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In Vivo Evidence that Defects in the Transcriptional Elongation Factors RPB2, TFIIS, and SPT5 Enhance Upstream Poly(A) Site Utilization†

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While a number of proteins are involved in elongation processes, the mechanism for action of most of these factors remains unclear primarily because of the lack of suitable in vivo model systems. We identified in yeast several genes that contain internal poly(A) sites whose full-length mRNA formation is reduced by mutations in RNA polymerase II subunit RPB2, elongation factor SPT5, or TFIIS. RPB2 and SPT5 defects also promoted the utilization of upstream poly(A) sites for genes that contain multiple 3′ poly(A) signaling sequences, supporting a role for elongation in differential poly(A) site choice. Our data suggest that elongation defects cause increased transcriptional pausing or arrest that results in increased utilization of internal or upstream poly(A) sites. Transcriptional pausing or arrest can therefore be visualized in vivo if a gene contains internal poly(A) sites, allowing biochemical and genetic study of the elongation process.

The regulation of eucaryotic gene expression can occur at different and multiple levels. Control of the elongation phase of transcription has been found to be important for a number of genes, most notably those in higher eucaryotes (14, 53). It is expected, therefore, that numerous biological controls are in place to ensure that elongation occurs to the extent and degree necessary to result in the proper levels of mRNA for the different genes in the cell. Most importantly, recent evidence has suggested that the elongation process is linked and critical to other posttranscriptional processes such as mRNA capping, splicing, polyadenylation and cleavage, and transport (7, 42, 48). Elongation can therefore be viewed as the center through which the whole quality control of mRNA formation can be integrated (33, 36, 42).

Recently a number of factors have been identified in yeast and other organisms as either components of the elongating polymerase or as possible modulators of the process of elongation. Biochemical and in vitro studies have demonstrated or suggested a requirement for several of these factors in elongation. As a result of the RNA Pol II structure being recently determined, it is clear that the RPB2 subunit of RNA Pol II may play multiple roles during elongation (16). In vitro evidence confirms a key role for RPB2 because RNA polymerase II (Pol II) containing either the rpb2-10 or rpb2-4 protein fails to elongate well in vitro (41). In particular, the rpb2-10 protein causes arrest at known mammalian pause sites and requires elongation factor TFIIS to overcome the arrest. Moreover, the *rpb2-10* allele in combination with a *dst1* (encoding TFIIS) deletion causes severe defects in the synthesis of most mRNA in vivo (29) and reduces the induction of a number of other genes (56). These observations suggest that in vivo rpb2-10 actually causes promiscuous arrest of RNA Pol II that requires TFIIS to suppress and overcome.

Other important factors involved in elongation are the yeast proteins SPT5 and SPT4, whose higher eucaryotic orthologs are components of the DSIF complex. SPT5 and SPT4 have been found to be both activators and repressors of elongation. The DSIF complex is known to be important in repressing elongation in vitro and appears to do so by its interaction with the hypophosphorylated form of the RNA Pol II carboxy-terminal domain (54, 55, 59, 60). In the control of *hsp70*, *Drosophila* SPT5 is known to be recruited to the transcribing polymerase, although it may also have a role in forming the paused RNA Pol II complex at *hsp70* (1, 27). Also, depleting SPT5 in a TAT-dependent system promotes pausing and transcriptional termination, indicating that SPT5/SPT4 can have positive roles on elongation in addition to previously described negative roles (6). It has also recently been shown that SPT4 plays a positive role in elongation in yeast (43). Various genetic analyses have implicated SPT5 and SPT4 in controlling elongation in vivo (22) and initiation (50). In addition, certain *spt5* alleles can be suppressed by *rpb2-10* or by the presumed slowing of elongation (22). The physical association of the SPT5/SPT4 complex with RNA Pol II further confirms its importance in RNA Pol II function (22).

In the yeast *Saccharomyces cerevisiae*, the principal limitation in characterizing the action of factors involved in elongation has been the lack of identification of specific genes whose expression is clearly affected by defects in these elongation factors. For instance, no particular gene whose elongation is affected by these factors has been identified (29, 45, 56), although it has been suggested that transcription of long genes and genes with high G+C content is defective with certain elongation defects (9, 43). Similarly, whole genome microarray analysis with *dst1* and *rpb9* alleles did not yield particular genes controlled at the level of elongation (23). Moreover, chromatin

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TABLE 1. Yeast strains used

Strain	Genotype
FY1642	*MAT***a** *his4-912δ lys2-128δ leu2Δ1 ura3-52 SPT5-FLAG*
FY1668-uH	*MAT***a** *his4-912δ lys2-128δ spt5-4 ura3::HIS3*
FY1635	*MATα his4-912δ lys2-128δ leu2Δ1 ura3-52 spt5-242*
L615	*MAT***a** *his4-912δ lys2-128δ ura3-52 ade2-1 trp5 can1-100 spt5-25*
FY300	*MAT***a** *his4-912δ lys2-128δ leu2Δ1 ura3-52 spt5-194*
GHY180	*MATα ura3-52 leu2Δ1 his4-912δ lys2-128δ spt4Δ2::HIS3*
FY276-uT	*MAT***a** *leu2Δ1 ura3::TRP1 his4-9128 lys2-128δ spt5-8*
AYW3-1B1	*MAT***a** *leu2 trp1 ura3 his3*
AYW3-3D1	*MAT***a** *leu2 his3 ura3 leu2-k::ADE2-URA3-leu2-k hpr1Δ3::HIS3*
Z96	*MAT***a** *ura3-52 leu2-3,112 his3Δ200 rpbΔ297::HIS3* [*pRP214* (*LEU2 RPB2*)]
Z100	Isogenic to Z96 except [*pRP2-4L* (*LEU2 rpb2-4*)]
Z106	Isogenic to Z96 except [*pRP2-10L* (*LEU2 rpb2-10*)]
Z103	Isogenic to Z96 except [*pRP2-7L* (*LEU2 rpb2-7*)]
DY106-u	Isogenic to Z96 except *dstl::hisG*
DY108	Isogenic to Z106 except *dst1::hisG*

immunoprecipitation analysis was unable to verify in vivo that deletion of TFIIS resulted in increased RNA Pol II occupancy even with a gene whose transcriptional elongation was apparently impaired (28). These results suggest that the pausing that is presumed to be occurring in vivo with elongation defects is difficult to detect either as effects upon gene expression or in enhanced RNA Pol II occupancy. Because many putative elongation factors (SPT5/SPT4, PAF1 complex, TFIIE/IIF/IIH, RPB subunits, TFIIS, and CCR4-NOTs) (13, 14, 17, 19, 45, 49–51) can also play roles in affecting initiation, it is of paramount importance to identify genes in yeast that are regulated at the level of elongation.

Previously, the *lacZ* gene, albeit an *Escherichia coli* gene, has been found to be regulated at the level of elongation when expressed in yeasts that are defective in the *HPR1* gene (8). The *hpr1* deletion reduces transcription through *lacZ*, apparently because of both its high G+C content and its extreme length (9). We have consequently examined whether other defects in elongation factors also affected expression through *lacZ*. Our results show that defects in SPT5 and RNA Pol II subunit RPB2 impair transcription through the *E. coli lacZ* gene but by a different mechanism than that found for *hpr1*. We show that transcription through genes containing internal polyadenylation sequences is particularly sensitive to *spt5-4* and *rpb2-10* defects. Most importantly, we identify several bona fide yeast genes containing naturally occurring internal poly(A) sites whose elongation is impaired by *rpb2-10*, *spt5-4*, and *dst1* defects. Our model is that *spt5-4*, *rpb2-10*, and *dst1* alleles, which would be expected to cause pausing or arrest during elongation, result in increased usage of internal poly(A) sites. These results are consistent with those of other studies linking downstream pause sites to poly(A) site utilization (2, 5, 61, 62) and imply that elongation pausing or arrest can be studied biochemically and genetically in vivo by using genes containing internal poly(A) sites.

MATERIALS AND METHODS

Yeast strains, growth conditions, and enzyme assays. The yeast strains used are listed in Table 1. Strains containing *spt5* or *spt4* alleles are all isogenic to FY1642 (wild type) except as indicated (22). The Z96 set of strains has been described previously (41). Yeast were grown on medium containing 1% yeast extract–2% Bacto Peptone, minimal medium, or CAA-U$^-$ medium (31) supplemented with an appropriate carbon source, as indicated in the figures. β-Galactosidase activities were determined as described previously (31).

RNA analyses. Quantitative S1 nuclease protection assays were conducted as previously described (13) using the oligonucleotides listed in Table 2. Control experiments in each case indicated that at the concentration of S1 nuclease used no radioactively labeled oligonucleotide remained if no RNA was present and that the S1 nuclease assay was linear over the concentration of RNAs used.

Total RNA and poly(A) mRNA were purified and Northern blotting was conducted as described previously (15, 18). mRNA was verified as being enriched in polyadenylated RNA because only 1% of the total RNA was recovered, rRNA was substantially reduced as evidenced by ethidium bromide staining, and one-fifth the abundance of poly(A) was required to give a much stronger mRNA signal following Northern analysis than that required for total RNA. Oligonucleotides were radiolabeled at their 5′ end with T4 polynucleotide kinase as previously described (10).

The rates of degradation for *GAL1-lacZ*, *RP51-lacZ*, *RP51-ADH2*, and *GAL1* RNA were determined following growth of yeast on galactose-containing medium for 3 h (15 min for *GAL1*) and shifting to medium containing glucose, as described previously (10). For the analysis of the polyadenylated species of *GAL1* mRNA, *GAL1* gene expression was induced for 15 min by shifting yeast from raffinose-containing medium to galactose-containing medium. The newly synthesized *GAL1* mRNA was detected by using an RNase H assay in which an 18-nt DNA probe (5′-GCCATTTGGGCCCCCTGG-3′) complementary to the sequences 133-bp upstream of the *GAL1* translation stop codon was hybridized to total yeast RNA prior to RNase H cleavage (52). The resultant *GAL1* 3′ polyadenylated species were detected by Northern analysis by using a probe that was complementary to the 3′ end of *GAL1* (5′-GCCCAATGCTGGTTTAGAG ACGATGATAGCATTTTCTAGCTCAGCATCAGTGATCTTAGGG-3′).

