

of the input precursor RNA is processed correctly (Fig. 4A, pIVP144). The product is the size expected for cleavage at the in vivo poly(A) site followed by poly(A) addition. Substrate RNAs containing the D2 and D3 deletions were also tested. RNA with the D2 deletion showed a reduction in processing efficiency to 21% of that of precursor with the wild-type sequence. With the D3 deletion, there was no processing at all. These in vitro results emphasize the importance of the TA repeat in the coupled cleavage and polyadenylation reaction.

It is possible to examine only the cleavage reaction in vitro by using partially purified cleavage factors. In *S. cerevisiae*, two factors, CF I and CF II, are sufficient for cleavage (Chen & Moore, 1992). Under these conditions, 42% of the pIVP144 substrate is cleaved, giving a cleavage product consistent with cleavage at the major *FBP1* poly(A) site (Fig. 4B). The partially purified factors contain an exonuclease that degraded the uncapped downstream cleavage product, such that only the upstream fragment is detected. We also examined the effects of the 5′ deletions on the cleavage reaction, and used the 397 fragment as the wild-type control for these experiments. Fifty percent of the input transcript (pIVP397) is processed and, as expected, gives a cleavage product of the same size as that of pIVP144. In contrast, only 8% of a similar substrate, but with the D3 deletion, is cleaved. Substrate with the 5′-328 or 5′-307 deletions were also tested. Processing of these RNAs

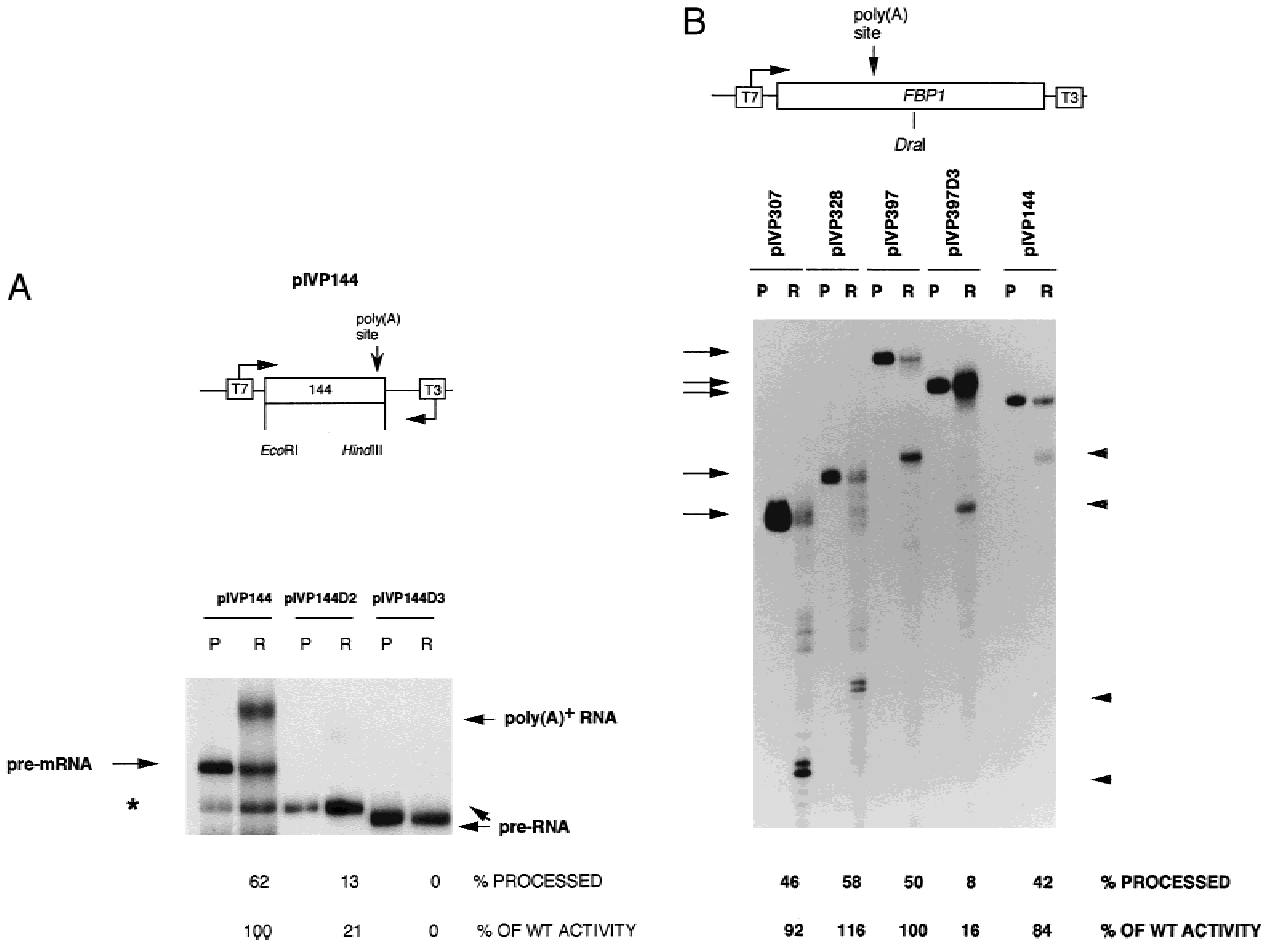


FIGURE 4. In vitro cleavage and polyadenylation of precursor mRNAs. **A:** Comparison of the in vitro polyadenylation efficiencies of 3′-144 derivatives carrying internal deletions of the TA repeat. A scheme of the pIVP144 plasmid is shown on the upper part. One microgram of *Hin*d III-linearized plasmid was used for in vitro transcription in the presence of α^{32}P-UTP in order to generate the precursors for processing. The reaction of in vitro cleavage and polyadenylation was performed as described in Materials and Methods and the samples were analyzed on a 5% polyacrylamide-urea gel. P lanes show the precursor RNA and R lanes show the products of the processing reaction. Band indicated by an asterisk in pIVP144 lanes is not due to processing of the precursor. The amount of processed RNA is indicated at the bottom of each lane, and the processing efficiency given as a percentage of the activity of the pIVP144 processing. **B:** Analysis of the in vitro cleavage activity of *FBP1* substrates. In this case, plasmids carrying *FBP1* sequences were transcribed by T7 polymerase after digestion with *Dra* I to generate the precursors. Cleavage reactions were performed as previously, but adding 2 μL of CF I and CF II-containing fractions instead of whole-cell extract. Arrows on the left indicate the positions of the RNA precursors and arrowheads on the right indicate the positions of the main 5′ cleavage products. In all cases, the values shown were calculated as an average from three different experiments. The standard deviation error was lower than 3% in all cases. Complete description of the pIVP plasmids can be found in Materials and Methods.

