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T7 RNA polymerase-directed transcripts are processed in yeast and link 3′ end formation to mRNA nuclear export

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ABSTRACT

We have characterized transcripts synthesized in vivo by bacteriophage T7 RNA polymerase to investigate yeast mRNA processing. T7 transcripts are not capped, consistent with capping being tightly coupled to RNA polymerase II (pol II) transcription. In contrast to higher eukaryotic non-pol II transcripts, yeast T7 transcripts are spliced as well as cleaved and polyadenylated. However, T7 and pol II transcripts are affected differently in cleavage and polyadenylation mutant strains, indicating that pol II may have a role in yeast 3′ end formation. T7 transcripts with 3′ ends directed by a polyadenylation signal are exported from the nucleus, and this export is dependent on the canonical cleavage and polyadenylation machinery. Importantly, transcripts with T7 terminator-directed 3′ ends are unadenylated and predominantly nuclear in wild-type cells. Our results suggest that transcription by pol II is required for neither the nuclear export of an in vivo-transcribed mRNA nor for the retention of transcripts with aberrant 3′ ends. Moreover, proper 3′ end formation may be necessary and sufficient to promote mRNA export in yeast.

Keywords: 3′ end formation; nuclear export; T7; yeast

INTRODUCTION

Transcription in eukaryotic cells is carried out by three multisubunit RNA polymerases: RNA polymerase I (pol I), RNA polymerase II (pol II), and RNA polymerase III (pol III). Although similar in sequence and composition, these RNA polymerases direct transcription of distinct classes of RNA. Ribosomal RNA (rRNA) and transfer RNA (tRNA) transcription is carried out by pol I and pol III, respectively. Most spliceosomal U snRNA transcription and all messenger RNA (mRNA) transcription are directed by pol II. Primary mRNA transcripts undergo multiple processing reactions that impact mRNA function. These include modification of the 5′ end of the transcript (capping), removal of intervening noncoding sequences (splicing), 3′ end formation, and export from the nucleus for translation by cytoplasmic ribosomes. Results in higher eukaryotic systems indicate that several of these events are coupled to RNA polymerase, that is, pol II transcription is required for efficient post-transcriptional mRNA processing (reviewed in Neugebauer & Roth, 1997; Bentley, 1999; Hirose & Manley, 2000; Proudfoot, 2000).

Shortly after transcription initiation, the 5′ end of a nascent pol II transcript is modified by the addition of a 7-methyl guanosine (m^7G) cap. Capping is carried out by three enzymatic activities. RNA triphosphatase cleaves the triphosphate end to generate a 5′ diphosphate to which guanosine is added by RNA guanylyl transferase. Cap-guanine is then methylated at position 7 by RNA methyl transferase. Components of the capping machinery physically interact with the distinctive pol II carboxy-terminal domain (CTD; McCracken et al., 1997; Cho et al., 1997, 1998; Ho et al., 1998; Schroeder et al., 2000). Capping is consequently tightly coupled to pol II in both yeast and higher eukaryotes (Benton et al., 1990; Gunnery & Mathews, 1995; Lo et al., 1998; McCracken et al., 1998). Subsequent cap recognition by cap-binding proteins is a critical step in ribosome recruitment and mRNA translation (reviewed in Gunnery & Mathews, 1995).

Most higher eukaryotic genes and a small percentage of yeast genes give rise to precursor mRNA (pre-mRNA) from which noncoding intron sequences are removed by splicing. Pre-mRNA splicing is also coupled to pol II transcription in higher eukaryotes. Intron-

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containing reporter genes whose transcription is driven by either pol I or pol III are not spliced efficiently in tissue culture (Sisodia et al., 1987; McCracken et al., 1998). Furthermore, mammalian factors implicated in splicing interact with the pol II CTD, and recombinant CTD stimulates splicing in vitro (Yuryev et al., 1996; Hirose et al., 1999). These results argue that pol II has a direct role in higher eukaryotic splicing. In yeast, less is known about the relationship between pol II and pre-mRNA splicing. However, removal of yeast introns in which the splicing signals are initially masked from transcribing pol II suggests that pre-mRNA alone can recruit the splicing machinery (Lopez & Seraphin, 2001).

3′ end formation of nearly all pol II transcripts is carried out by the cellular cleavage and polyadenylation machinery. Transcripts are cleaved to generate a 3′ end to which a tail of adenosine residues is added (the poly(A) tail). Cleavage and polyadenylation is directed by polyadenylation signals within the transcript (Graber et al., 1999; van Helden et al., 2000; reviewed in Keller & Minvielle-Sebastia, 1997; Zhao et al., 1999). Mammalian 3′ end formation factors interact with the pol II CTD, and pol II has been implicated in higher eukaryotic 3′ end formation both in vitro and in vivo (Sisodia et al., 1987; Hirose & Manley, 1997; McCracken et al., 1998). In yeast, pol II may assist in 3′ end formation but is not required (Lo et al., 1998; McNeil et al., 1998).

Fully processed mRNA is exported from the nucleus to the cytoplasm where bulk translation occurs. mRNA export is a regulated process, and the signals directing export are thought to be provided by proteins that first bind mRNA within the nucleus (reviewed in Stutz & Rosbash, 1998; Reed & Magni, 2001). Recent data in vertebrate systems indicate that splicing has a prominent role in promoting mRNA export (Le Hir et al., 2000; Zhou et al., 2000). Given that ~95% of yeast genes do not have introns, however, it is unlikely that such a relationship offers a complete explanation for yeast mRNA export. It is also more generally unclear to what extent yeast posttranscriptional mRNA processing is coupled to pol II. Recent results indicate that mRNA export factors are recruited cotranscriptionally in yeast (Lei et al., 2001). A pol II requirement for export of an in vivo transcribed mRNA, however, remains largely unexplored.

To address these issues, we have analyzed posttranscriptional processing and steady-state localization of T7 transcripts in vivo in the budding yeast *Saccharomyces cerevisiae*. Bacteriophage T7 RNA polymerase (T7 RNAP) is a single-subunit, ~100-kDa RNA polymerase that directs transcription from a well-defined T7 promoter element when expressed ectopically in both yeast and higher eukaryotes (Chen et al., 1987; Benton et al., 1990; McCracken et al., 1998). Consistent with previous results, we find T7 transcripts are not capped. Intron-containing T7 transcripts are spliced, albeit with reduced efficiency. We attribute much of this effect to the lack of a cap and its well-described role in splicing (Colot et al., 1996; Lewis et al., 1996; Zhang & Rosbash, 1999). T7 transcripts are efficiently processed at a pol II polyadenylation signal, and this processing requires canonical components of the cleavage and polyadenylation machinery. However, T7 and pol II transcript 3′ end formation are affected differently in mutant strains, suggesting a contribution of pol II to yeast 3′ end formation. Finally, we observe a strong correlation between proper 3′ end formation and the export of T7 transcripts.