RESULTS

The *rpb2-10*, *rpb2-4*, *spt5-4*, and *spt4* alleles affect *lacZ* reporter expression irrespective of the promoter. A previous study of the *hpr1* deletion showed that it affected expression of the *PHO5-lacZ* or *GAL1-lacZ* gene in yeast although *hpr1* had no effect on endogenous *PHO5* or *GAL1* expression (8). These and other results led to the conclusion that HPR1 was required for efficient transcriptional elongation through the *lacZ* gene (8). Based on the observation that the *lacZ* gene might contain specific sequences or structures that interfered with elongation, we conducted an analysis to determine whether defects in other factors known or presumed to play roles in transcriptional elongation also failed to properly express *lacZ*. We used three *lacZ* reporter constructs, each of which contained a different promoter: *ADH2-lacZ*, *FKS1-lacZ*, or *GAL1-lacZ* (31). These promoters were chosen to represent genes that are constitutively expressed (*FKS1*), inducible (*GAL1*), or subject to derepression by nonfermentative growth (*ADH2*). We subsequently analyzed the effects of defects in transcription elongation and initiation factors with each of these three reporters. As was found for *hpr1*, the *rpb2-10*, *rpb2-4*, *spt5-4*, and *spt4* alleles conferred large decreases in *lacZ* expression for all reporters tested (Table 3). These effects were not likely due to effects on the respective promoters, as *spt5-4* does not reduce *GAL1* or *ADH2* expression (data not shown; see Fig. 4A). *rpb2-10* is known to reduce *GAL1* steady-state mRNA but did so by only 1.4-fold (56), and it had no effect on *ADH2* derepression (see Fig. 4A).

Another *rpb2-7* allele, which like *rpb2-4* and *rpb2-10* confers 6AU sensitivity (41), did not display a consistent major effect on the *lacZ* reporters (Table 3). Other alleles of *SPT5* (*spt5-8* and *spt5-242*) had much less effect on *lacZ* expression (Table 3). Moreover, defects in the elongation factor ELP1 (38) or SPT16 (37) did not affect *lacZ* expression (data not shown). Similarly, defects in a number of transcription initiation fac-

TABLE 2. Characteristics of oligonucleotide probes used

Probe	Sequence	Location (nt)
ADH2		
5′	5′-GTATTACGATATAGTTAATAGTTGATAGTTGATTGTATGCTTTTTGTAGCTTGATATTCTATTTA CCAAGAAGAAAC-3′	−80 to −4
3′	5′-GGCATACTTGATAATGAAAACTATAAATCGTAAAGACATAAG-3′	+1126 to +1167
NOT1		
5′	5′-GCGGCCTGTTTTTCCTTTGATGTTTCTAAATTAGATGCTGTGTTCAAATCACGGGTCC-3′	+18 to +71
3′	5′-GGCGGATTGGTCATCTTGTTCACTGGTTGTGTATTTTTTGG-3′	+6245 to +6285
YAT1		
5′	5′-GGCGCTCGTCCTGCAGGGGTTCCACGCGTGCCAGGTAGCGG-3′	+51 to +91
3′	5′-GCGGTCGATCTCCAGCAGCGACTTTTCCATGAGCGACGCAAACCGAGCAGTCTGGCG-3′	+1876 to +1932
RNA14		
5′	5′-GGCTCTGCGACTTTGTCCGCAGAGGGATATAGTAAATCAGGAGTCGTAGAG-3′	+5 to +59
3′	5′-GCGCATCGAGTAAATTTGTATTAAAATATTGACGTTTTGG-3′	+1927 to +1966
CBP1		
5′	5′-CCCTGCTGCTGTGGTTGATTCGTCGCAAGGTCCTGGTAGGTACCATTTTTATAAACCTCTCGG-3′	+32 to +94
3′	5′-GCCGTTCATCTTAAGTAACGTTTGACAGCCGACACACCATGC-3′	+1929 to +1970
AEP2		
5′	5′-CGGGGATAGACAGAATGACATATTGTGCTATTCGGCAGTACACCAAATTCACGCAGCG-3′	+86 to +143
3′	5′-CCCCGGTTTGAAACTCCTTAAAACATCAATTCCAAGCGGG-3′	+1401 to +1440
lacZ		
5′	5′-GGATCCGGTCATTATTAATTTAGTGTGTGTATTTGTGTTTGCGTGTCTATAGAAGTATAGTA-3′	−49 to +12
0.5-kb	5′-GCGCTCAGGTCAAATTCAGACGGCAAACGACTGTCCTGGCC-3′	+487 to +527
1.5-kb	5′-CGGGAAGGGCTGGTCTTCATCCACGCGCGCGTACATCGGGC-3′	+1502 to 1542
2.3-kb	5′-CGCCAATGTCGTTATCCAGCGGTGCACGGGTGAACTGATCGC-3′	+2345 to +2386
3′	5′-CCGCGTGCAGCAGATGGCGATGGCTGGTTTCCATCAGTTGC-3′	+2898 to +2938

tors, such as CCR4-NOT complex components, RNA Pol II holoenzyme components SRB9, SRB10, SRB11, SIN4, and GAL11, and SAGA components ADA2 and GCN5, did not display decreases in *lacZ* gene expression for each reporter plasmid (4, 11, 12, 31; data not shown). We conclude that the *rpb2-10*, *rpb2-4*, *spt5-4*, and *spt4* alleles are affecting an apparently postinitiation step in the expression of the *lacZ* gene. Importantly, the rpb2-4- and rpb2-10-containing RNA Pol IIs have been shown in vitro to display increased transcriptional arrest, whereas rpb2-7–RNA Pol II did not (41).

The *rpb2-10*, *rpb2-4*, *spt5-4*, and *spt4* alleles reduce the ability of RNA Pol II to form full-length *lacZ* mRNA. To address whether transcriptional elongation through the *lacZ* gene was being impaired by the above-described *rpb2*, *spt5*, and *spt4* alleles, we used the quantitative S1 nuclease protection assay (13) to identify the abundance of *lacZ* transcripts that were full length. To do this, we compared the abundance of total *lacZ* mRNA to the abundance of full-length *lacZ* mRNA. Previously, it was shown by using Northern analysis that an *hpr1* deletion blocked the synthesis of *lacZ* mRNA (8). In our assay system, *hpr1* similarly displayed an inability to form full-length *lacZ* mRNA regardless of the promoter. Comparing an *hpr1* strain to its isogenic parent, we observed fourfold less full-length *lacZ* mRNA (corresponding to 3′-end bands) than total *lacZ* RNA (corresponding to 5′-end bands) (Fig. 1A and data not shown).

As shown in Fig. 1B and C, the *rpb2-10*, *rpb2-4*, *spt5-4*, and *spt4* alleles which displayed consistent reductions in overall *lacZ* reporter expression concomitantly displayed reduced levels of full-length *lacZ* mRNA formation compared to the quantity of total *lacZ* mRNA that was present (Fig. 1B and C). In contrast, *rpb2-7* (Fig. 1B), *spt5-8*, *spt5-25*, and *spt5-194* (Fig. 1C) had no significant effect or less effect on formation of full-length *lacZ* mRNA. The same results obtained with *FKS1-*

TABLE 3. Effects of elongation defects on *lacZ*-reporter activities

Strain	Relative β-galactosidase activity with[a]:		
	FKS1-lacZ	*ADH2-lacZ*	*GAL1-lacZ*
WT	100	100	100
hpr1	2.3	1.6	2.2
rpb2-4	20	12	5.5
rpb2-10	24	13	15
rpb2-7	ND	290	56
spt5-4	23	2.9	4.1
spt5-8	65	35	56
spt5-242	ND	59	51
spt4	1.5	4.9	0.50

[a] β-Galactosidase activities were determined for each allele and its corresponding isogenic wild-type parent containing the *lacZ* reporter as indicated. In order to ease comparison between strains, all wild-type parent values were set to 100 for each reporter. *FKS1-lacZ* was assayed on medium containing 4% glucose, *ADH2-lacZ* was assayed on 2% ethanol–2% glycerol, and *GAL1-lacZ* was assayed on 2% galactose–2% raffinose. All values represent the averages of the results for five separate transformants. The SEMs were less than 20%. ND, no data.