gave doublets that may correspond to cleavage in positions 1188 and 1195, and several minor bands. This result indicates that the wild-type poly(A) sites are used on the last two substrates in vitro, although the preference for position 1188 has been lost, as was observed for 5′-328 in vivo. The 5′-328 and 5′-307 substrates are processed at close to wild-type efficiency—116% and 92%, respectively. This result is different from the one found in vivo, in which the processing efficiency in 5′-307 was greatly reduced relative to 5′-328 because of elimination of part of the TA repeat. It seems that, in vitro, the remaining TA stretch and downstream sequence on 5′-307 are sufficient for recognition and cleavage of *FBP1* RNA precursor. In addition, these observations confirm that the TATGT or AAAAA upstream elements are not required for efficient processing, but are involved in selection of the cleavage site. 3′ Deletions were also tested using the cleavage reaction, and the results are in good accordance with the ones obtained in vivo. Again, a progressive reduction in levels of processing was seen upon deletion of 3′ sequences (data not shown).

Transcription termination can be uncoupled from efficient 3′-end processing

The results obtained with the D3 construct using in vivo poly(A) signal competition experiments suggest that transcription termination may be separated from efficient 3′-end formation. To further analyze the sequences involved in transcription termination, we used a plasmid stability assay in which transcription is induced from a *GAL1* promoter positioned upstream of the *CEN3* element. By taking advantage of the fact that transcription from this promoter reduces the mitotic stability of the plasmid (Hill & Bloom, 1987), this assay has been used to demonstrate that yeast sequences necessary for 3′-end formation are also involved in transcription termination (Russo & Sherman, 1989; Russo, 1995). For our experiments, fragments containing sequences from the *FBP1* 3′ region were inserted between the *GAL1* promoter and *CEN3* of the pAB610 plasmid containing the *URA3* marker. Transformants containing these plasmids (the pTER series) were grown in glucose-containing medium without uracil, then shifted to growth in medium containing uracil and either glucose or galactose. Aliquots of cells were taken at different time points and plated on solid medium containing uracil to determine total cell numbers and, without uracil, to determine the number of cells with plasmids. The percentage of cells carrying the plasmid was plotted against the number of generations (Fig. 5), and the efficiency of transcription termination was deduced from the rate of plasmid loss per generation (Table 1).

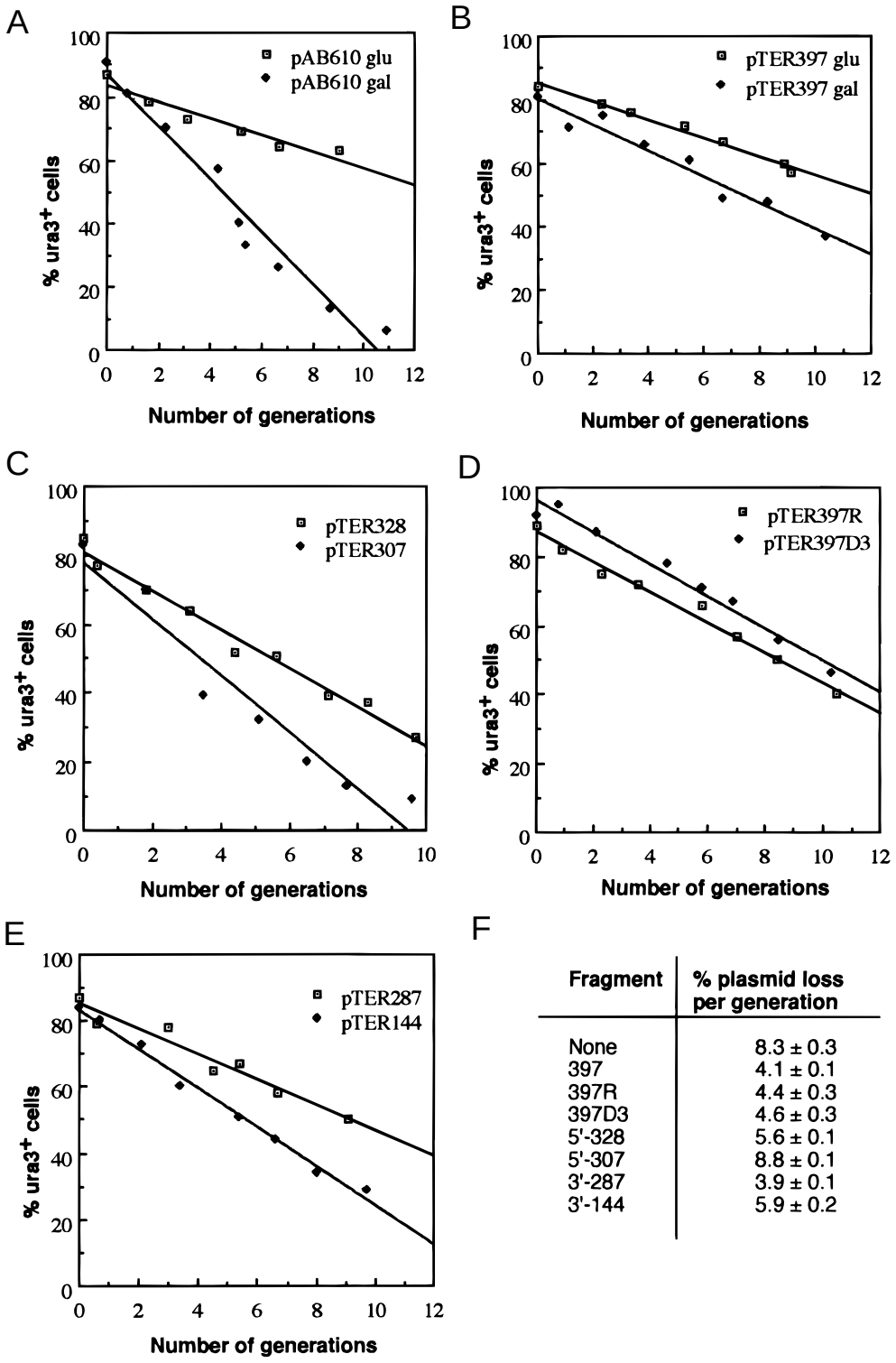
The pAB610 plasmid without an insert exhibited a 3% plasmid loss per generation when cells were grown in glucose, compared to 8.3% in galactose, values similar to the ones reported previously (Russo & Sherman, 1989). When the 397-bp *FBP1* region was inserted in the forward orientation, the plasmid stability in galactose increased significantly, with a loss per generation of 4.1%, confirming that this sequence can specify transcription termination. These experiments have given some interesting information regarding sequences that can promote transcription termination (Fig. 5; Table 1). The 397 fragment in either orientation exhibits a low rate of plasmid loss, implying efficient transcription termination. Thus, in contrast to what was observed for *CYC1* sequence (Russo & Sherman, 1989), the termination signal as well as the polyadenylation signal of *FBP1* is bidirectional. The high termination efficiency of the 3′-287 fragment (Fig. 1) demonstrates that no more than 153 bases downstream of the poly(A) site are required for transcription termination. However, the 3′-144 insert, which has nine nucleotides of *FBP1* sequence beyond the poly(A) site, showed only a moderate decrease in efficiency to 69%.