RESULTS

T7 RNAP transcription in yeast

We utilized a previously described system to generate T7 transcripts in *S. cerevisiae* (Chen et al., 1987; Benton et al., 1990). Bacteriophage T7 RNAP containing an N-terminal SV40 nuclear localization signal (nls) was constitutively expressed in yeast. A schematic of the pol II and T7 green fluorescent protein (GFP) reporter genes is shown in Figure 1A. There are two T7 promoter elements, ~20 nt apart, due to a T7 promoter element upstream of the polylinker. Transcription from the downstream promoter was designed to generate a transcript with a 5′ leader sequence identical to its pol II counterpart. T7 RNAP directs transcription from both T7 promoter elements in vivo, as shown by primer extension of total RNA (Fig. 1B, lane 3). Despite comparable steady-state levels of pol II and T7 transcripts, translation of T7 GFP transcripts is not detectable by α-GFP western blotting (data not shown). Failure to translate T7 transcripts in vivo is consistent with previous reports (Chen et al., 1987; Benton et al., 1990).

The absence of translation has been attributed to the lack of a cap on T7 transcripts (Benton et al., 1990). To confirm this previous report, we performed immunoprecipitation of total RNA with antibody against the cap structure prior to primer extension (Fig. 1B, lanes 4–9). As the α-cap antibody also binds the trimethyl cap structure of U snRNAs, primer extension of U2 snRNA was included as a control for immunoprecipitation. In contrast to pol II GFP transcripts, T7 GFP transcripts are not detectably immunoprecipitated with α-cap antibody. The lack of a cap is consistent with capping being tightly coupled to pol II transcription.

Splicing of T7 transcripts

To address the splicing of intron-containing T7 transcripts, we introduced the ~150-nt pre-U3 snoRNA intron immediately downstream of the ATG start codon in GFP to generate intron-containing U3GFP. Splicing was assayed by primer extension of total RNA with an oligonucleotide directed downstream of the U3 intron (Fig. 2A). Primer extension for transcripts from the in-

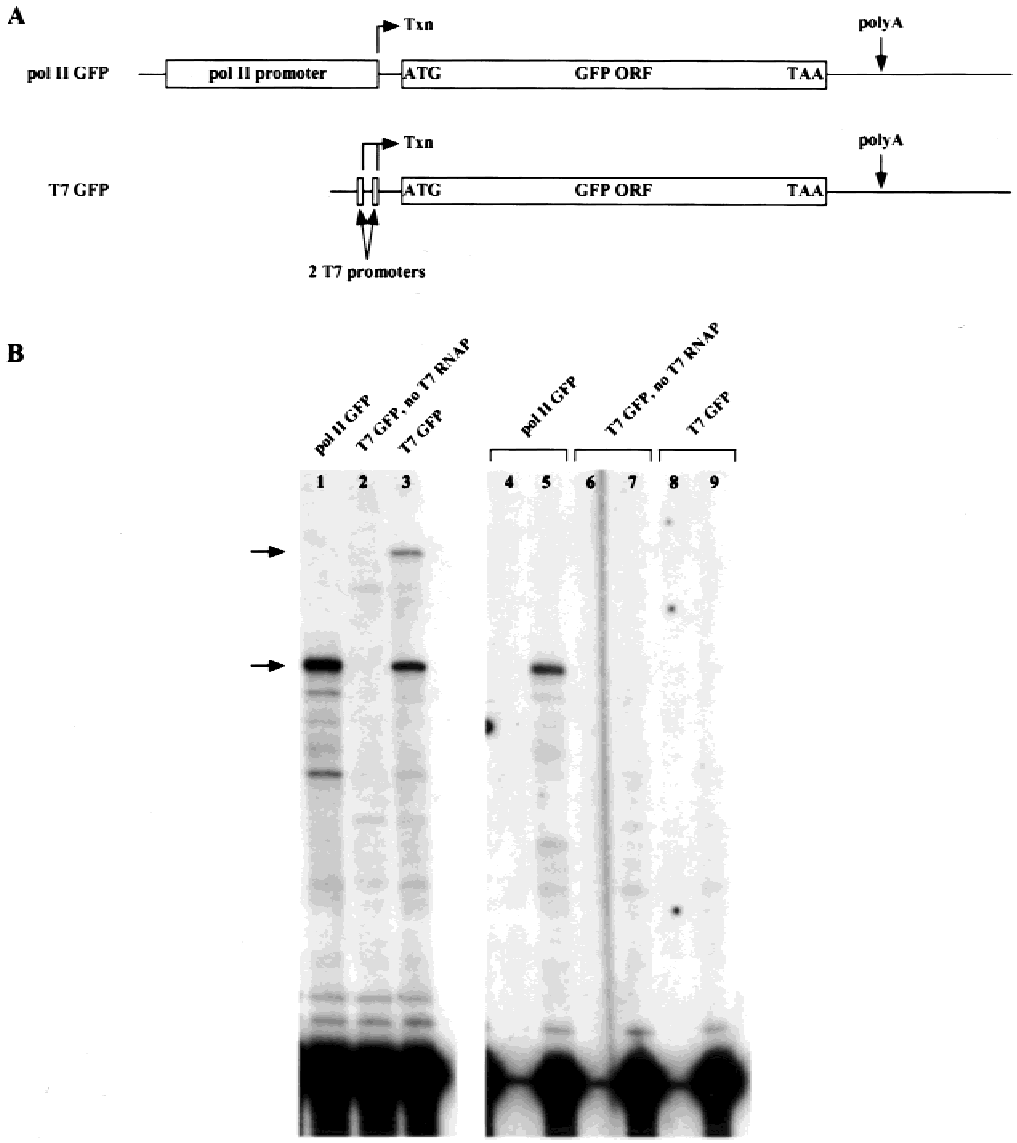

FIGURE 1. T7 RNAP-directed transcription in yeast. **A:** Schematic of pol II and T7-promoter driven GFP reporter constructs. The T7 GFP reporter contains two promoters, ~20 nt apart, due to a T7 promoter element upstream of the polylinker region. Transcription from the downstream promoter generates a transcript with a 5′ leader sequence identical to pol II GFP transcripts. Txn: Transcription start site; poly A: TDH3 polyadenylation signal, within the context of TDH3 3′ flanking region. **B:** Lanes 1 and 3: Primer extension of 5 μg of total RNA for pol II GFP and T7 GFP transcripts, respectively. Lane 2: Cells transformed with T7 GFP reporter in the absence of T7 RNAP. Arrows indicate extension products for transcripts directed from pol II GFP and the two T7 promoters in T7 GFP. Lanes 4–9: Primer extension of a 250 μg equivalent of total RNA immunoprecipitated with α-cap antibody. The α-cap antibody binds both the mRNA monomethyl cap and the trimethyl cap structure of U snRNAs. Primer extension for U2 snRNA is included as a control for immunoprecipitation. Immunoprecipitations were performed with and without α-cap antibody, as indicated.

tronless pol II and T7 GFP reporters are shown as a reference for splice products (Fig. 2A, lanes 1 and 4). As expected, pol II U3GFP transcripts are efficiently spliced (Fig. 2A, lane 2). Splicing of both T7 U3GFP transcripts, directed from the two T7 promoter elements, is also detectable (Fig. 2A, lane 6). These products disappear when the 5′ splice site sequence is destroyed, confirming that they arise from splicing (Fig. 2A, lane 7). Similar results were obtained using a synthetic RP51A intron instead of the pre-U3 intron (data not shown).