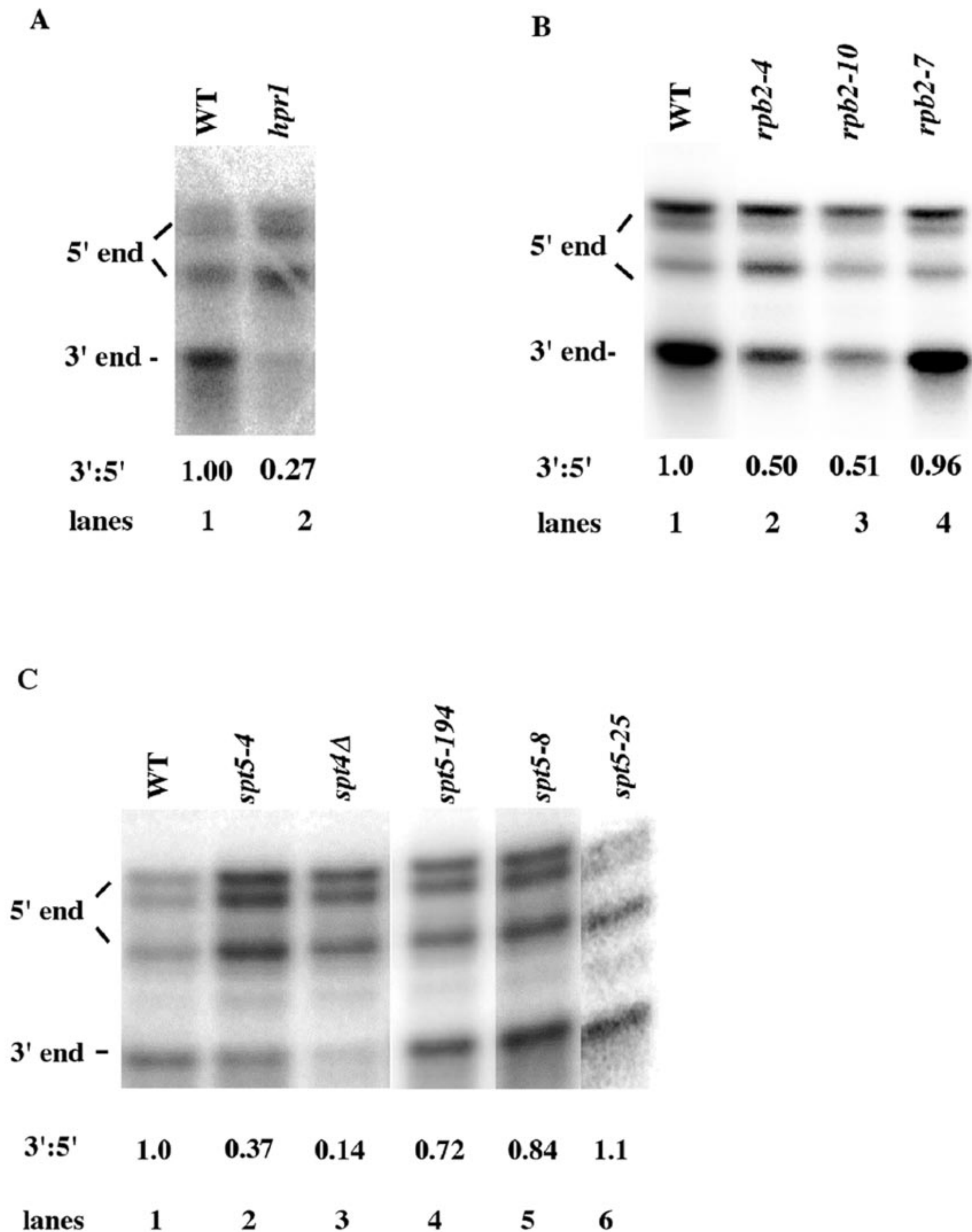


FIG. 1. S1 nuclease protection analysis of the effects of HPR1, RPB2, SPT4 and SPT5 defects on full-length *lacZ* mRNA formation. *lacZ* 5′- and 3′-end RNA levels were quantified by using an S1 nuclease protection assay with probes directed against the 5′ end of *lacZ* RNA and the site 2.9 kb downstream of the *lacZ* initiation site, respectively (Table 2). Total RNA was extracted, and the ratios of 3′ to 5′ RNA levels were quantified by using a phosphorimager. The values were normalized based on the ratio for the wild type, and the average ratio is given below each panel except as indicated. The multiple 5′ends that are visualized resulted from multiple initiation sites. Changes in the ratios of these 5′ band intensities appear to vary somewhat with the strain background. (A) *hpr1* reduces full-length *lacZ* mRNA formation. Strains AYW3-1B1 (WT, lane 1) and AYW3-3D1 (*hpr1*, lane 2) containing plasmids expressing *FKS1-lacZ* were grown on 4% glucose-containing CAA-U$^-$ medium. The values represent the averages of the results of five determinations, and the SEM for *hpr1* was 20%. (B) *rpb2-4* and *rpb2-10* alleles reduce full-length *lacZ* mRNA formation. The growth conditions with *FKS1-lacZ* and the calculations for the results were as described for panel A. Lane 1, WT strain Z96; lane 2, *rpb2-4* strain Z100; lane 3, *rpb2-10* strain Z106; and lane 4, *rpb2-7* strain Z103. The values represent the averages of the results of five determinations for *rpb2-4* with an SEM of 4%, four determinations for *rpb2-10* with an SEM of 7%, and a single determination for *rpb2-7*. Similar results for the analysis of *ADH2-lacZ* mRNA expression were observed for *rpb2-7*. (C) *spt5-4* and *spt4* alleles reduce full-length *lacZ* mRNA formation. The growth conditions with *FKS1-lacZ* and the calculations for the results were as described for panel A. Lane 1, WT strain FY1642; lane 2, *spt5-4* strain FY1668-uH; lane 3, *spt4* strain GHY180; lane 4, *spt5-194* strain FY300; lane 5, *spt5-8* strain FY276-uT; and lane 6, *spt5-25* strain L615. The *spt5-4* value represents the average of the results for eight determinations, with an SEM of 8%. The other values represent the results for single determinations, although similar results were obtained for these defects with the analysis of *ADH2-lacZ* mRNA expression.

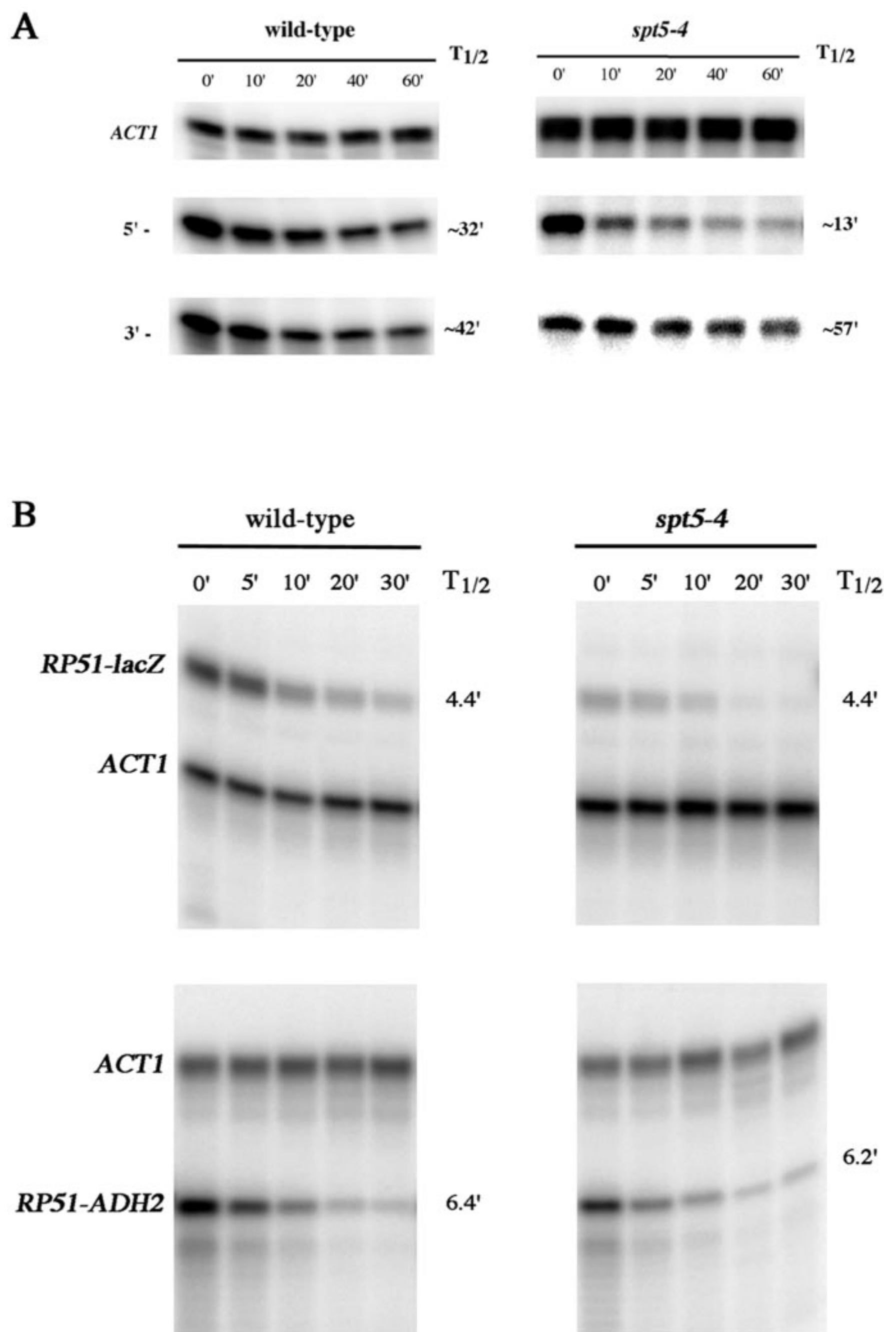


FIG. 2. *spt5-4* does not preferentially enhance full-length *lacZ* and *RP51-lacZ* mRNA half-lives. mRNA half-life determinations were conducted as described in Materials and Methods (10). (A) *GAL1-lacZ* mRNA was detected at the 5′ end with the 0.5-kb *lacZ* probe and at the 3′ end with the 2.3-kb *lacZ* probe (Fig. 3). (B) *RP51-lacZ* and *RP51-ADH2* RNAs were detected, respectively, with the 0.5-kb *lacZ* probe 2 (Fig. 5A) and with the probe 1 corresponding to the *RP51-ADH2* 5′ intron junction (Fig. 5A). Quantitation of *ACT1* RNA levels was used to standardize for loadings. The wild-type strain was FY1642, and the *spt5-4* strain was FY1668.

lacZ (Fig. 1) were obtained with *ADH2-lacZ* (data not shown), indicating that these effects were independent of the promoter. These data confirm a direct correlation between the effect these elongation factors have on *lacZ* reporter expression and the formation of full-length *lacZ* mRNA. Importantly, only the *rpb2* alleles which displayed reduced ability to transcribe through elongation blocks in vitro (41) failed to form full-length *lacZ* mRNA in vivo.

Note that, in agreement with the results of other studies (32, 58), these S1 analyses indicate that, due to differences in length and sequence, oligonucleotide probes can display different degrees of stable binding to the same mRNA and hence different apparent levels of mRNA. Because of these inherent differences in stability of binding between the individual probes and a given mRNA, only the ratios of 5′ to 3′ mRNA levels can be compared between a particular mutant and its isogenic parent.

Importantly several of these mutants can affect the overall abundance of a particular mRNA that results in either an increased or reduced level of total mRNA levels relative to that of the wild type (for example, see *spt5-4*, Fig. 1C and 3B). The *rpb2-4* and *rpb2-10* alleles also reduced total *FKS1-lacZ* expression (Fig. 1B). However, as we were interested in comparing the relative abilities of different elongation defects to reduce the formation of the full-length mRNA, alterations in the level of initiation of the transcript did not affect these comparisons.

The effect on *lacZ* mRNA formation is not due to increased mRNA 3′-end degradation. The reduced level of formation of full-length *lacZ* mRNA could be attributed either to a block in expression through the *lacZ* gene or to increased degradation of the 3′ end of the mRNA in comparison to the degradation of the 5′ end. To address this latter possibility, we utilized the *GAL1-lacZ* reporter to determine the rate of degradation of the 5′ and 3′ ends of the *lacZ* mRNA following the shutting off of *GAL1-lacZ* transcription by growth on glucose-containing medium. As shown in Fig. 2A, the *spt5-4* allele had little effect on the stability of the full-length *lacZ* mRNA (3′-end probe). Note that with the 5′ probe, in the *spt5-4* strain, the rate of degradation of the *lacZ* RNA was actually enhanced relative to that in the wild-type strain. This finding may be the result of higher rates of degradation for RNAs that are not full length, as identified with the 5′ probe, and which are in greater abundance in the *spt5-4* background. A similar analysis showed that in the *rpb2-10* background full-length *lacZ* RNA had a half-life of 50 min, whereas the 5′-end RNA had a half-life of 20 min, and that in the *rpb2-4* background full-length RNA had a half-life of 41 min, while the 5′-end RNA had a half-life of 26 min. These data indicate that the *spt5-4*, *rpb2-10*, and *rpb2-4* alleles are not reducing full-length *lacZ* RNA expression by enhancing the degradation rate of the full-length mRNA relative to all RNAs containing the 5′ end.