Results with other fragments indicate that there is not always a correlation between signals required for efficient polyadenylation and those for transcription termination. For example, the 397D3 fragment, with its low efficiency of polyadenylation in vivo and in vitro, still conferred a termination efficiency of 88%, confirming our hypothesis that the $(TA)_{14}$ element is not required for transcription termination downstream of the *FBP1* poly(A) site. The 5′-328 fragment, which gives efficient but inaccurate polyadenylation, has a termination efficiency lower than that of 397D3 (64% versus 88%). Finally, the 5′-307 fragment, in which the positioning element and half of the TA stretch are deleted, is ineffective in both termination and polyadenylation.

TRO experiments show that RNA polymerase stops shortly after the poly(A) site in wild-type *FBP1* sequence

To obtain more information about the region in which RNA polymerase II stops and to analyze the possibility of a pause site for transcription termination, we performed TRO experiments. This analysis measures the level of nascent transcript synthesis by incubating permeabilized cells for 2 min with α-^{32}P-UTP. Quantitation of the amount of radioactive RNA that hybridizes with immobilized strand-specific templates gives an indication of the density of active RNA polymerase complexes on the DNA template.

For these experiments, the plasmids used in the polyadenylation signal competition assays, pVPA397 and pVPA397D3, were used. Cells were transformed with the respective plasmids and grown in media containing 2% glucose. In these conditions, the chromosomal copy of the *FBP1* gene is regulated by catabolite repression



F

Fragment	% plasmid loss per generation
None	8.3 ± 0.3
397	4.1 ± 0.1
397R	4.4 ± 0.3
397D3	4.6 ± 0.3
5'-328	5.6 ± 0.1
5'-307	8.8 ± 0.1
3'-287	3.9 ± 0.1
3'-144	5.9 ± 0.2

FIGURE 5. Plasmid stability assay. Yeast strain J17 containing the pAB610 or pTER plasmids (see Materials and Methods for details about these plasmids) were grown in medium lacking uracil and inoculated into medium containing uracil and either glucose or galactose. Portions of the growing cultures were diluted and plated on glucose medium (for determining the total cell number and hence the number of generations) and on minus-uracil medium (for detecting the ura$^+$ cell number). An average from three different experiments is shown in each case. **A:** pAB610 grown in glucose and galactose medium. **B:** pTER397 grown in glucose and galactose medium. **C:** pTER328 and pTER307 grown in galactose medium. **D:** pTER 397R and pTER397D3 grown in galactose medium. **E:** pTER287 and pTER144 grown in galactose medium. **F:** Table showing the percentage of plasmid loss per generation.

TABLE 1. Summary of mutational analysis of the *FBP1* polyadenylation/termination signal.

Fragment	Polyadenylation efficiency[a]		Termination efficiency[b]	A_{11}[c]	TGTATATG	TA #1	TA #2	ACCGTAAG	Poly(A) site
5′-397	98	100	100	x	x	x	x	x	x
5′-328	89		64			x	x	x	x
5′-307	<10		0				x	x	x
D2	98	61	nd	x	x	x		x	x
D3	60	16	88	x	x			x	x
3′-144	98		69	x	x	x	x	x	x
3′-122	85		nd	x	x	x	x	x	
3′-114	53		nd	x	x	x	x		
3′-94	47		nd	x	x	x	x		
3′-84	<10		nd	x	x	x			

[a]Values to the left are ones determined by the polyadenylation signal competition assay, and are taken from Figure 2. As discussed in the text, this calculation does not take into account the possibility that transcription termination occurs downstream of the *FBP1* poly(A) site. Values on the right were determined relative to *ACT1* mRNA levels and, in these cases, are probably a more accurate determination of polyadenylation efficiency.