There is less splice product when transcription is directed by T7 RNAP than when transcription is directed by pol II (Fig. 2A, compare lanes 2 and 6). Two immediate explanations are that either pol II makes a major contribution to pre-mRNA splicing or T7 transcripts lack another feature required for efficient splicing. An obvious possibility for the latter is the cap, which T7 transcripts lack (see Fig. 1B). In the nucleus, the cap structure is bound by nuclear cap binding complex (CBC), a heterodimer of Cbp20p and Cbp80p. Cbp20p interacts with the cap, and Cbp80p binds pre-mRNA

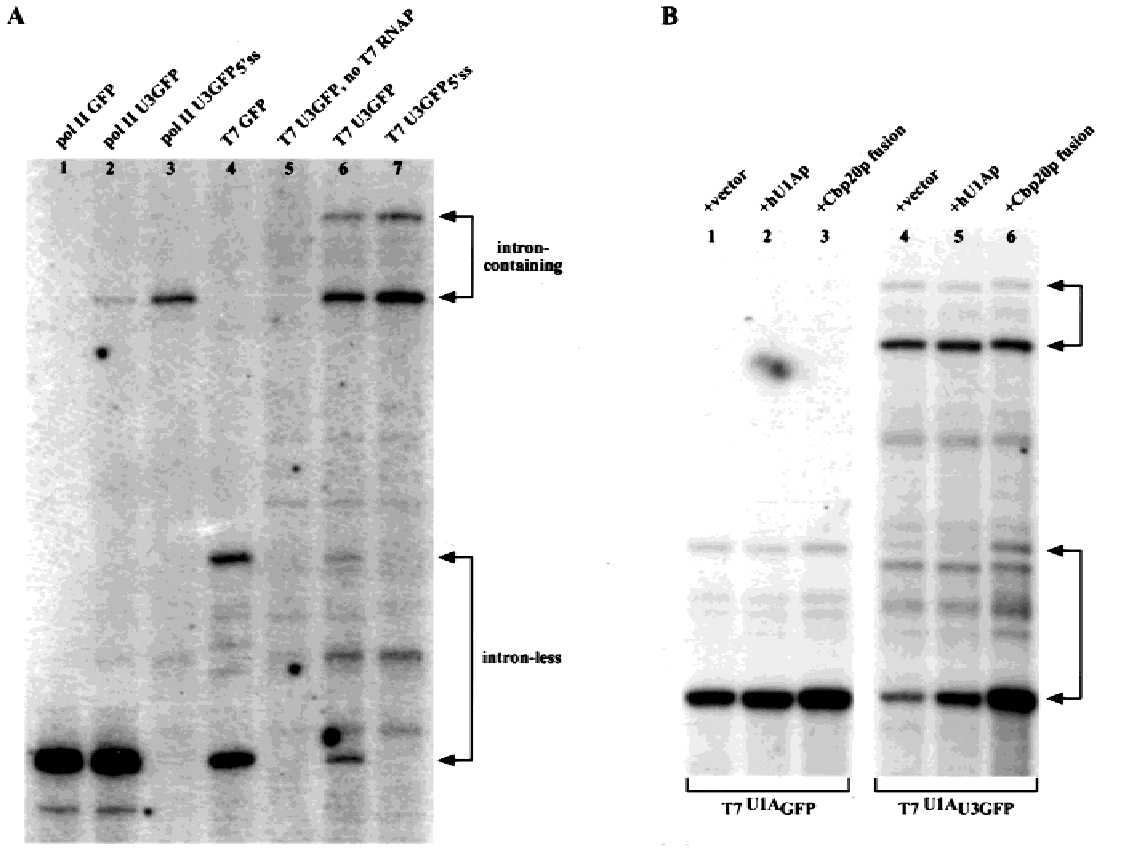

FIGURE 2. Intron-containing T7 transcripts are spliced. The ~150-bp pre-U3 snoRNA intron was introduced immediately downstream of the ATG start codon in the GFP open reading frame to generate U3GFP. **A:** Primer extension for transcripts generated from pol II and T7 versions of intronless GFP (lanes 1 and 4), intron-containing U3GFP (lanes 2 and 6), and 5′ splice site mutant U3GFP$_{5'ss}$ (lanes 3 and 7) reporters. Lane 5: Cells transformed with T7 U3GFP reporter in the absence of T7 RNAP. Arrows indicate extension products for intron-containing and intronless transcripts. There are multiple products for T7 transcripts due to the two T7 promoters that are ~20 nt apart (see Fig. 1). **B**: Reduced splicing efficiency is largely due to cap status. A 5′ proximal U1A stem-loop has been introduced to generate intronless T7 ^{U1A}GFP and intron-containing T7 ^{U1A}U3GFP reporter constructs. The effect on splicing of coexpressing a fusion of the RNA-binding domain of hU1Ap and Cbp20p (Cbp20p fusion) was assayed by primer extension. Lanes 1–3: Primer extension for T7 ^{U1A}GFP transcripts. Lanes 4–6: Primer extension for T7 ^{U1A}U3GFP transcripts. Cells were transformed with empty plasmid (lanes 1 and 4), or a plasmid expressing either hU1Ap (lanes 2 and 5) or the CBp20p fusion (lanes 3 and 6). Extension products corresponding to intron-containing and intronless transcripts are indicated by arrows as in **A**. Samples were normalized by quantitating total RNA prior to primer extension. A U2 snRNA control was included as in Figure 1B (not shown).

near the 5′ splice site and interacts with components of the splicing machinery (Fortes et al., 1999a, 1999b; Zhang & Rosbash, 1999). We reasoned that the artificial targeting of cap-binding Cbp20p to the 5′ end of intron-containing T7 transcripts might improve splicing. A 5′ proximal hU1A stem loop was engineered into the intron-containing T7 transcript, and the effect of coexpressing a fusion of the RNA-binding domain of hU1A and CBP20 (Cbp20p fusion) was assayed by primer extension (Fig. 2B). Coexpressing the Cbp20p fusion has no effect on stem-loop-containing transcripts from the intronless T7 reporter (Fig. 2B, lanes 1–3) or on T7 pre-mRNA (intron-containing transcripts; Fig. 2B, lanes 4–6). However, steady-state levels of splice products are increased (intronless transcripts; Fig. 2B, lanes 4–6). We observe a slight increase in splicing efficiency by introducing the hU1A stem loop structure alone. Combined with the further increase when the Cbp20p fusion is coexpressed, this results in splice product accumulation of T7 transcripts that compares favorably to that from an identical pol II transcript (data not shown; compare Fig. 2A, lane 2, and Fig. 2B, lane 6). The results suggest that much of the pol II effect on splicing is explained by cap status.