The block to transcriptional elongation in *lacZ* occurs at multiple sites. We subsequently examined where in the *lacZ* gene the block to elongation was occurring by using the S1 nuclease protection assay with probes spaced across the *lacZ* gene. In backgrounds with mutations in *spt5-4*, *rpb2-4*, and *rpb2-10*, *lacZ* mRNA synthesis appeared unimpeded through the first 500 bp of the *lacZ* gene (Fig. 3). However, with probes at 1.5, 2.3, and 2.9 kb, decreased levels of *lacZ* 3′-end mRNA formation became apparent. In moving from 1.5 to 2.9 kb, at each step an additional decrease in *lacZ* 3′-end mRNA formation was observed, suggesting the existence of multiple sites for blockage of mRNA synthesis (Fig. 3).

***spt5* and *rpb2* defects may enhance use of cryptic poly(A) sites in *lacZ*.** The above observations indicate that *lacZ* mRNA formation is particularly sensitive to SPT5 and RPB2 defects. One model to explain these data is that transcriptional pausing or arrest caused by the *spt5-4*, *spt4*, *rpb2-10*, and *rpb2-4* alleles results in increased utilization of known cryptic poly(A) sites located within the *lacZ* gene (8, 44). An alternative hypothesis for how *hpr1* affects *lacZ* has been suggested: that the long length of *lacZ* or its high G+C content contributes to its impaired transcription (9). *spt5-4* and *rpb2-10* had no effect on transcription through the native *YAT1* gene, which is extremely G+C rich (Fig. 4A). *hpr1* and *spt4* have both been shown to reduce overall *YAT1* gene expression, presumably by effects on elongation (9, 43). We also found that *spt5-4* reduced the overall expression of *YAT1* relative to that of the wild type by about threefold (data not shown). Our results (Fig. 4A), in contrast, indicate that the *spt5-4* allele does not specifically reduce the abundance of full-length *YAT1* mRNA relative to the levels of the initiated mRNA. Likewise, *spt5-4* and *rpb2-10* did not affect transcription through *NOT1*, a 6.5-kb transcript (Fig. 4A), suggesting that it is not the length per se of *lacZ* which impedes expression. Finally, *rpb2-4*, *rpb2-10*, and *spt5-4* did not affect full-length *ADH2* expression (Fig. 4A), although they all affected *ADH2-lacZ* expression (Table 3 and data not shown).

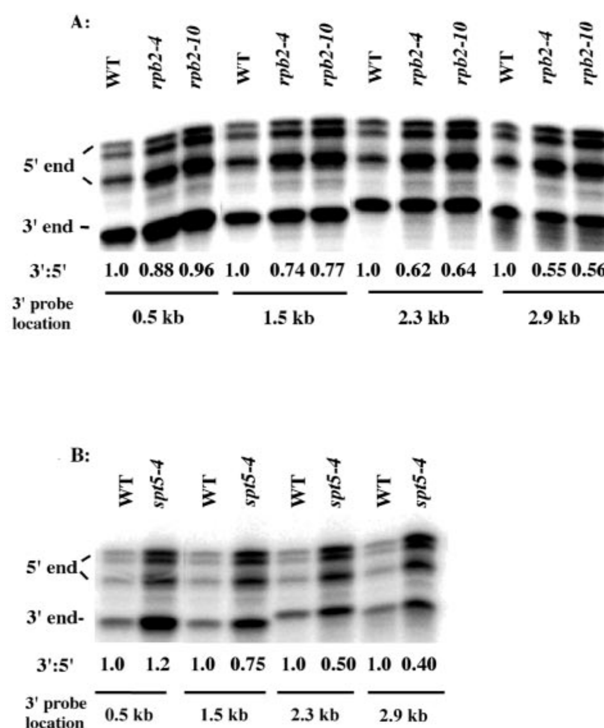


FIG. 3. *spt5-4* and *rpb2* alleles affect full-length *lacZ* RNA formation at multiple sites. The 3′ *lacZ* probes for conducting the S1 nuclease protection assays are listed in Table 2. In all cases, the 5′ probe corresponded to sequences at the initiation codon of *lacZ*. Growth conditions, assays, and analyses were as described for Fig. 1. The *rpb2* (A) and *spt5-4* (B) strains contained *FKS1-lacZ*. The values represent the averages of two experiments, and the SEMs were 10% or less. Note that the *rpb2-4* and *rpb2-10* alleles resulted in less total *FKS1-lacZ* gene expression than for the wild type, which necessitated loading fourfold more *rpb2-4* and *rpb2-10* RNA than for the wild type in panel A. Equivalent amounts of total RNA were analyzed in panel B for the wild type and *spt5-4*.

If the poly(A) usage model were correct, the incompletely formed *lacZ* RNA would be polyadenylated. We would expect, therefore, that when polyadenylated enriched RNA is analyzed, both *spt5-4* and *rpb2-10* would reduce the formation of full-length *lacZ* mRNA to the same extent that they reduce full-length *lacZ* RNA formation when total RNA is analyzed. As shown in Fig. 4B, *rpb2-10* and *spt5-4* affected the formation of full-length *lacZ* polyadenylated RNA to the same degree as they affected the formation of full-length *lacZ* RNA isolated from total RNA. These results support the model that *spt5-4* and *rpb2-10* enhance utilization of the cryptic poly(A) sites in *lacZ* (44) and do not simply cause RNA Pol II to cease tran-

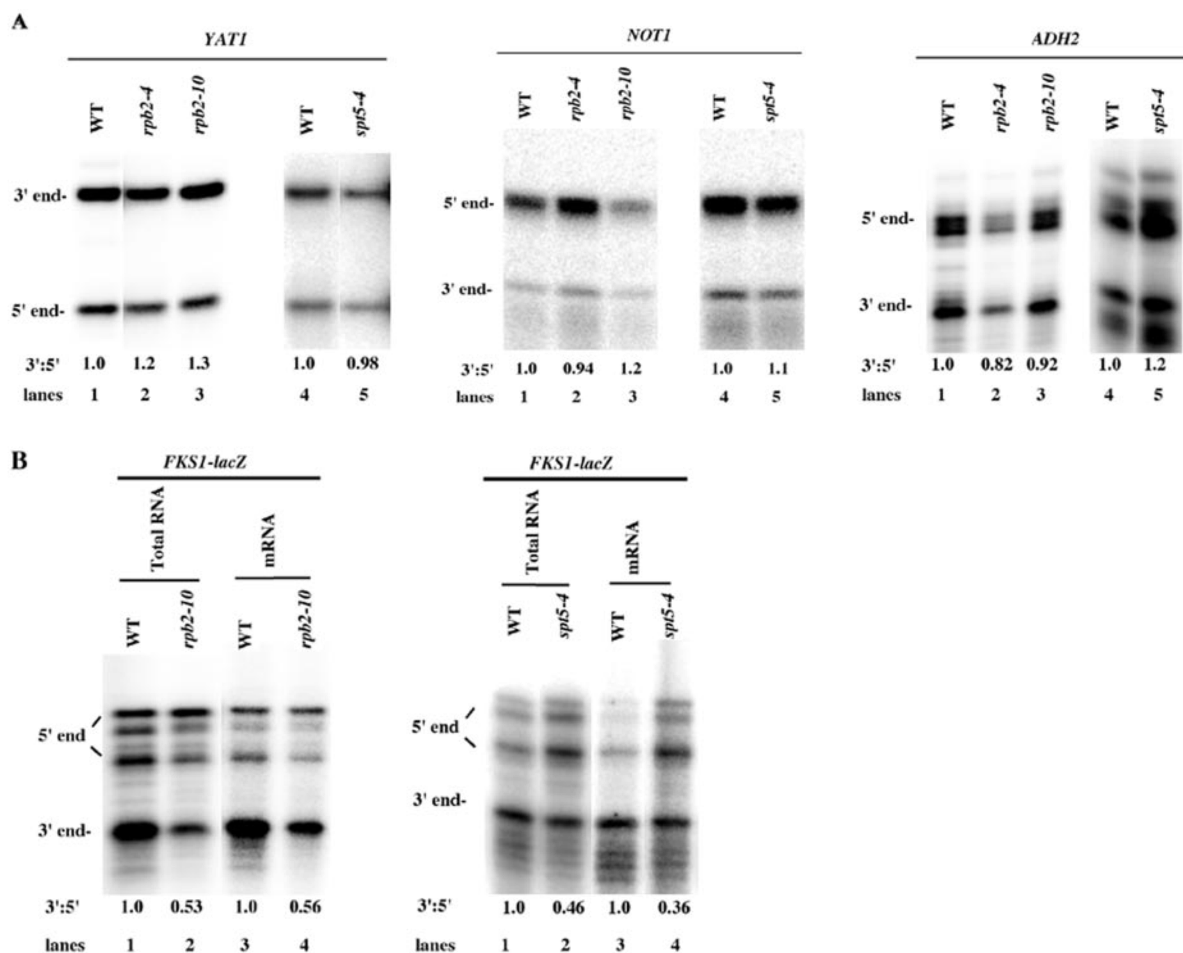
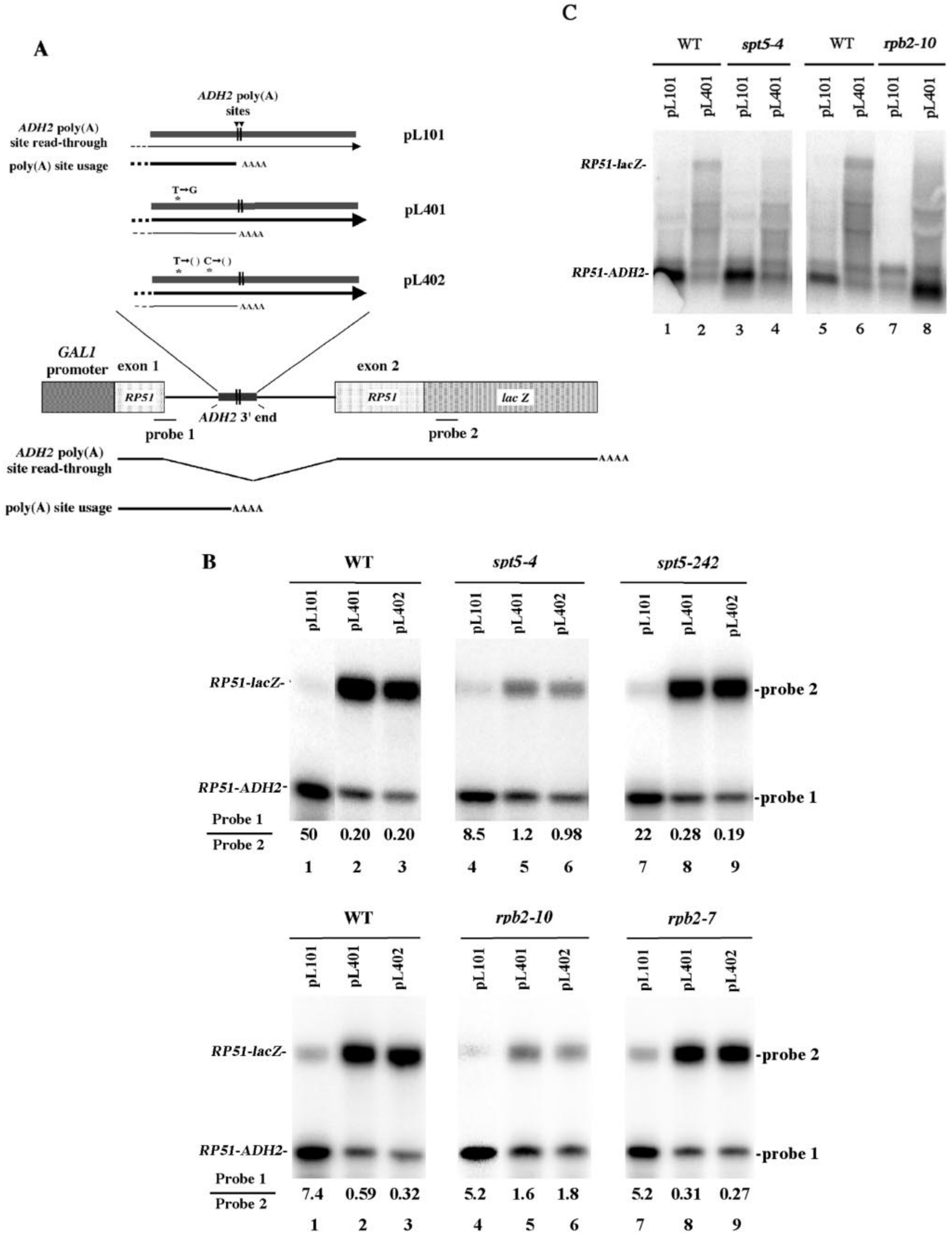