[b]Efficiency of transcription termination is calculated from the data in Figure 5. Termination efficiency is considered 100% for the 397 fragment and 0% for the 5′-307 deletion. The termination efficiency for each fragment is determined from the difference between its percentage of plasmid loss per generation and the corresponding value of the 397 fragment.

[c]Presence of the various elements on the DNA fragments are indicated. An x is shown when an element is present in a fragment. A_{11} element is located between positions 1100 and 1110, TGTATATG between positions 1113 and 1121, TA #1 in between 1125 and 1138, TA #2 in between 1141 and 1152, and finally ACCGTAAG localizes between positions 1168 and 1175 (see Fig. 1).

and is not transcribed, meaning that all of the *FBP1*-labeled RNA will come from plasmid transcription. Uniformly labeled transcripts prepared with T7 polymerase were used as a control for the hybridization signal expected for each template. Three hybridization templates covering most of the 397-nt fragment were used. The first one (I) corresponds to the 3′ UTR of the *FBP1* gene, except for 41 nt upstream of the poly(A) site. The second one (II) includes the 41 bases upstream of the poly(A) site and 61 bases downstream. The last one (III) has sequences farther downstream. Single-stranded plasmids were prepared containing these sequences in either the sense or anti-sense orientation. An additional template corresponding to the *URA3* marker on the plasmid was used as an internal standard.

When hybridization was performed using labeled RNA from cells with the pVPA397 plasmid, fragment II gives a signal 9.5 times higher than fragment I (Fig. 6), suggesting an accumulation of RNA polymerases in this region. In fragment III, the signal is 25-fold lower than that of fragment II, indicating that the level of transcription drops off in this region. According to this data, most of the polymerases must pause and/or stop within the 61 nt downstream of the *FBP1* poly(A) site. Labeled RNA from the strain with the pVPA397D3 plasmid was also examined. The level of transcription of the *FBP1* sequence corresponding to fragment I compared to the *URA3* sequence is similar to that found with the pVPA397-derived RNA (Fig. 6B), confirming that the D3 deletion does not affect transcription rate. With this construct, an accumulation of polymerases is again detected in the region covered by fragment II.

DISCUSSION

The formation of mRNA 3′ ends has been studied extensively in the yeast *S. cerevisiae*, yet there are still many unanswered questions regarding the sequences and factors involved in this processing event and how they interact to promote polyadenylation and transcription termination. The aim of this work was to identify *cis*-acting elements necessary for these two events in the *S. cerevisiae FBP1* gene. Our deletion analysis has shown that 83 nt of sequence upstream of the poly(A) site and 9 nt downstream of this site are sufficient for accurate and efficient polyadenylation.

This region of *FBP1* has several important features. Similar to many yeast genes (Heidmann et al., 1992), the major poly(A) site is found at a TAA sequence with minor sites in the immediate vicinity of this predominant one. An element important for selecting this poly(A) site is found between 68 and 83 nt upstream of the major poly(A) site, limited by ends of the 5′-343 and 5′-328 deletions. Analysis of this type of signal in the *CYC1* gene (Russo et al., 1993; Guo & Sherman, 1995) suggests that a tract of adenosines in this region of the *FBP1* gene is the most likely candidate for a positioning element. Also in agreement with these studies, deletion of this region in *FBP1* or the region containing the natural poly(A) sites results in the usage of other sites scattered over a broader region without much effect on the overall efficiency of processing. The positioning element of a synthetic polyadenylation signal directs cleavage in yeast whole-cell extract (Guo & Sherman, 1995). Our analysis extends this study by show-