T7 transcripts are exported from the nucleus

We next used message-specific fluorescent in situ hybridization (FISH) to determine the steady-state localization of T7 transcripts (Fig. 3). To obtain strong and reproducible results, the T7 GFP reporter was introduced on a high copy 2μ plasmid. Cells were fixed after a 42 °C heat shock, which further increases steady-state T7 transcript levels (data not shown; see Fig. 4).

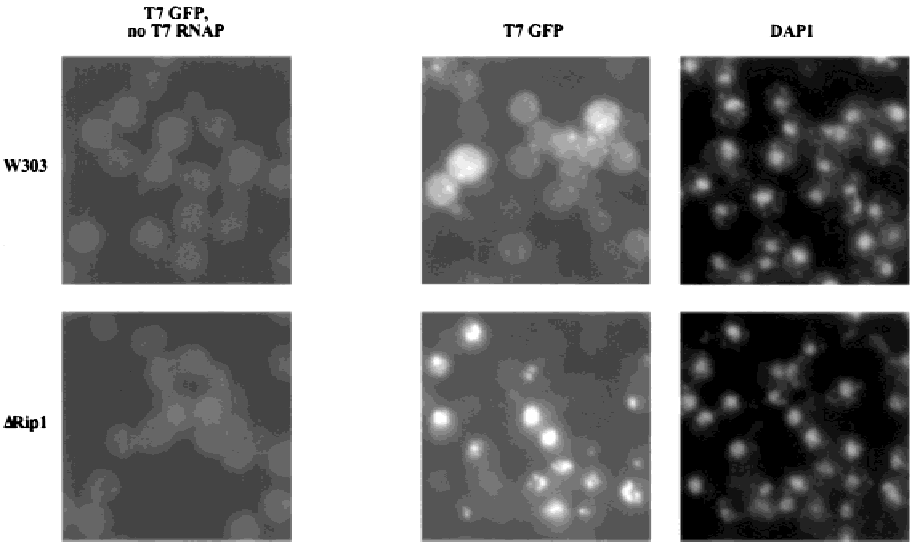

FIGURE 3. T7 transcripts are exported from the nucleus. Fluorescent in situ hybridization (FISH) for T7 GFP transcripts in wild-type and ΔRip1 cells after 30 min incubation at 42 °C. Cells were transformed with high copy 2μ T7 GFP reporter in the absence (left column) and presence (middle column) of T7 RNAP. DAPI (4′,6′-diamidino-2-phenylindole) staining for the middle column is shown to the right. Only approximately half the cells in a field show the representative staining pattern, possibly due to the plasmid-based system and a consequent variability in expression.

To address RNA export issues, T7 transcripts were also localized in the ΔRip1 mutant strain. Rip1p is an inessential nucleoporin-like protein, and cells lacking Rip1p have an mRNA export block at 42 °C (Saavedra et al., 1996, 1997; Stutz et al., 1997; Vainberg et al., 2000). In situ hybridization for T7 GFP transcripts stains wild-type W303 cells faintly and diffusely, with what appear to be some cytoplasmic aggregates (top). In contrast, there is a strong nuclear signal in ΔRip1 cells. T7 transcript levels are comparable in wild-type and ΔRip1 cells by primer extension analysis (data not shown). T7 transcripts are therefore exported from the nucleus in wild-type cells, and, as with pol II transcripts, their export under these conditions requires Rip1p.

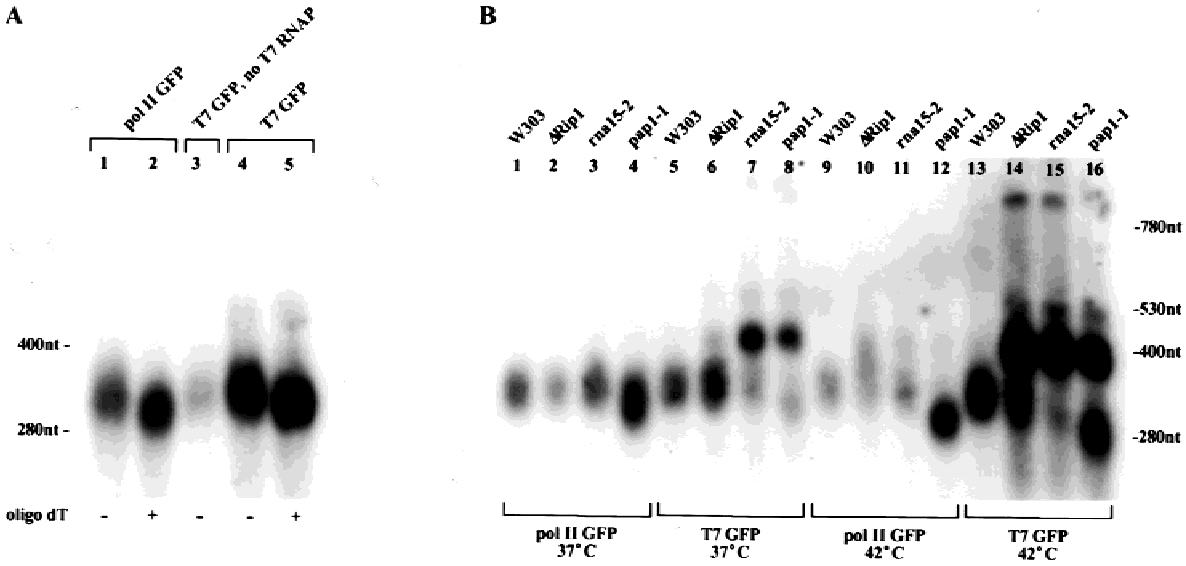

FIGURE 4. T7 transcripts are cleaved and polyadenylated. Total RNA was subjected to RNase H cleavage with an oligonucleotide directed ~300 bp upstream of the polyadenylation signal prior to northern blot analysis. **A**: Northern blot analysis for pol II GFP (lanes 1 and 2) and T7 GFP (lanes 4 and 5) 3′ end fragments with and without oligo dT included in the RNase H treatment, as indicated. Lane 3: Cells transformed with T7 GFP reporter in the absence of T7 RNAP. Cells were incubated at 37 °C for 30 min prior to total RNA isolation. **B:** Northern blot analysis for pol II and T7 GFP 3′ end fragments in wild-type W303, ΔRip1, rna15-2, and pap1-1 strains. Cells were incubated at 37 °C or 42 °C for 30 min prior to total RNA isolation, as indicated. Two strong cryptic stops for T7 readthrough transcripts are observed under restrictive conditions. By size, the first (~400 nt) is in the TDH3 3′ flanking region, ~100 nt downstream of the polyadenylation signal, and the second (>780 nt) is in vector sequence.