FIG. 4. *spt5* and *rpb2* alleles do not affect genes lacking internal poly(A) sites and do affect *lacZ* containing internal poly(A) sites. (A) *spt5* and *rpb2* alleles do not affect elongation through the *ADH2*, *NOT1*, and *YAT1* genes. Cells were grown on 1% yeast extract–2% Bacto Peptone medium containing 4% glucose (for *NOT1*), 2% galactose–2% raffinose (for *YAT1*), or 2% ethanol (for *ADH2*). 5′- and 3′-end RNA levels were quantitated as described for Fig. 1 by using 5′ and 3′ probes specific to *ADH2*, *NOT1*, and *YAT1*, respectively (Table 2). To quantitate *YAT1* 5′ and 3′ RNA levels, yeast strains were transformed with a *GAL1-YAT1* plasmid (9). The values represent the data as presented, but repetitions showed no significant differences. (B) *rpb2-10* and *spt5-4* alleles reduce full-length *lacZ* mRNA formation by increasing shortened *lacZ* mRNA formation. Both total RNA (left panel, lanes 1 and 2; right panel, lanes 1 and 2) and poly(A) RNA (left panel, lanes 3 and 4; right panel, lanes 3 and 4) were extracted and analyzed by using an S1 nuclease protection assay. S1 nuclease protection assays, growth conditions, and calculations were as described for Fig. 1. The values represent the data as presented. Shown are wild-type (WT) strain Z96 (left panel, lanes 1 and 3), *rpb2-10* strain Z106 (lanes 2 and 4), wild-type strain FY1642 (right panel, lanes 1 and 3), and *spt5-4* strain FY1668-uH (lanes 2 and 4).

scription, resulting in shortened RNAs lacking poly(A) tails. Northern analysis, however, was unable to detect discrete shortened RNAs, probably because of multiple diffuse ends (data not shown).

***spt5-4* and *rpb2-10* enhance utilization of defective *ADH2* poly(A) sites.** If *spt5-4* and *rpb2-10* were causing increased pausing or arrest that results in subsequent upstream poly(A) site utilization, then it would be expected that these mutations should be able to enhance the use of known defective poly(A) sites internal to genes. To test this hypothesis, we utilized the previously described *ADH2* defective poly(A) sites that have been used in a poly(A) site usage assay (26). For this assay, as shown in Fig. 5A, the 3′ end of the *ADH2* gene containing its poly(A) signaling sequences was embedded within the *RP51* intron. Usage of the *ADH2* poly(A) cleavage site promotes the formation of a short mRNA that does not include downstream *RP51* sequences that are fused to *lacZ* (26). Read-through of the poly(A) site, on the other hand, promotes splicing of the intron and synthesis through *lacZ*. We monitored these two alternative events by quantitating with an S1 nuclease protection assay the relative amount of mRNA that is expressed by using either probe 1, which overlaps the 5′ *RP51* junction of the exon and intron [thus measuring mRNA resulting from *ADH2* poly(A) site usage], or probe 2, which is at 150 bp within the *lacZ* gene [thus measuring RNA in which the poly(A) site has not been used and splicing has occurred]. As shown in Fig. 3, transcription through the first 500 bp of *lacZ* is unaffected by *spt5-4* or *rpb2-10* alleles.

Three plasmids containing this setup were used: pL101, which has a wild-type *ADH2* 3′-end sequence; pL401, which contains a T to G alteration upstream of the *ADH2* poly(A) site that blocks *ADH2* poly(A) site usage by 10-fold; and pL402, which contains two single nucleotide deletions upstream of the *ADH2* poly(A) site that also block poly(A) site usage (Fig. 5A).


A
ADH2 poly(A) sites
ADH2 poly(A) site read-through
poly(A) site usage
AAAA
pL101
T→G
pL401
T→( ) C→( )
pL402
GAL1 promoter
exon 1
RP51
ADH2 3' end
exon 2
RP51
lac Z
probe 1
probe 2
ADH2 poly(A) site read-through
poly(A) site usage
B
WT
spt5-4
spt5-242
pL101
pL401
pL402
RP51-lacZ-
RP51-ADH2-
-probe 2
-probe 1
Probe 1 / Probe 2
50 0.20 0.20 8.5 1.2 0.98 22 0.28 0.19
1 2 3 4 5 6 7 8 9
WT
rpb2-10
rpb2-7
7.4 0.59 0.32 5.2 1.6 1.8 5.2 0.31 0.27
1 2 3 4 5 6 7 8 9
C
WT
spt5-4
WT
rpb2-10
pL101
pL401
1 2 3 4 5 6 7 8

As shown in Fig. 5B (upper and lower panels, lane 1), in a wild-type strain, with pL101 the *ADH2* poly(A) site is used and very little read-through mRNA is made. With both pL401 and pL402 (lanes 2 and 3, respectively), poly(A) site usage is substantially weakened at *ADH2* and corresponding read-through into the *lacZ* gene occurs. These results are the same as those previously obtained by Hyman et al. (26).

In an *spt5-4* strain, however, the defective poly(A) sites in pL401 and pL402 display increased usage. The ratio of *ADH2* poly(A) site usage RNA (probe 1) to that of read-through RNA (probe 2) increased about five- to sixfold in both cases relative to the ratio observed in the wild type (Fig. 5B, upper panel; compare lanes 5 and 6 to lanes 2 and 3). In contrast, the *spt5-242* allele, which does not affect *lacZ* expression, did not have a corresponding effect on poly(A) site usage in pL401 and pL402 (Fig. 5B, upper panel, lanes 8 and 9). Exactly the same results were obtained for the *rpb2* alleles as were observed for *spt5*. *rpb2-10*, which reduces *lacZ* expression, increased the ratio of poly(A) site usage RNA (probe 1) to that of read-through RNA (probe 2) by about three- and fivefold relative to that for the wild-type ratio in plasmids pL401 and pL402, respectively (Fig. 5B, lower panel; compare lanes 5 and 6 to lanes 2 and 3). *rpb2-7*, which does not affect *lacZ*, also did not affect pL401 and pL402.

Northern analysis was used to confirm this increased utilization of the *ADH2* poly(A) sites in *rpb2-10* and *spt5-4* backgrounds (Fig. 5C). The *spt5-4* and *rpb2-10* alleles resulted in increased utilization of the internal *ADH2* poly(A) site for pL401 relative to that observed for the wild type (Fig. 5C; compare lanes 4 and 8 to lanes 2 and 6, respectively). As shown in Fig. 5C, lanes 4 and 8, the full-length *RP51-lacZ* RNA becomes diminished relative to that of the wild type without the formation of any smaller RNA products other than those of the *RP51-ADH2* species. These data indicate that the *spt5-4* and *rpb2-10* alleles enhance utilization of an upstream, partially defective poly(A) site.

One alternative explanation for these results with the *ADH2* poly(A) usage assay is that *spt5-4* and *rpb2-10* decrease the rate of degradation of the short *RP51-ADH2* mRNA that terminates in the intron relative to that of the read-through *RP51-lacZ* mRNA. This result is unlikely because, as observed in Fig. 5B, upper and lower panels, lane 4, *spt5-4* and *rpb2-10* do not cause a corresponding augmentation of the levels of the short mRNA relative to the those of the long mRNA. We did, however, determine the mRNA half-lives for the short and long mRNA and found that *spt5-4* had no effect on the half-life of either mRNA (Fig. 2B).

***rpb2-10* reduces full-length mRNA formation from yeast genes that contain internal poly(A) sites.** Since *rpb2-10* has been shown to increase pausing or arrest in vitro, the most likely interpretation of the above results is that increases in pausing downstream of the *ADH2* poly(A) site caused by the *rpb2-10* allele promote greater utilization of the upstream *ADH2* poly(A) sites. These results also imply that *rpb2-10* pausing will become manifest in reduced full-length gene expression of native yeast genes if partially functional internal poly(A) sites are present. In yeast, three genes, *RNA14*, *AEP2*, and *CBP1*, have been shown to contain internal inefficiently utilized poly(A) sites (46, 47). *RNA14* contains two such sites, whereas *AEP2* and *CBP1* each contain one. We therefore assayed the effects of *rpb2-10* and *rpb2-7* on expression through *RNA14*, *AEP2*, and *CBP1* (Fig. 6A through C). With 5′ and 3′ probes to the *RNA14* mRNA, *rpb2-10* reduced the level of the 3′ RNA relative to the amount of 5′ RNA (Fig. 6A, compare lanes 4 and 5). Over seven such experiments, the average drop in the 3′/5′ ratio in an *rpb2-10* mutant was 0.62 ± 0.045 (mean $\pm$ standard error of the mean [SEM]). The *rpb2-7* allele again displayed much less of an effect (Fig. 6A, lane 6), with an average decline in the 3′/5′ ratio for five determinations of 0.91 ± 0.082.