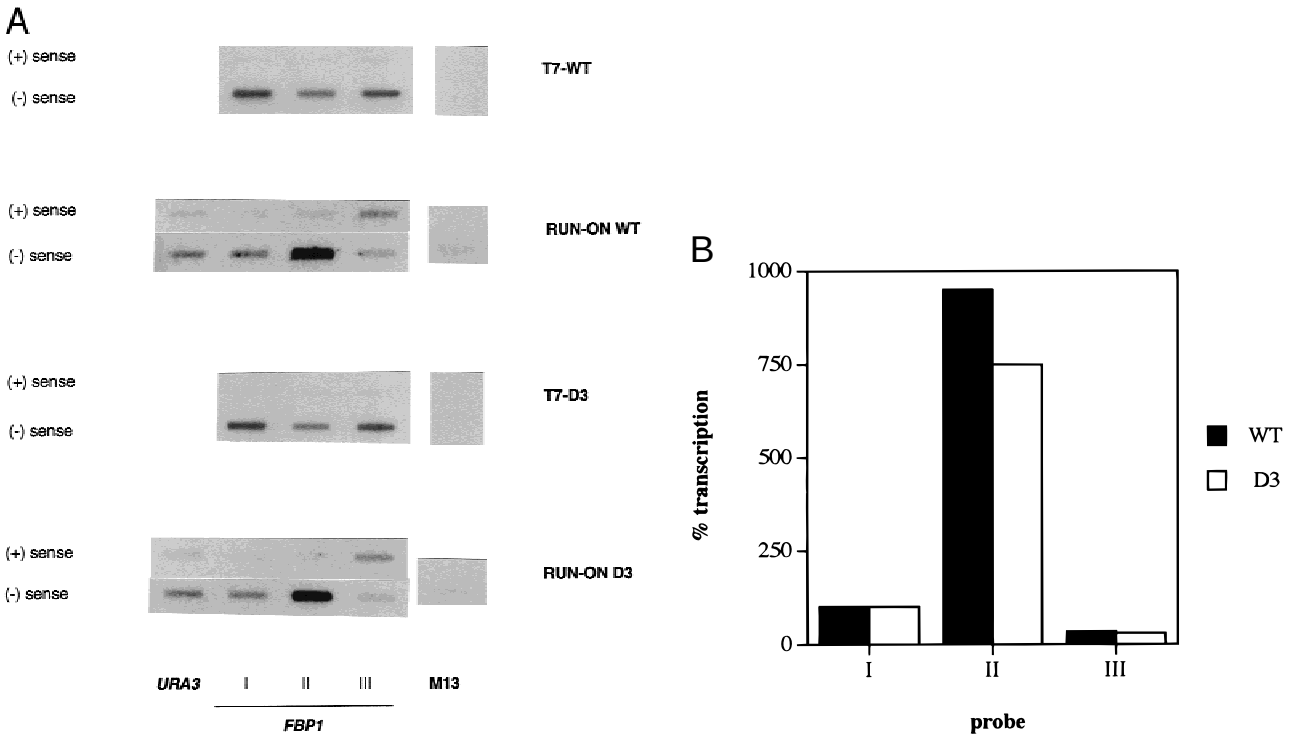


FIGURE 6. TRO experiments. **A:** Hybridization pattern across the indicated probes for RNAs derived from strains carrying the pVPA397 (Run-on WT) or the pVPA397D3 (Run-on D3) plasmids, or uniformly labeled transcript generated by T7 RNA polymerase (T7-WT, T7-D3). **B:** Graph indicating the percentage of transcription (relative to probe 1) in each fragment, when wild-type sequences or D3 sequences are used. To obtain these values, a normalization has been made with the T7 transcripts. The data correspond to an average of three different experiments. The standard deviation error was lower than 3% in all cases.

ing that the positioning element also functions in a reconstituted system using partially purified cleavage factors.

The *FBP1* 3′-end is unusual in that a critical positioning element is located upstream of several elements that act in concert to promote efficient polyadenylation (Table 1). A more typical organization of a yeast polyadenylation signal places the positioning element between the efficiency element(s) and the poly(A) site (Guo & Sherman, 1996). We cannot discard the existence of an additional positioning element just upstream of the major poly(A) site. However, if it is present, it does not function on its own, in the absence of the far upstream element. By comparison with the 3′ ends of other yeast genes that have been examined using the poly(A) site competition assay (Irniger et al., 1991), the *FBP1* gene has a strong polyadenylation signal. The efficient utilization of polyadenylation elements arranged as they are in *FBP1* suggests that there may be more flexibility than expected in how factors recognize polyadenylation signals and interact with each other to assemble a processing complex.

Compilation of the deletion analysis data from our study (Table 1) shows that at least three efficiency elements must be present for efficient processing of *FBP1* precursor (at levels of 50% or greater), but that not all combinations of these elements are equally effective. Two of these elements in the *FBP1* gene are composed of stretches of TA pairs located immediately adjacent to each other. A TATATA hexamer is thought to be the strongest version of an efficiency element (Irniger & Braus, 1994), but, as demonstrated by the 5′-307 deletion, one of these is not sufficient for *FBP1* 3′-end formation. However, this deletion, which removed 5′ sequence including the first TA repeat, functioned more poorly than the 397D2 deletion, which removed only the first TA repeat. The most reasonable explanation for this result is that a third sequence, further upstream of the TA tract, can also serve as an efficiency element. This region encompasses a stretch of alternating thymines and purines, TGTATATG, which contains the TGTATA hexamer thought to function as an efficiency element in *DEG1* and *GCN4* yeast sites (Egli et al., 1995; Brambilla et al., 1997). A related motif, TATGTA, is found between the TA tract and the ACCGTAAG element. A further decrease in polyadenylation efficiency was not observed when this region was removed in fragment 3′-94, and it is not clear whether it too can contribute to efficient processing. Finally, the decline in the polyadenylation efficiencies seen in comparing the 3′-122 and the 3′-114 deletions revealed a fourth element. This short stretch has the sequence ACCGTAAG,

which does not correspond to any of the previously reported efficiency elements. Surprisingly, none of these elements are a good match to a consensus binding sequence, U-G-C/U-G-U-A-U-U-C/U-U-C-C, derived by selection amplification for the RNA recognition motif (RRM) of Rna15 (Takagaki & Manley, 1997). This protein is a subunit of the CF IA cleavage/polyadenylation factor of *S. cerevisiae* (Kessler et al., 1996) and is essential for yeast mRNA 3′ end formation (Minvielle-Sebastia et al., 1994).

We also investigated the sequences involved in transcription termination downstream of the *FBP1* poly(A) site. As described in the Introduction, previous studies in yeast and mammals have shown that the efficiency of termination directly correlates with the strength of the upstream polyadenylation signals as well as the presence of a strong polymerase pause site downstream of the poly(A) site. By TRO analysis with wild-type *FBP1* DNA, we found that there is pausing within the first 61 nt downstream of major poly(A) site. Other studies have also demonstrated that transcription termination in *S. cerevisiae* stops within the first 100 nt beyond the poly(A) site (Osborne & Guarente, 1989; Russo & Sherman, 1989), a phenomenon consistent with the dense packing of genes on yeast chromosomes (Dujon, 1996). The pause sites suggested by in vivo and in vitro studies of *S. cerevisiae* and *S. pombe* genes are further away from the poly(A) site (Russo & Sherman, 1989; Hyman & Moore, 1993; Birse et al., 1997).

The most unexpected finding in this study concerns the relationship between polyadenylation and transcription termination. In general, our observations did not support a strict correlation between the efficiency of polyadenylation and termination (Table 1). In the case of the 5′-307 deletion, a fragment that was extremely poor at 3′-end processing, termination was equally ineffective. In contrast to this example, the 397D3 fragment, with a deletion of the entire TA repeat, is an efficient terminator in the plasmid stability assay and also induces pausing in the normal position, but it is a poor processing signal both in vivo and in vitro. It is also interesting that transcription termination was affected more adversely by removal of the positioning element in the 5′-328 fragment than it was by deletion of the TA repeat. Additional evidence for sequence in this upstream region being important for transcription termination can be derived from the competition assays using the 3′-84 and 3′-40 deletions (Fig. 2B). Although these fragments have not been analyzed in the plasmid stability assay, it is clear that removal of the A_{11} tract, the TATATG motif, and the remaining TA repeat in 3′-40 greatly increased the level of readthrough transcripts.