T7 transcripts are cleaved and polyadenylated

To investigate 3′ end formation of T7 transcripts, total RNA was subjected to RNase H cleavage with an oligonucleotide directed ~300 nt upstream of the polyadenylation signal. 3′ end fragments were then analyzed by northern blotting (Fig. 4). The fragments of T7 as well as pol II GFP transcripts are the predicted size for 3′ end formation directed by the provided polyadenylation signal (Fig. 4A). To determine if the transcripts are polyadenylated, samples were incubated with oligo dT and RNase H to remove poly(A) tails. This results in higher mobility RNAs for both sets of transcripts, consistent with polyadenylation (Fig. 4A, lanes 2 and 5). T7 transcripts are therefore efficiently cleaved and polyadenylated at or near the polyadenylation signal, indistinguishably from pol II transcripts within the resolution of these experiments.

To verify that 3′ end formation of T7 transcripts requires canonical components of the cellular cleavage and polyadenylation machinery, transcripts were analyzed in strains with temperature-sensitive mutations in cleavage factor Rna15p (rna15-2) and poly(A) polymerase (pap1-1; Mandart & Parker, 1995). Interestingly, 3′ end formation of pol II and T7 transcripts is affected differently. In both mutant strains, T7 transcripts with extended 3′ ends arise from a cryptic stop downstream of the polyadenylation signal (Fig. 4B, lanes 7, 8, 15, and 16). We attribute the accumulation of this larger fragment to a more severe cleavage defect of T7 transcripts at the upstream polyadenylation signal. This result suggests that there may be a polymerase dependence that is revealed under mutant conditions, despite normal T7 transcript 3′ end formation in wild-type cells. Because of recent results in which pol II transcripts with aberrant 3′ ends were observed in ΔRip1 cells at 42 °C, we also analyzed 3′ end formation in ΔRip1 cells (Jensen et al., 2001). T7 transcript cleavage is also affected in ΔRip1 cells at the nonpermissive temperature of 42 °C (Fig. 4B, lane 14).

Proper 3′ end formation and export

T7 transcripts that are cleaved and polyadenylated are cytoplasmic by FISH (see Fig. 3). As results from this and other laboratories indicate a role for 3′ end formation in mRNA export in higher eukaryotes (Eckner et al., 1991; Huang & Carmichael, 1996; McCracken et al., 1998; Nemeroff et al., 1998; Custodio et al., 1999) and in yeast (Kessler et al., 1997; Brodsky & Silver, 2000; Hilleren et al., 2001; Jensen et al., 2001), we investigated a cleavage and polyadenylation requirement for T7 transcript export. To this end, we localized T7 transcripts in rna15-2 and pap1-1 strains (Fig. 5). T7 transcripts become nuclear in both strains after a shift to 42 °C. Similar results were observed with a strain mutated for the cleavage factor Hrp1p, hrp1-5 (data not shown), suggesting an important role for 3′ end formation in the export of T7 transcripts.

To further support the relationship between 3′ end formation and export, we generated a construct in which 3′ end formation is directed by a T7 terminator, an element that causes T7 RNAP transcription termination (Macdonald et al., 1994). A schematic of the construct is shown in Figure 6A. Northern blot analysis reveals a terminator-directed 3′ end fragment and an

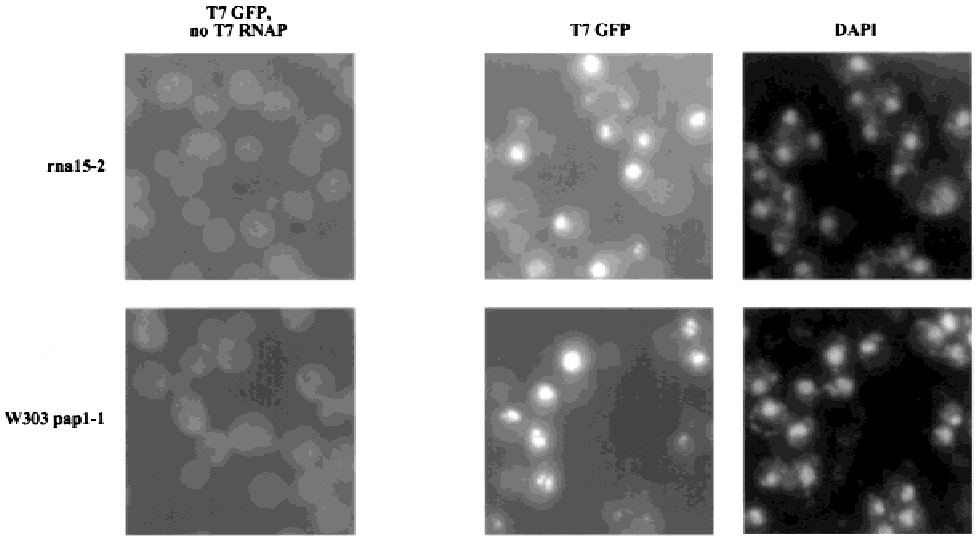

FIGURE 5. T7 transcripts are nuclear in 3′ end processing mutant strains. FISH for T7 GFP transcripts in rna15-2 and W303 pap1-1 strains after 30 min incubation at 42 °C. Cells were transformed with high copy 2μ T7 GFP reporter in the absence (left column) and presence (middle column) of T7 RNAP . DAPI staining for the middle column is shown to the right. The results shown were obtained in parallel to those in Figure 3.