We reexamined these results using Northern analysis to verify whether one or both of the *RNA14* internal poly(A) sites were being preferentially affected. As shown in Fig. 7A, lanes 3 and 5, *rpb2-10* resulted in diminished full-length mRNA formation and augmented levels of the shortest (1.1-kb) mRNA. This result agrees with the S1 analysis shown in Fig. 6A and establishes that the *rpb2-10* allele is directly enhancing upstream poly(A) usage. It suggests further that the two internal poly(A) sites in *RNA14* might behave differently or that there are specific sequences across the *RNA14* gene that display different levels of responsiveness to *rpb2-10*. In addition, *rpb2-10* also reduced *CBP1* (Fig. 6C, lane 4) and *AEP2* (Fig. 6B, lane 4) full-length formation. As mentioned above, *rpb2-10* did not affect expression through several other genes analyzed, *ADH2*, *NOT1*, and *YAT1*, indicating that it specifically affects expression through genes containing internal poly(A) sites. However, *hpr1* had no effect on *RNA14* full-length expression (Fig. 6A, lane 8) or that of *AEP2* (Fig. 6B), confirming that *hpr1* operates by a different mechanism than *rpb2-10* in affect-

FIG. 5. *rpb2-10* and *spt5-4* enhance utilization of defective *ADH2* poly(A) sites embedded within the *RP51* gene. (A) Results of the internal *ADH2* poly(A) site assay as previously described (26). Plasmid pL101 contains the *RP51* locus fused to the *lacZ* gene in which the 3′ end of *ADH2* [containing the signals for poly(A) cleavage and adenylation] is inserted into the *RP51* intron. *ADH2* poly(A) site usage results in a short transcript ending in the intron that will be detected with probe 1 (5′-GCCTCCTTTAGTCCATATTAACATACCATTTTGTTATTGC-3′; see panel B, lane 1). Plasmids pL401 and pL402 contain the same *ADH2* sequence inserted into the intron of *RP51* except that each contains mutations in the *ADH2* poly(A) recognition sequences resulting in substantial read-through and the formation of a full-length mRNA transcript that is detectable with probe 2 (5′-GCGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCAGAA-3′; see panel B, lanes 2 and 3). (B) S1 nuclease protection analysis of the relative levels of read-through and short transcripts. Strains were pregrown in 4% glucose-containing CAA-U$^-$ medium at 30°C for 20 h and then shifted to CAA-U$^-$ medium with 2% galactose–2% raffinose at 30°C for 4 h. RNA was subjected to S1 nuclease protection assays as described for Fig. 1 using probes 1 and 2 (as described for panel A). The values represent the data as presented. Other repetitions resulted in the same trends. Note that because of differential effects of the *spt5-4* and *rpb2-10* alleles on *RP51-lacZ* gene expression from the pL401 and pL402 plasmids compared to those from pL101, only conclusions about the relative abundances of *RP51-lacZ* and *RP51-ADH2* RNA can be made. (C) Northern analysis of the effect of elongation defects on full-length *RP51-lacZ* and truncated *RP51-ADH2* RNA levels. Total RNA was isolated as described for panel B, and probe 1 and *RP51* probe 2 were used to detect RNA. The RNA band just above the *RP51-ADH2* RNA species most likely represents the native *RP51* RNA, and the two faint bands above that represent nonspecific hybridization to the 18S and 25S rRNA species.

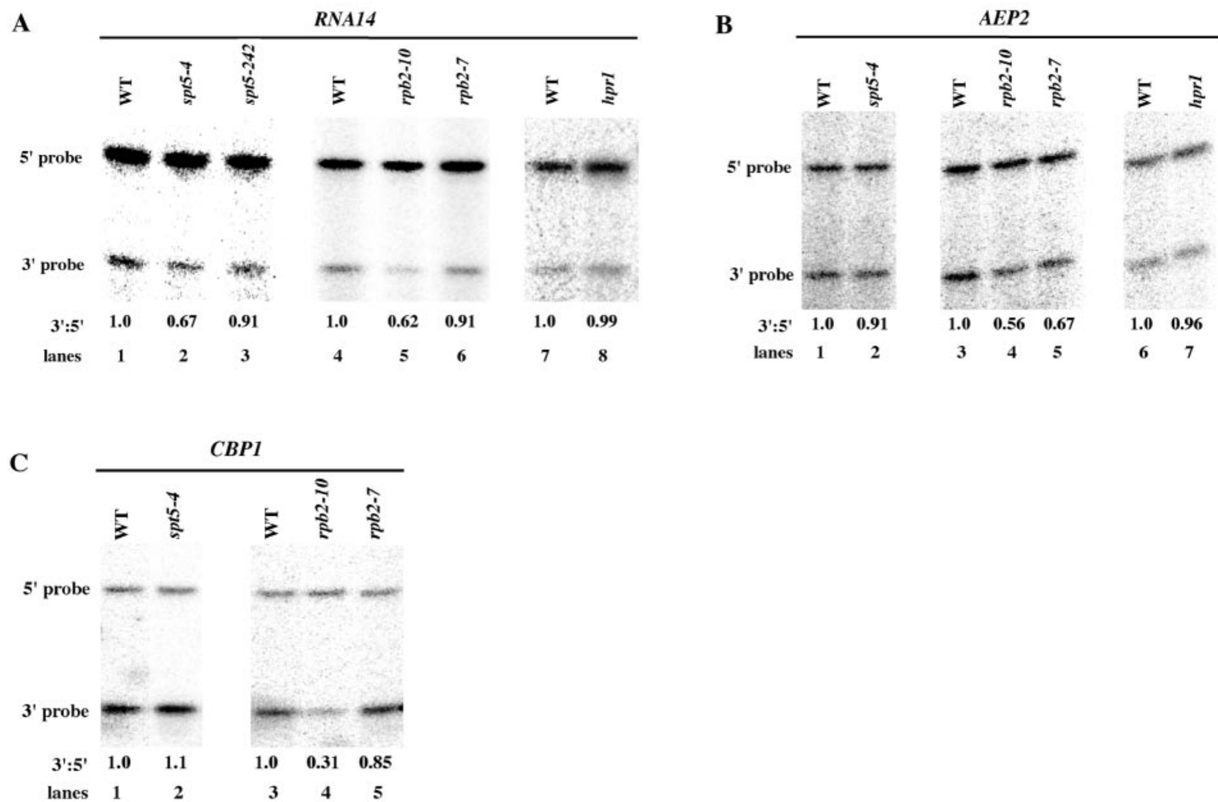


FIG. 6. *rpb2-10* and *spt5-4* reduce full-length expression of yeast genes containing internal poly(A) sites. (A through C) Cells were grown on minimal medium with 4% glucose overnight and shifted to 2% glycerol-containing minimal medium for 6 h. S1 nuclease protection assays were conducted as described for Fig. 1 using probes specific to the 5′ and 3′ ends of *RNA14*, *CBP1*, and *AEP2* (Table 2). The values represent the averages of the results for three to seven repetitions except for the values for *hpr1* and the *rpb2-7* effect on *CBP1*, which are the results for single experiments. SEMs not indicated in the text were less than 10% except that for *rpb2-10* on *CBP1*, which was 20%. (A) S1 analysis of *RNA14*. (B) S1 analysis of *AEP2*. (C) S1 analysis of *CBP1*.

ing elongation. Note that *rpb2-7* did display some enhanced internal poly(A) site utilization at *RNA14* (Fig. 7A, lane 3) and at *AEP2* (Fig. 6B, lane 5). While these effects are consistently less than those observed with *rpb2-10*, they do indicate that the *rpb2-7* allele, which confers a 6AU phenotype in vivo (41) consistent with an elongation defect, can affect elongation under certain in vivo conditions.

spt5-4 also resulted in decreased full-length formation for *RNA14* (Fig. 6A, lane 2) with an average decrease in the 3′/5′ ratio of 0.67- $\pm$ 0.046-fold for six experiments. In contrast, *spt5-242* had little apparent effect on full-length *RNA14* expression (Fig. 6A, lane 3) with an average drop in the 3′/5′ ratio of 0.91 $\pm$ 0.12 for four experiments. These results were also confirmed by Northern analysis (Fig. 7A). *spt5-4*, however, displayed no effect on *CBP1* or *AEP2* expression (Fig. 6B and C), suggesting that there is sequence specificity to its pausing, its effects are weaker than *rpb2-10*, or the multiple poly(A) sites within *RNA14* allow greater amplification of its putative pausing.

We further tested the model that *rpb2-10* enhances utilization of upstream poly(A) sites by examining the effect of deleting the internal poly(A) sites present in the *CBP1* gene on *CBP1* RNA formation. When the internal poly(A) region of the *CBP1* gene was deleted (47), only full-length *CBP1* RNA was formed in both wild-type and *rpb2-10* backgrounds (Fig. 7B, lanes 3 and 4). These data establish that the *rpb2-10* allele enhances upstream poly(A) site usage and does not just result in a blockage to elongation.

Deletion of TFIIS also reduces full-length expression of *RNA14*. The above results indicate that defects in transcriptional elongation in vivo can be visualized by monitoring the use of internal poly(A) sites. It had already been shown previously that a *dst1* deletion blocks *GAL1-lacZ* expression, presumably by affecting its elongation (28). We subsequently examined the effect of *dst1* by using the defective *ADH2* poly(A) signal inserted into the *RP51* gene. As shown in Fig. 8A (compare lanes 2 and 4), *dst1*, like *rpb2-10* and *spt5-4* (Fig. 5B), caused the formation of increased truncated *RP51-ADH2* RNA (for *dst1*, lane 4, about a twofold-more-shortened transcript relative to the read-through transcript than that found in the wild type, shown in lane 2), consistent with *dst1* augmenting internal *ADH2* poly(A) site utilization. To further test this hypothesis, we subsequently examined the effect of a TFIIS deletion on *RNA14* expression. As shown in Fig. 8B, a *dst1* deletion reduced full-length RNA expression by 1.6-fold. This result was confirmed by Northern analysis, in which the shortest transcript was increased in abundance relative to the full-length transcript by twofold (Fig. 8C). Combining a *dst1* deletion with an *rpb2-10* allele did not result, however, in any significantly worse effects than those observed with the *rpb2-10*