A straightforward explanation for our observations is that signals specifying transcription termination in the *FBP1* sequence are distinct from, but overlap with those necessary for polyadenylation. But do these results represent a true uncoupling of termination and 3′-end processing? An alternative view is that polyadenylation factors can assemble on a transcript with certain combinations of *cis*-acting elements, but that this complex cannot efficiently carry out polyadenylation. Assembly of a partial or unstable complex may be sufficient to provoke a change in the processivity of pol II that is still adequate to precipitate pausing at the normal termination site. In yeast, three factors (CF IA, CF IB, and CF II) are necessary for cleavage of mRNA precursor (Kessler et al., 1996; Zhao et al., 1997). These factors have been shown to interact with RNA and are probably involved in the initial recognition of RNA substrate. The binding of the Rna15p, as a subunit of CF IA, does not require a UA-type efficiency element (Kessler et al., 1996), but the A-rich positioning element found in the *GAL7* polyadenylation signal (Guo & Sherman, 1995) is essential for this interaction (A. Baughn, M. Kessler, & C. Moore, unpubl.). The ultraviolet crosslinking of the Cft2p component of CF II requires the efficiency element and sequence at or immediately downstream of the cleavage site (Zhao et al., 1997). CF IB is the Hrp1 protein and binds to the efficiency element (Kessler et al., 1997). Furthermore, Hrp1p has two RRM-type RNA binding motifs (Henry et al., 1996), and thus the potential to contact RNA in two places. The CF IA, CF IB, and CF II factors can interact with the precursor independently of each other, supporting the possibility that partial processing complexes could form on nascent RNA transcripts.

A recent report has shown that mammalian splicing and polyadenylation factors bind to the carboxy-terminal domain of the pol II large subunit and co-purify with pol II in a high-molecular mass complex (McCraken et al., 1997). This has led to a model in which 3′-end processing factors release from the transcriptional complex once polyadenylation signals on the nascent transcript become available and, in doing so, make the polymerase more sensitive to terminating transcription at pause sites. There is currently no evidence for such an mRNA factory in yeast. However, our findings with the *FBP1* sequence would be consistent with such a model, and suggest that the generation of a termination-competent pol II may require only release of polyadenylation factors and not assembly of a functional polyadenylation complex.

The extreme compactness of the yeast genome (Dujon, 1996) probably necessitates efficient transcription termination to occur immediately downstream of poly(A) sites. Because of the short distance between the end of the *FBP1* gene and the start of the coding sequence of the downstream *YRL376c* gene, it is quite possible that *FBP1* sequences required for polyadenylation and termination also serve as *YRL376c* promoter elements. For example, the $(TA)_{14}$ repeat is 100 bp upstream of the *YRL376c* start codon, and could contain the TATA

box for this gene. A transcriptional terminator has also been mapped to the promoter region of the *URA3* gene (Yarger et al., 1986). Moreover, the association of transcription termination signals and *cis*-acting elements important for replication origin function reported by Chen et al. (1996) represents a similar overlap. This situation should be very common in the yeast genome, and raises the question of whether termination-competent RNA polymerases can interfere with the assembly of downstream transcription initiation complexes in the same way that has been observed for processively elongating polymerases. Further analysis is required to understand the mechanisms used to avoid transcriptional interference.

MATERIALS AND METHODS

Reagents

All restriction enzymes and modifying enzymes were purchased from Amersham, Boehringer Mannheim, Pharmacia, or Promega and used according to the manufacturer's directions. Radioactive nucleotides ^{35}S-dATP, ^{32}P-dATP, and ^{32}P-UTP were from Amersham and DuPont-NEN Research Products. The nylon transfer membrane Hybond-N™ was from Amersham. Synthetic oligonucleotides were purchased from MWG or GenoSys. Sodium N-lauryl sarcosine sulfate was from Sigma. RNA marker was from Gibco BRL. Rediprime™ kit for random primer labeling was from Amersham. Sequenase sequencing kit was from USB.

Plasmid constructions and mutagenesis

The previously described pFBP391 plasmid, which has a *Sau*3A fragment containing 397 bp of the 3′ flank of the *S. cerevisiae FBP1* gene cloned into the *Bam*H I site of pUC18 (del Olmo & Pérez-Ortín, 1993), was used as a substrate for nested deletions. These were created by using the exonuclease III/nuclease S1 protocol (Henikoff, 1984). To generate 5′ deletions, the *Sac* I and *Sma* I restriction enzymes were used. To generate 3′ deletions, the plasmid was digested with *Kpn* I and *Xba* I. The resulting deletions were characterized by sequence analysis using the dideoxynucleotide chain-termination method (Sanger et al., 1977). Derivative plasmids have been named according to the length of *FBP1* sequence remaining in the original pFBP391.

Derivatives of the pFBP391 plasmid carrying internal deletions or mutations in the *FBP1* sequence (pFBP391DM, pFBP391D1, pFBP391D2, and pFBP1391D3) have been described previously (Aranda et al., 1997).

pFBP144 plasmid (one of the plasmids resulting from the nested deletions) was used as a substrate for in vitro mutagenesis using the oligos and conditions described previously (Aranda et al., 1997). The resulting plasmids have been named pFBP144D2 and pFBP144D3. pUC2083 plasmid (Aranda et al., 1997), which contains the whole *FBP1* gene as a 2,083-bp *Sma* I–*Xba* I fragment, was also a substrate for the D3 deletion. The resulting 2083D3 fragment was also cloned into the pRS316 vector (Sikorski & Hieter, 1989), giving pRS2083D3. pRS2083 is a derivative of pRS316 containing the wild-type 2083 fragment.