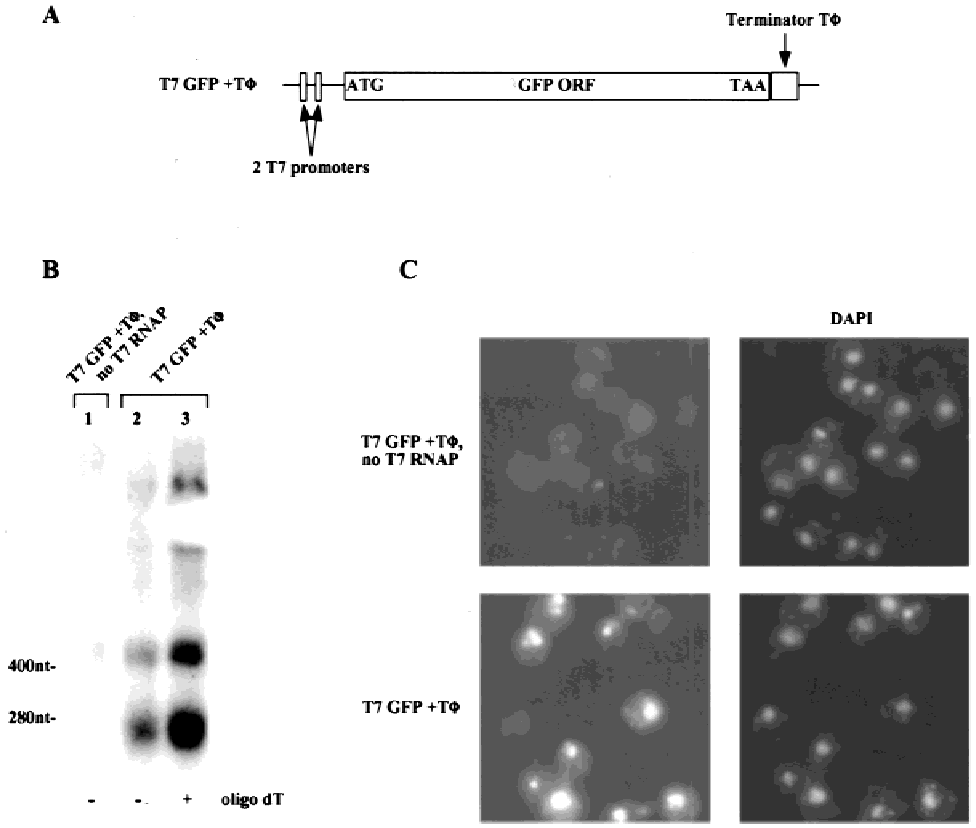

FIGURE 6. Transcripts with terminator-directed ends are nuclear in wild-type cells. **A**: Schematic of the construct with a ~50-bp T7 terminator element (T7 GFP +TΦ). Vector sequence immediately follows the terminator element. **B:** Northern blot analysis for T7 GFP +TΦ 3′ end fragments, with and without oligo dT in the RNase H digestion, as indicated (lanes 2 and 3). Lane 1: Cells transformed with T7 GFP +TΦ reporter in the absence of T7 RNAP. The <280-nt fragment corresponds to a terminator-directed 3′ end, and is absent when the terminator is removed (data not shown). The ~400-nt readthrough fragment arises from a cryptic transcription stop in vector sequence. **C**: FISH for T7 GFP +TΦ transcripts in wild-type cells after 30 min incubation at 42°C. Cells were transformed with high copy 2μ T7 GFP +TΦ reporter in the absence (top) and presence (bottom) of T7 RNAP. DAPI staining is shown to the right.

additional readthrough transcript (Fig. 6B, lane 2). This profile persists in rna15-2, pap1-1, and ΔRip1 cells under restrictive conditions (data not shown). Neither product is polyadenylated, as a mobility shift is not observed after oligo dT/RNase H cleavage under conditions where such a shift is readily apparent (Fig. 6B, lane 3; data not shown). By FISH, T7 transcripts directed from this reporter are nuclear accumulated in wild-type cells (Fig. 6C). As cleaved and polyadenylated T7 transcripts are cytoplasmic (see Fig. 3), the results suggest that proper 3′ end formation may be sufficient as well as necessary to promote RNA export.

DISCUSSION

The emerging picture in higher eukaryotes is that pol II has a prominent role in posttranscriptional mRNA processing. Non-pol II transcripts are inefficiently processed, and factors involved in capping, splicing, and 3′ formation physically interact with the pol II CTD (see Introduction). Pol II is therefore likely to be directly involved in higher eukaryotic mRNA processing through interactions mediated by the CTD. We have characterized transcripts synthesized by ectopically expressed bacteriophage T7 RNAP to investigate pol II and mRNA processing in yeast. In contrast to higher eukaryotes, a non-pol II yeast transcript undergoes multiple processing steps in vivo, with the notable exception of capping (Fig. 1B). The absence of a cap and the importance of cap recognition in translational initiation also explains the lack of T7 transcript translation.

Intron-containing T7 transcripts are spliced in yeast, albeit less efficiently than when transcription is directed by pol II (Fig. 2A). Much of the reduced splicing efficiency of T7 transcripts is probably due to the absence of a cap, as artificially targeting Cbp20p to the 5′ end of intron-containing T7 transcripts increases splicing efficiency (Fig. 2B). We note, however, that we do not observe a decrease in pre-mRNA levels concomitant with T7 splice product accumulation in this experiment. It is therefore possible that splice product accumulation results from stabilization of the pre-mRNA, allowing for

the splicing of transcripts that would otherwise be degraded (Bousquet-Antonelli et al., 2000). The results, however, are consistent with those showing that the cap enhances but is not required for yeast pol II transcript splicing (Fresco & Buratowski, 1996; Schwer & Shuman, 1996). Much of the requirement for pol II in yeast splicing may therefore be explained by the coupling of capping and pol II, and the well-described role of cap-binding proteins in splicing (see Introduction). This scenario probably does not explain results in higher eukaryotes, especially as recombinant CTD stimulates splicing in mammalian extracts (Hirose et al., 1999). Furthermore, a class of higher eukaryotic splicing factors called SR proteins physically interact with the CTD (Yuryev et al., 1996). Notably, there are as yet no bona fide yeast counterparts of SR proteins.

Differences in pol II requirements between yeast and higher eukaryotes extend to mRNA 3′ end processing. Consistent with previous yeast results, our data show that 3′ end formation does not require pol II transcription (Lo et al., 1998; McNeil et al., 1998). T7 transcripts are efficiently cleaved and polyadenylated at a polyadenylation signal and are affected in rna15-2 and pap1-1 mutant strains, indicating that the canonical cellular cleavage and polyadenylation machinery is utilized (Fig. 4). However, we observe a polymerase-dependent effect on 3′ end formation in both mutant strains. An effect of pol II is therefore revealed under mutant conditions. Indeed, modest defects in yeast 3′ end formation have been reported when transcription is directed by pol I or by CTD-less pol II (Lo et al., 1998; McNeil et al., 1998). It is unlikely that these defects are an indirect consequence of cap status. Uncapped yeast pol II transcripts are cleaved and polyadenylated efficiently, and yeast 3′ end processing factors associate with pol II (Fresco & Buratowski, 1996; Rodriguez et al., 2000; Komarnitsky et al., 2000; Barilla et al., 2001). Furthermore, tethering the Cbp20p fusion to the 5′ end of T7 transcripts had no effect (data not shown). Interactions between the pol II CTD and the 3′ end formation machinery are probably stabilizing, giving rise to less severe mutant effects on pol II transcripts than on T7 transcripts.