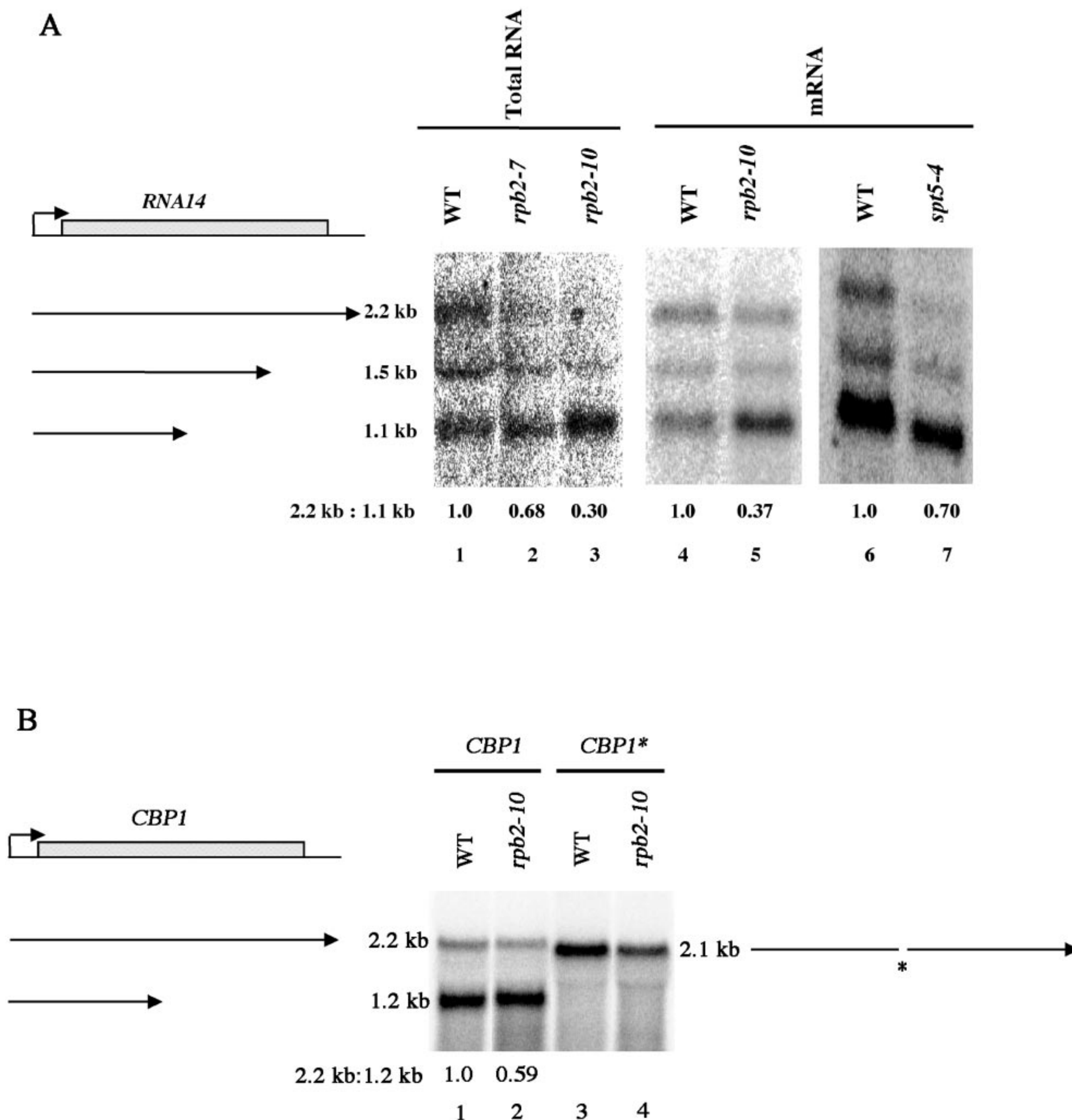


FIG. 7. (A) Northern analysis of *rpb2-10* and *spt5-4* effects on full-length formation of *RNA14* RNA. Cells were grown as described for Fig. 6A. Both total RNA (lanes 1 to 3) and poly(A) RNA (lanes 4 to 7) were extracted and subjected to Northern analysis using the 5′ probe from *RNA14*. The values represent the data as presented. Repeat experiments gave similar data. (B) Effect of deleting the *CBP1* internal poly(A) site on *rpb2-10*-enhanced usage of upstream poly(A) sites. Northern analysis of *CBP1* RNA levels was conducted as described for Fig. 6. Plasmid pG::-26 (*CBP1*) contains the *CBP1* gene under the control of the *GAL10* promoter (47); plasmid pdeltaGM (*CBP1**) is the same as pG::-26 except that the 98-bp region encompassing the internal poly(A) site of *CBP1* has been removed (47). An asterisk indicates the location of the internal poly(A) sites. The apparently reduced abundance of *CBP1** RNA in lane 4 (*rpb2-10*) compared to that in lane 3 (wild type) was not borne out by other analyses.

allele alone (Fig. 8C). These observations identify the first native gene in vivo whose elongation is impaired by deletion of TFIIS.

***rpb2-10* and *spt5-4* affect poly(A) site usage at the 3′ end of genes.** Differential poly(A) site choice at the 3′ ends of genes can be regulated in response to a variety of biological influences (20). The above effects of elongation defects on internal upstream poly(A) site utilization suggest that elongation may also play a role in regulating poly(A) site choice. To test this prediction, we analyzed poly(A) site utilization for the *GAL1* gene, which contains two poly(A) sites about 50 and 160 bp downstream of its translation stop codon (21, 35). Newly syn-

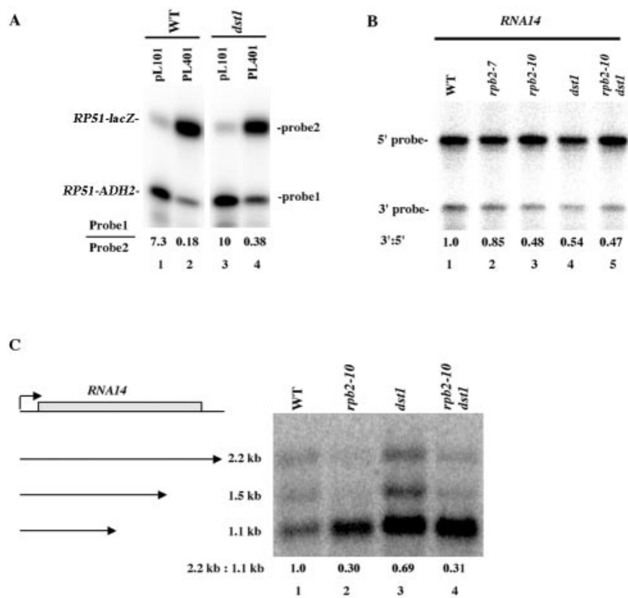


FIG. 8. *dst1* has effects on full-length RNA formation similar to those for *rpb2-10*. (A) Wild-type (WT) strain Z96 (lanes 1 and 2) and *dst1* strain DY106-u (lanes 3 and 4) were analyzed for their effects on *ADH2* poly(A) site usage as described for Fig. 5B. Plasmids pL101 and pL401 are the same as for Fig. 5. (B) *RNA14* full-length RNA expression was analyzed as described for Fig. 5A. Total RNA was extracted from strains Z96 (WT), Z103 (*rpb2-7*), Z106 (*rpb2-10*), DY106-u (*dst1*), and DY108 (*dst1 rpb2-10*). The values represent the data as presented. Repeat experiments gave similar data. (C) Northern analysis of RNA14 mRNA levels in the strain backgrounds described above was conducted as described for Fig. 7A. The values represent the data as presented. Repeat experiments gave similar data.

thesized *GAL1* polyadenylated mRNA was created by inducing *GAL1* expression for 15 min in galactose-containing medium followed by repression of *GAL1* mRNA synthesis with the addition of glucose. The 3′ ends of *GAL1* mRNA were detected by using an RNase H assay (52) and a DNA probe that was complementary to sequences present in both species (Fig. 9, top panel). Two polyadenylated species migrating at about 380 and 275 nucleotides (nt) that corresponded to poly(A) sites at about 160 bp and 50 bp, respectively, downstream of the *GAL1* stop codon were identified (Fig. 9, bottom panel). Each mRNA species contained 80 nt of poly(A), as determined by a deadenylation assay (52; data not shown). As shown in Fig. 9, lanes 1 and 4, the downstream site is preferred by about twofold over the upstream site in the wild-type strain. In a *rpb2-10* background, use of the *GAL1* upstream poly(A) site was increased by about twofold (Fig. 9, compare lanes 1 and 3), whereas the *rpb2-7* allele had no effect on *GAL1* poly(A) site utilization (Fig. 9, lane 2). Similarly, in an *spt5-4* or *spt4* strain background, the use of the upstream site is increased by 1.6-fold and twofold, respectively (Fig. 9, lanes 5 and 6). The *spt5-242* allele, which does not affect internal poly(A) site utilization (see above), had no effect on augmenting *GAL1* upstream poly(A) site usage (data not shown). *rpb2-10*, *spt5-4*, and *spt4* also did not enhance the stability of the short *GAL1* mRNA relative to that of the long *GAL1* mRNA (Table 4). These results confirm our prediction that elongation defects enhance utilization of upstream poly(A) sites and indicate that regulating the elongation process will be critical to 3′-end poly(A) site choice.

DISCUSSION

In this report, we have identified native yeast genes whose full-length mRNA formation is significantly reduced by defects in the elongation factors RPB2, SPT5,and TFIIS. These experiments represent the first demonstration in vivo of genes whose transcriptional elongation is regulated by these elongation factors, as previous studies, including microarray analyses, were unsuccessful in clearly identifying such genes (23, 45, 56). In addition, we have shown that expression through other chimeric yeast gene constructs and that of the *E. coli lacZ* gene are also affected by RPB2, SPT5,and TFIIS defects. The common feature of these genes and constructs is that they contain internal poly(A) sites. These observations should aid the study of elongation in vivo and allow the development of genetic

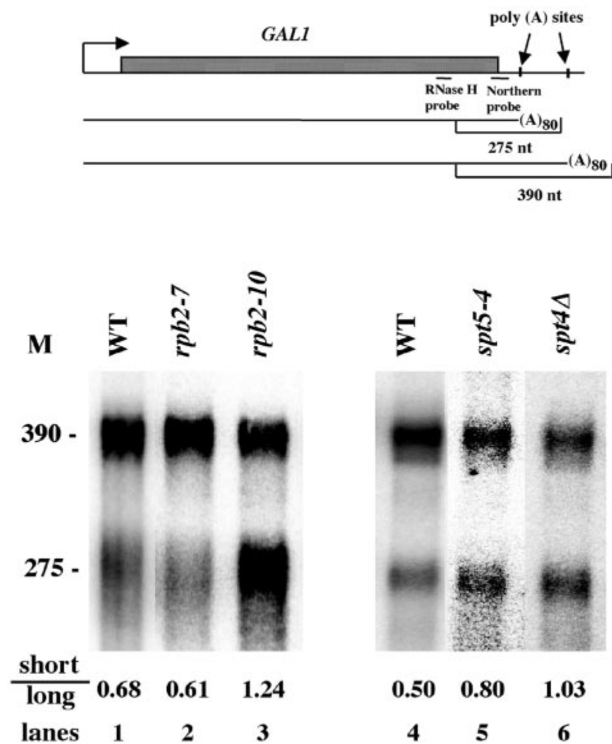


FIG. 9. *rpb2-10*, *spt5-4* and *spt4* affect 3′-end poly(A) site choice at *GAL1*. (Top panel) Diagram of *GAL1* gene RNA. The two poly(A) sites located 50 and 160 bp downstream of the stop codon are indicated, as are the RNase H probe and the Northern probe. (Bottom panel) Northern analysis of *GAL1* mRNA. Yeast were grown on galactose-containing medium for 15 min, followed by glucose addition, upon which total RNA was extracted. Newly synthesized *GAL1* polyadenylated mRNA species were detected by using an RNase H assay (52). *GAL1*-specific transcripts were identified by Northern analysis. The larger RNA species (migrating at about 390 nt) corresponds to a poly(A) cleavage site about 160 bp downstream of the *GAL1* gene translation stop codon, and the smaller RNA species (migrating at about 275 nt) corresponds to a cleavage site about 50 bp downstream of the stop codon. Each mRNA species carries a poly(A) tail measured at about 80 nt by a deadenylation assay (data not shown). The values represent the average ratios of the results for the 275-nt species to those for the 390-nt species of two to five determinations, with SEMs less than 20%.