Derivatives of the plasmid pME729 (Irniger et al., 1992) were used for testing the in vivo polyadenylation ability of fragments of the 3′ region of the *FBP1* gene. Fragments of *FBP1* 3′ end were inserted into the filled-in *Xho* I site located in the vector. The 397-bp original fragment and its derivatives DM, D1, D2, and D3 were subcloned in this vector as a *Sma* I–*Hin*c II fragment generated from the corresponding pFBP391 plasmids. Other fragments were isolated as *Eco*R I–*Hin*d III fragments from the pFBP391 derivatives carrying the deletions and were blunt ended by the Klenow fragment of *E. coli* DNA polymerase I. The resulting plasmids have been named pVPA with a number indicating the length of the inserted fragment. When fragments were inserted in reverse orientation, with regard to their natural transcription, they are indicated by the letter R.

The 28-bp sequence corresponding to the region removed in the D3 deletion described above was also cloned into pME729. For this purpose we designed the oligonucleotides dTCGA$(TA)_7$TTT$(AT)_6$ and dTCGA$(AT)_6$AAA$(TA)_7$. Hybridization of these oligos to each other gives a double-stranded region flanked by three unpaired bases that are cohesive with *Xho* I ends. This allows the cloning of this fragment directly into the vector. The hybridization was performed by mixing 1 μg of each phosphorylated oligo in a total volume of 10 μL of 0.4 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. The solution was heated for 5 min at 95 °C, then for 10 min at 65 °C, and finally cooled slowly to room temperature.

Derivatives of the pHZ18Δ2SMA plasmid (Hyman et al., 1991) were used for determining the mRNA stability of several fragments. Blunt-ended fragments were cloned in the *Sma* I site of this plasmid.

pT7T3 18U/19U vectors from Pharmacia were used to prepare the precursors for the in vitro processing assay. In this case, the selected fragments were isolated from the pFBP derivatives by *Eco*R I–*Hin*d III digestion and were introduced in the same cutting sites in the polylinker of the vector. The resulting plasmids have been named pIVP with a number indicating the length of the sequence in the fragment introduced.

To analyze the ability of several sequences to induce transcription termination, we prepared several derivatives of the pAB610 plasmid (Russo, 1995). First of all, we subcloned the *Eco*R I–*Bam*H I fragment of this vector (containing the *GAL1* and *CEN3* sequences) into pT7T3 18U plasmid to generate pT7T3GALCEN. This plasmid was digested with *Xho* I, filled in with Klenow, and dephosphorylated. Several regions of the *FBP1* gene were isolated from the previously described pFBP plasmids as *Eco*R I–*Hin*d III fragments and were introduced into the *Xho* I site of the pT7T3GALCEN vector after filling with Klenow. These plasmids were called pGALCEN, including a number indicating the length of the *FBP1* sequence introduced. To reconstitute pAB610 derivatives, we isolated *Pin*A I–*Sma* I fragments containing a portion of *GAL1*, the *FBP1* insert, and most of the *CEN3* sequence, from the pGALCEN plasmids and subcloned them into pAB610 digested with the same restriction enzymes. The resulting plasmids have been named pTER, with a number indicating the length of the *FBP1* sequence introduced.

To measure the polymerase density by TRO, several fragments from the 3′ end of the *FBP1* gene were subcloned into

M13mp18 and M13mp19 in order to prepare single-strand templates. Fragment I contains the 99-bp region between the *Kpn* I site in the polylinker of pFBP391 to the *Dra* I site in position 1147 of the *FBP1* gene and was introduced into M13mp18 and M13mp19 digested with *Kpn* I and *Sma* I enzymes. Fragment II corresponds to the 102-bp region between the *Dra* I sites in positions 1147 and 1248 of the *FBP1* gene, and it was introduced in both orientations into M13mp18 vector digested with *Sma* I. Finally, fragment III contains the 142-bp fragment located between the *Dra* I sites in positions 1248 and 1391 and was subcloned in both orientations into M13mp18 vector digested with *Sma* I. In order to compare the levels of expression with another gene, we also subcloned in both orientations the 123-bp fragment contained between the restriction sites for *Eco*R V and *Sca* I from the *URA3* coding region in M13mp18.

Yeast strains, media, and culture methods

Yeast strain pRG1-5d (a, *fbp1-2::LEU2, ura3, gal7*) was used for the complementation experiments. This strain was a gift of Dr. J.M. Gancedo and contains an internal deletion in the *FBP1* gene that prevents the growth on gluconeogenic substrates. This strain was transformed with pRS2083 and pRS2083D3 plasmids by the protocol of Ito et al. (1983).

Yeast strain SEY2101 (a, *ade2-101, leu2-3,112, ura3-52, suc2*Δ*9, gal2*) was a gift of Dr. S. Emr and was used for the analysis of in vivo polyadenylation. It was transformed with the pVPA plasmids by the same procedure.

Yeast strain W303-1a (a, *leu2-3,112, trp1-1, can1-100, ura3-1, ade2-1, his3-11,15*) was transformed with the pHZ18Δ2SMA plasmid and its derivatives.

Yeast diploid strains BJ2168 (a/α, *leu2/+, trp1/trp1, ura3-52/+, prb1-1122/prb1-1122, pep4-3/pep4-3, prc1-407/prc1-407, +/his1*) (from Dr. E. Jones) and 1097/930 (a/α, *leu2/+, trp1/trp1, prb1-1122/prb1-1122, pep4-3/pep4-3, prc1-407/prc1-407, his1/+*) were used for preparing the whole-cell extracts for the in vitro processing reactions.

Yeast strain J17 (α, *his2, ade1, trp1, met14, ura3*) from Dr. F. Sherman was transformed with pTER plasmids to perform the assays of plasmid stability.

All yeast culture methods were those described by Sherman et al. (1986).

Plasmid stability assay

The plasmid stability assay was performed as described by Russo (1995). J17 strain transformed with pAB610 or pTER plasmids was grown in minus uracil liquid media containing glucose and then shifted to growth in medium containing uracil and either glucose (to determine the stability of plasmids under noninduced *GAL1* conditions) or galactose (to determine plasmid stability under induced *GAL1* conditions). Aliquots from the growing cultures were removed and were plated on solid medium containing uracil (to determine total cell numbers) and without uracil (to determine the number of cells with plasmids). These experiments were performed three times with each strain and averaged. The stability was determined as the plasmid loss rate per generation calculated as the slope of the graphs in which the number of cells is plotted against the number of generations.