Our results therefore suggest that, with the exception of capping, posttranscriptional processing in yeast differs from that in higher eukaryotes in that it is less dependent on pol II transcription. The CTD contains 26 nearly identical heptad repeats in yeast and 52 in higher eukaryotes. Yet repeats 1–26 from higher eukaryotes are more similar to the yeast CTD (89% identity) than to repeats 27–52 (77% identity). Moreover, recent results demonstrated that repeats 27–52 are sufficient for the capping, splicing, and 3′ end formation of pol II transcripts in tissue culture, but repeats 1–26 only supported capping (Fong & Bentley, 2001). Perhaps the yeast CTD functions similarly to repeats 1–26 of higher eukaryotes. We suggest that the complexity of a typical metazoan transcription unit requires a more orchestrated series of interactions between processing machineries and transcription.

A surprising result from these experiments is an observed defect in 3′ end cleavage in cells deleted for the nucleoporin-like protein Rip1p (Fig. 4B). A role for Rip1p in 3′ end cleavage is perhaps less unexpected, because of recent work from this laboratory demonstrating that pol II transcripts acquire aberrantly long poly(A) tails in ΔRip1 cells at 42 °C (Jensen et al., 2001). The Rip1p effect on 3′ end cleavage could be indirect, due, for example, to the failure of critical proteins to recycle back to nascent RNAs (Jensen et al., 2001).

Cleaved and polyadenylated T7 transcripts are cytoplasmic under heat-shock conditions. More convincing are the results in the export mutant ΔRip1 strain: steady-state levels of T7 transcripts are comparable in wild-type and ΔRip1 cells, but there is nuclear accumulation in ΔRip1 cells after a shift to 42 °C (Fig. 3; data not shown). We also observe a strong correlation between proper 3′ end formation and RNA localization: T7 transcripts with aberrant 3′ ends are nuclear by FISH, indicating that RNA export requires proper cleavage and polyadenylation. Aberrant 3′ ends were induced either *in trans*, by temperature-shifting mutant strains (Fig. 5), or *in cis* by using a T7 terminator element to direct nonphysiological 3′ end formation in wild-type cells (Fig. 6). The latter approach is important, because it is not subject to the potentially indirect effects of temperature-sensitive mutant strains. Therefore, the recruitment of factors required for mRNA export, and of factors required for the retention of transcripts with aberrant 3′ ends, may not require pol II.

The nuclear accumulation we observe by FISH is typically granular and may reflect RNA accumulation at or near transcription sites of the high copy 2μ reporter (Jensen et al., 2001). Indeed, FISH for T7 transcripts directed from a low copy CEN reporter reveals one to three discrete nuclear spots when the T7 terminator directs 3′ end formation (data not shown). We suggest that the nuclear retention of T7 transcripts with aberrant 3′ ends is related to the similar phenomenon recently described for aberrant pol II transcripts (Hilleren et al., 2001; Jensen et al., 2001).

We localized T7 transcripts at 42 °C for technical reasons. T7 transcripts are more abundant after heat shock, and illegitimate transcription of the GFP reporter by cellular polymerases is reduced (data not shown; see Fig. 4). We were also interested in using the ΔRip1 strain for characterizing T7 transcripts, and the ΔRip1 phenotype is only revealed at 42 °C. It is therefore possible that the link between 3′ end formation and export of T7 transcripts only exists under these extreme heat-shock conditions. This is unlikely, however, as pol II transcripts with 3′ ends directed by a self-cleaving ribozyme element are unadenylated and nuclear accumulated in wild-type cells at 30 °C (data not shown).

Moreover, a relationship between 3′ end formation and nuclear export has been previously established in studies on pol II transcripts (Eckner et al., 1991; Huang & Carmichael, 1996; Kessler et al., 1997; McCracken et al., 1998; Nemeroff et al., 1998; Custodio et al., 1999; Brodsky & Silver, 2000; Hilleren et al., 2001; Jensen et al., 2001). When compared to T7 transcripts that are cleaved and polyadenylated (Fig. 3), the results with the T7 terminator (Fig. 6) suggest further that proper 3′ end formation may be sufficient to promote mRNA export.

Of particular interest to the role of 3′ end formation are recent results providing evidence for cotranscriptional recruitment of mRNA export factors and the retention of transcripts with aberrant 3′ ends at or near their site of synthesis (Hilleren et al., 2001; Jensen et al., 2001; Lei et al., 2001). Additionally, results from *Xenopus* microinjection experiments demonstrate that the introduction of unstructured RNA sequences (i.e., mRNA-like) into otherwise structured RNAs such as tRNA and U snRNAs results in mRNA export factor recruitment to the chimeric RNAs (I.W. Mattaj, pers. comm.). It is therefore possible that subtle features of mRNA structure may be sufficient for the recruitment of mRNA export factors. Such a mechanism may be especially important in yeast, where mRNP formation during splicing is unlikely to provide a general explanation for mRNA export. In this view, some aspect of proper 3′ end formation may then serve to remove factors that actively retain the transcripts. The distinction between promoting export and relieving retention should be clarified by defining discrete physical links between 3′ end formation and factors involved in mRNA export and retention.

MATERIALS AND METHODS

DNA manipulations and yeast transformations were performed using standard protocols (Ausubel et al., 1994). Yeast strains used in this study are described in Table 1. All yeast transformations were of the wild-type W303 strain unless otherwise noted.

Parental constructs

pRS315GPD: The glyceraldehyde-3-phosphate dehydrogenase 3 (TDH3) locus, from ~670 bp upstream of the start codon to ~500 bp downstream of the stop codon, was cloned into pRS315. *Bam*HI and *Sal*I cloning sites replace the TDH3 open reading frame (ORF).

pRS316ADH: The alcohol dehydrogenase I (ADH1) locus, from ~500 bp upstream of the start codon to ~300 bp downstream of the stop codon, was cloned into pRS316. *Bam*HI and *Sal*I cloning sites replace the ADH1 ORF.

pRS314ADH$_{GPD}$: The ADH1 promoter region and TDH3 locus, including the TDH3 5′ leader and ~500-nt TDH3 3′ flanking region, were cloned into pRS314. *Bam*HI and *Sal*I cloning sites replace the TDH3 ORF. Transcription from the ADH1 promoter starts at the TDH3 5′ UTR as determined by primer extension.

pRS304 2μ: The 2μ region of Yep24 was cloned into the *Aat*II site of pRS304.

Expression constructs

pRS315 nlsT7RNAP: T7 RNAP was PCR amplified from BL21(DE3) bacterial cells (Novagen) and cloned into pRS315GPD. Sequence encoding the SV40 lT nuclear localization signal has been introduced immediately downstream of the ATG start codon in the T7 RNAP ORF.

pRS316 hU1Ap: The RNA-binding domain of hU1A (amino acids 1–102) was cloned into pRS316ADH. *Bam*HI and *Sal*I sites have been introduced immediately downstream of the hU1Ap ORF.

pRS316 Cbp20p fusion: The CBP20 ORF was cloned downstream of the hU1Ap ORF of pRS316 hU1Ap.