TABLE 4. Half-lives of *GAL1* mRNA[a]

Strain	Long *GAL1* RNA	Short *GAL1* RNA
WT[b]	5.2	11
spt5-4	8.8	20
spt4	8.5	17
WT[c]	6.0	9.8
rpb2-10	9.9	12

[a] The half-lives of *GAL1* RNAs were determined after yeast was grown on galactose-containing medium for 15 min, followed by the addition of glucose and harvesting of RNA for times up to 60 min. The two *GAL1* RNA species were detected as described in the legend to Fig. 9. The SEMs were less than 15%. The value for *rpb2-10* is the result for a single determination.

[b] Strain FY1642, which is isogenic to FY1668 (*spt5-4*) and GHY180 (*spt4*), except for the indicated allele.

[c] Strain Z96, which is isogenic to Z106 (*rpb2-10*), except as indicated.

assays for the identification of novel elongation factors and new relationships among known factors.

It has previously been shown in vitro and in vivo that transcriptional pausing downstream to a poly(A) site promotes polyadenylation and cleavage of the RNA in both yeast and mammalian cells (2, 5, 40, 61). These observations are consistent with several observations for the requirement of RNA Pol II in 3′ processing (24, 34, 42). We have established in this report that defects in SPT5, SPT4, RPB2, and TFIIS, all factors involved in transcriptional elongation, affect mRNA formation in vivo of genes containing internal poly(A) sites. We postulate, therefore, that transcriptional pausing caused by defects in elongation factors enhances internal poly(A) site usage and consequently increases truncated mRNA formation relative to that of full-length mRNA.

Several pieces of evidence support our hypothesis. First, mutations in *SPT5*, *SPT4*, and *RPB2* (such as *spt5-4*, *spt4*, *rbp2-4*, and *rpb2-10* alleles) reduced full-length RNA formation in *lacZ*, a gene known to contain cryptic poly(A) sites (44). Other *SPT5* or *RPB2* mutations had no effect on full-length *lacZ* RNA formation, indicating that this effect was allele specific. Second, the truncated *lacZ* RNAs were polyadenylated. Third, these SPT5 and RPB2 defects did not affect full-length mRNA formation for genes such as *YAT1* or *NOT1* containing high G+C content or excessive length that lacked internal poly(A) sites, indicating that their effects on elongation occurred by a mechanism different from or in addition to that ascribed to HPR1 (9). Fourth, we showed that *spt5-4*, *rpb2-10*, and *dst1* alleles can enhance the usage of defective *ADH2* poly(A) sites embedded within the *RP51* gene. The other *SPT5* or *RPB2* alleles, which did not affect *lacZ* expression, also had no effect on *ADH2*-defective poly(A) site usage. Importantly, the *rpb2-10* allele, which is known to cause increased pausing in vitro and to block in vitro transcriptional elongation, resulted in enhanced internal poly(A) site usage whereas the *rpb2-7* allele, which does not block in vitro elongation, did not display the same effects. Fifth, we showed that several yeast genes with known internal poly(A) sites displayed decreased full-length mRNA expression and increased internal poly(A) site usage with *rpb2-10* and to a lesser extent with *spt5-4* and *dst1*. Importantly, deleting the internal poly(A) site for *CBP1* in an *rpb2-10* background resulted in only full-length *CBP1* mRNA being visualized. Finally, the *rpb2-10*, *spt5-4*, and *spt4* defects affected 3′-end poly(A) site choice in which the usage of the upstream poly(A) site became preferred in strains carrying these defects.

That the usage of the upstream poly(A) site is always enhanced relative to that of the downstream site with these elongation mutations suggests that RNA Pol II pausing occurs throughout the gene and that poly(A) site usage becomes favored at the first available poly(A) signal. Our results also imply that at normal genes such as *ADH2*, *NOT1*, and *YAT1*, where *rpb2-10* does not have an apparent effect on 3′-end formation, or the first 1.0 kb of *lacZ*, *rpb2-10*-induced RNA Pol II pausing probably still occurs but is not significant enough in vivo to cause reduced levels of 3′ RNA formation relative to that of 5′ RNA levels. Only when a cryptic or defective poly(A) site is present can the pausing be visualized as reduced normal 3′-end formation due to increased poly(A) site usage. This interpretation is consistent with the observation that *rpb2-10* did not appear to affect transcription in vivo even when a known in vitro arrest site was introduced into a gene (57). Relatedly, *dst1* did not affect RNA Pol II occupancy at the *lacZ* gene (28), implying that enhanced pausing caused by *dst1* or *rpb2-10* may not have noticeable effects on measurable RNA Pol II association with the gene. The known effects of *rpb2-10*, *dst1*, *spt5-4*, or *spt4* on many genes' expression, either positive or negative (50, 56), must therefore be interpreted carefully as to whether they are due to transcriptional initiation or elongation defects. The observation that these defects did not result in reductions in full-length mRNA formation for long genes such as *NOT1* or genes with a very high G+C content such as *YAT1* does not imply that *spt5*, *rpb2*, or *dst1* strains were not impaired in transcribing through these genes. For example, *YAT1* total mRNA expression was decreased in an *spt5-4* or *rpb2-10* background, but we are unable to ascertain whether this effect is at the level of transcriptional initiation or not.

Interestingly, it has been shown that nonfermentative growth conditions promote increased poly(A) site cleavage at the *RNA14* 1.1-kb site and enhanced internal poly(A) site cleavage at *CBP1* and *AEP2* (46). These effects of nonfermentative growth on *RNA14*, *CBP1*, and *AEP2* expression are similar to those observed for *rpb2-10* and suggest that they occur by a similar mechanism, that is, by causing increased RNA Pol II pausing. Several stress conditions have also been observed to enhance *SUA7* upstream poly(A) site utilization (25). Nonfermentative growth and other stress conditions may impair elongation and thereby alter poly(A) site utilization. However, the effects of the several elongation defects on internal poly(A) site usage that we observed would occur in addition to the nonfermentative growth effect since all of our experiments were conducted under glycerol growth conditions.

While *rpb2-10* can result in decreased full-length RNA formation for several yeast genes containing internal poly(A) sites, such as *RNA14*, *CBP1*, and *AEP2* (46, 47), and differential 3′-end poly(A) usage at *GAL1*, *spt5-4* clearly affected only full-length *RNA14* RNA formation and 3′-end choice of *GAL1*. This difference in behavior may be due to sequence differences between *RNA14*, *CBP1*, and *AEP2*. While *CBP1* and *AEP2* each contain one internal poly(A) site, *RNA14* contains two internal poly(A) sites, which may allow a greater amplification of the effect caused by pausing or arrest. *RNA14* and the 3′ end of *GAL1* may also contain specific sequences

that can result in more pausing. Alternatively, the *spt5-4* allele may have a weaker effect on elongation than *rpb2-10*.

It could be argued that the *spt5*, *spt4*, *rpb2*, and *dst1* alleles affect full-length RNA formation not by blocking RNA elongation but by directly enhancing the polyadenylation and cleavage process at upstream poly(A) sites. While we cannot formally exclude this possibility, several factors suggest otherwise. First, these alleles have displayed a number of effects on elongation and are known to be involved in elongation. Second, in vitro evidence has shown that *rpb2-10* causes RNA Pol II to pause, while *rpb2-7* had no effect (41). Similarly, *rpb2-10* can strongly decrease full-length *lacZ* RNA formation and enhance defective *ADH2* poly(A) site usage, whereas *rpb2-7* had no effect in our experiments. Although *rpb2-7* did reduce *RNA14*, *CBP1*, and *AEP2* full-length RNA formation to a limited extent, *rpb2-10* displayed much stronger effects. In addition, while SPT5 has been indicated to play a role in transcription initiation, elongation, and mRNA capping (22, 39, 49, 50, 54, 55, 59, 60), it has not been identified as affecting polyadenylation or cleavage directly, although through its contacts to RNA Pol II it can immunoprecipitate with polyadenylation or cleavage factors (30). Finally, known defects in poly(A) cleavage or adenylation factors that reduce poly(A) site utilization have not been shown to enhance upstream poly(A) site use as presented herein (C. Moore, personal communication). However, in mammalian systems alternations in the activity or abundance of the 64-kDa subunit of CstF polyadenylation factor can in some circumstances influence poly(A) site choice (20). It remains possible, therefore, that the *rpb2-10*, *spt5-4*, and *dst1* defects could exert indirect effects on the activities of poly(A) cleavage or adenylation factors.

Many genes with alternative poly(A) sites have been identified and characterized in mammalian cells, yeast, and several types of viruses. Differential poly(A) site choice of some genes is regulated by development stages or in different tissues in mammalian cells (20). More than 5,000 human and 1,000 mouse genes with two or more poly(A) sites have been identified by using expressed sequence tag data (3). Therefore, regulation of poly(A) site choice is an important method for regulating certain mRNA levels in different cellular environments. The results presented herein indicate that the elongation process, through effects on transcriptional pausing or arrest, may also regulate mRNA levels by affecting poly(A) site choice. Since the same mRNA with different poly(A) site ends can display vastly different deadenylation rates and mRNA stabilities, altering poly(A) site choice could greatly influence protein translation and abundance in the cell. In addition, more than 1,000 yeast genes could contain internal poly(A) sites (21). It is obvious, therefore, that the proper regulation of pausing or arrest in vivo will be extremely important in maintaining both the formation and fidelity of full-length mRNA.

Not only do our results confirm an in vivo connection between transcription elongation and polyadenylation or cleavage, but they also provide a method to examine transcriptional elongation in vivo. The enhanced utilization of internal poly(A) sites that occurs as a result of defects in elongation factors can be used to develop appropriate genetic assays for identifying novel elongation factors and elucidating their mechanisms in transcriptional elongation and for analyzing the types of DNA sites that affect transcriptional elongation in vivo.

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