Measurement of enzymatic activities

Extracts for measurements of fructose-1,6-bisphosphatase (FBPase) activity were prepared according to Funayama et al. (1980). The enzymatic assays were performed according to Gancedo and Gancedo (1971). A unit of the enzyme is measured as the amount that catalyzes the conversion of 1 μmol of substrate per minute. FBPase specific activity is expressed in mU/mg of protein.

Isolation of RNA and northern analysis

Total RNA was isolated according to the method of Zitomer and Hall (1976) using glass beads to disrupt the yeast cells. Poly(A)$^+$ RNA was selected by oligo(dT) cellulose (Aviv & Leder, 1972). For northern (RNA) hybridizations, 10 μg of total RNA or 2 μg of poly(A)$^+$-enriched RNA was separated on a denaturing formaldehyde gel according to Sherman et al. (1986). After being transferred to nylon membranes, the bound RNA was hybridized and autoradiographed as described (Aranda et al., 1997). When needed, scanning and quantitation was done using a UVP Imagestore 5000 or a system of electronic autoradiography Instantimager (Packard).

RT-PCR

The 3′ ends of transcripts were determined precisely by sequencing of fragments generated by RT-PCR as described (Frohman, 1992). Ten micrograms of total RNA were used for these experiments. For reverse transcription, the oligo 5′-CGGTCGACTGCAGAAGC$(T)_{17}$-3′ was used. The amplification was performed with oligos 5′-CGGTCGACTGCAG AAGCTT-3′ and 5′-GGGGGAAAAGCGGTCAAC-3′ (when the poly(A) site is determined from constructs containing the whole *FBP1* sequence), or 5′-CTAGCGCTTGCACCATCCC-3′ (when the site is determined from pME729 derivatives).

The amplified DNA fragments were cloned into pUC18 after blunt ending by Klenow filling. Sequencing was performed by using universal primers according to the standard reaction conditions. Samples were analyzed in acrylamide-urea sequencing gels run in TBE buffer according to Sambrook et al. (1989).

TRO assays

We followed protocols described previously (Elion & Warner, 1986; Warner, 1991; Dammann et al., 1993; Birse et al., 1997), with the following modifications. Two-hundred-milliliter cultures were grown to an OD_{600} of approximately 0.1–0.15. Cells were harvested and washed with ice-cold TMN buffer (10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 100 mM NaCl). To permeabilize the cells, they were resuspended in 950 μL of ice-cold dH_2O, 50 μL of 10% (w/v) sodium N-lauryl sarcosine sulfate were added, and the mix was kept on ice for 20–30 min. The solution was spun down for 1–2 min and the pellet was resuspended in 100 μL of transcription buffer (50 mM Tris-HCl, pH 7.9, 5 mM $MgCl_2$, 100 mM NaCl, 1 mM $MnCl_2$, 2 mM DTT, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 10 mM creatine phosphate, 1.2 μM creatine phosphokinase). Transcription was allowed to proceed for 2 min at 30 °C following the addition of 100 μCi of α-^{32}P-UTP (3,000 Ci/mmol).

To stop the reaction, 1 mL of cold TMN buffer was added. Cells were harvested and total RNA was extracted as described previously. RNA was resuspended in 50 μL of dH_2O and the radioactivity, determined by Cerenkov counts, was measured in an LKB-Wallac 1409 scintillation counter. The RNA contained in an aliquot of 5×10^5 cpm was partially hydrolyzed for 5 min at 4 °C (0.4 M NaOH) and neutralized (0.4 M Tris-HCl, pH 7.2) and was used for hybridization. Immobilized single-stranded M13 probes, prepared as described (Osborne & Guarente, 1989; Sambrook et al., 1989), were prehybridized for 2 h in a solution containing 50% formamide, 0.5 M sodium phosphate buffer, pH 6.4, 0.8 M NaCl, 1 mM EDTA, pH 8, 0.1% SDS, 2.5 × Denhardt, 30 mg of heterologous denatured DNA, and tRNA 100 μg/mL, and then the labeled RNA was added. Uniformly labeled transcripts were also generated with T7 polymerase under manufacturer's conditions. Hybridization was performed overnight and filters were washed five times for 15 min at 60 °C with 50 mL of 50 mM NaCl, 20 mM sodium phosphate buffer, pH 6.4, 0.05% SDS; once for 30 min at room temperature with 5 μg/mL of RNase A in 50 mM NaCl, 20 mM sodium phosphate buffer, pH 6.4; and once for 15 min at room temperature with the last buffer without RNase. "Run-on" signals were then visualized and quantitated with a UVP Imagestore 5000.

Analysis of RNA secondary structures

For the analysis of RNA secondary structures, the MFOLD program was used. This program is from the GCG package (University of Wisconsin) (Devereux et al., 1984) and is based on the methods of Zuker and Stiegler (1981).

Yeast extracts, conditions for in vitro polyadenylation, and analysis of the samples

Yeast whole-cell extracts were prepared according to Chen and Moore (1992), except that the spheroplasting step was omitted and the cells were lysed by using glass beads.

Cleavage and polyadenylation assays were performed as described previously (Chen & Moore, 1992; Kessler et al., 1995). Cleavage reactions were performed with the same substrates and in the same conditions, but using partially purified CF I and CF II factors from yeast instead of whole-cell extracts. Fractions containing CF I and CF II were obtained by Mono Q chromatography (Chen & Moore, 1992).

To determine the efficiency at which various transcripts were processed, the amount of RNA in product and unprocessed precursor RNAs was obtained by phosphorimager. The percentage of processed RNAs was calculated by dividing the amount of RNA in the products (cleaved RNA for reactions using only cleavage factors; polyadenylated RNA for reactions using extracts) by the total amount of RNA (processed and unprocessed).

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