Pol II reporter constructs

pRS314 pol II GFP: The GFP ORF was PCR amplified from pJK19-1 (kindly provided by P.A. Silver) and cloned into pRS314ADH$_{GPD}$.

pRS314 pol II U3GFP: The ~150-nt pre-U3 snoRNA intron has been introduced immediately downstream of the ATG start codon in the GFP ORF of pRS314 pol II GFP.

pRS314 pol II U3GFP$_{5'ss}$: The 5′ splice site of the pre-U3 snoRNA intron in pRS314 pol II U3GFP has been mutated from GTATGT to ATGTAT.

T7 reporter constructs

pRS314 T7 GFP: The GFP ORF, with TDH3 5′ leader and ~500-nt TDH3 3′ flanking region, was cloned into pRS314. A T7 promoter element (TAA TAC GAC TCA CTA TAG GG) has been introduced immediately upstream of the TDH3 5′ leader sequence.

TABLE 1. Yeast strains

Strain	Genotype	Source
W303	MAT a ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 canR1-100	
W303 ΔRip1	MAT a ΔRIP1::KAN^r; otherwise isogenic to W303	Stutz et al., 1997
rna15-2	MAT a rna15-2 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1	R. Parker
pap1-1	MAT a pap1-1 cup1::LEU2PM lys2-201 ura3-52	R. Parker
W303 pap1-1	MAT a or α pap1-1 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 or -52	This study

pRS314 T7 U3GFP: The ~150-nt pre-U3 snoRNA intron has been introduced immediately downstream of the ATG start codon in the GFP ORF of pRS314 T7 GFP.

pRS314 T7 U3GFP$_{5'ss}$: The 5′ splice site of the pre-U3 snoRNA intron in pRS314 T7 U3GFP has been mutated from GTATGT to ATGTAT.

pRS314 T7 ^{U1A}GFP: Similar to pRS314 T7 GFP, with sequence encoding a U1A stem loop (ACA GCA TTG TAC CCA GAG TCT GTT CCC AGA CAT TGC ACC TGG CGC TGT) ~10 nt downstream of the T7 promoter element.

pRS314 T7 ^{U1A}U3GFP: Similar to pRS314 T7 U3GFP, with sequence encoding a U1A stem loop as described above.

pRS304 2μ T7 GFP: High copy 2μ version of pRS314 T7 GFP.

pRS304 2μ T7 GFP + TΦ: Similar to pRS304 2μ T7 GFP, except that the 3′ flanking sequence has been replaced by a T7 terminator element (TAG CAT AAC CCC TTG GGG CCT CTA AAC GGG TCT TGA GGG GTT TTT TG) that is immediately downstream of the TAA stop codon in the GFP ORF.

Yeast cultures and RNA analysis

When using mutant strains, early log phase 25 °C cultures were temperature shifted by adding an equal volume of 49 °C or 59 °C media to rapidly raise the temperature to 37 °C and 42 °C, respectively. For experiments using only the wild-type strain, cultures were maintained at 30 °C unless otherwise noted. Total RNA was isolated by hot acid-phenol extraction. Primer extension analysis was performed on 5–10 μg total RNA using AMV reverse transcriptase (Promega) and oligonucleotides KD182 (GGA ACA GGT AGT TTT CCA GTA GTG) for GFP RNA and DT58 (GCC AAA AAA TGT GTA TTG TAA) for U2 snRNA. Samples were resolved by 5% polyacrylamide denaturing gel electrophoresis, and bands were visualized by autoradiography. For northern blot analysis, 10–20 μg of total RNA were cleaved with RNase H using oligonucleotide KD290 (GAA CGC TTC CAT CTT CAA TGT TGT) and, where applicable, oligo dT_{18}. Oligonucleotide KD290 is complementary to the GFP ORF, ~200 nt upstream of the stop codon. Samples were resolved by 2.5% formaldehyde-agarose gel electrophoresis. Membranes were probed with radiolabeled oligonucleotide KD282 (GCA GCC AGA TCC TTT GTA TAG TTC ATC CAT GCC ATG), and washed in 2× SSC/0.1% SDS for 15 min twice at 25 °C and twice at 42 °C. Bands were visualized by autoradiography.

α-cap pulldowns

Ten μL of α-cap antibody H20 (kindly provided by R. Lührmann) were incubated with 10 μL GammaBindJ Plus SepharoseJ slurry (Amersham Pharmacia Biotech) for 1 h at 4 °C in 150 μL NET-150 (50 mM Tris-HCl, pH 7.5, 0.05% NP-40, 150 mM NaCl). Beads were washed three times in 1 mL NET-150 prior to incubation with 250 μg total RNA for 2 h at 4 °C in 250 μL NET-150. Beads were washed three times in 1 mL NET-150, and immunoprecipitated RNA was isolated by phenol/chloroform extraction and ethanol precipitation.

Yeast strain construction

Yeast strain W303 was transformed with pRS316 and mated to yeast strain pap1-1 (kindly provided by R. Parker). Diploids were selected on Leu$^-$ Ura$^-$ dropout plates, and cured of pRS316 on 5-FOA plates prior to sporulation and dissection. Spores were tested for growth at 37 °C and on the appropriate dropout plates to select strain W303 pap1-1. Strain details are provided in Table 1.

Fluorescent in situ hybridization

Early log-phase 25 °C cultures were shifted to 42 °C with an equal volume of 59 °C media prior to fixation. A mixture of 4 oligonucleotides complementary to the GFP ORF was used for FISH. Oligonucleotides KD209 (GT*G CCC ATT AAC AT*C ACC ATC TAA TT*C AAC AAG AAT* TGG GAC AAC T*CC AGT), KD210 (GTA CAT* AAC CTT CGG GCA T*GG CAC TCT T*GA AAA AGT CAT* GCC GTT TCA T*AT), KD211 (GAT TCC AT*T CTT TTG TT*T GTC TGC CAT* GAT GTA TAC AT*T GTG TGA GTT* ATA), and KD212 (CCC AGC AGC T*GT TAC AAA CT*C AAG AAG GAC CAT* GTG GTC T*CT CTT TTC GT*T), where T* represents 5′-dimethoxytrityl-S-[*N*-(trifluoroacetylaminohexyl)-3-acrylimido]-2′-deoxyuridine, 3′-[(2-cyanoethyl)-(*N*,*N*,-diisopropyl)]-phosphoramidite (Amino-Modifier dT, Glen Research), were coupled to Cy3 fluorochrome (Amersham) and used for FISH as described (Vainberg et al., 2000).

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