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TRANSCRIPTIONAL ELONGATION DEFECTS ENHANCE UPSTREAM POLY (A) SITE UTILIZATION AND SPT5 AFFECTS mRNA DEGRADATION THROUGH ITS PHYSICAL AND FUNCTIONAL INTERACTION WITH CCR4-NOT COMPLEX

BY

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DISSERTATION

Submitted to the University of New Hampshire

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the Requirements for the Degree of

Doctor of Philosophy

in

Biochemistry

May, 2003

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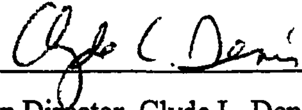

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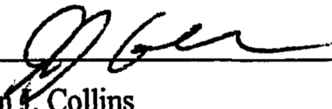

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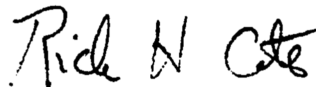

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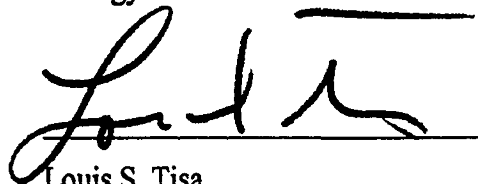

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Dedicated to

My Beloved Wife Xuelei Wang

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ABSTRACT

TRANSCRIPTIONAL ELONGATION DEFECTS ENHANCE UPSTREAM POLY (A) SITE UTILIZATION AND SPT5 AFFECTS mRNA DEGRADATION THROUGH ITS PHYSICAL AND FUNCTIONAL INTERACTION WITH CCR4-NOT COMPLEX

by

Yajun Cui

University of New Hampshire, May 2003

While a number of proteins are involved in elongational processes, the mechanism of action of most of these factors remains unclear primarily because of the lack of suitable in vivo model systems. We have identified in yeast several genes, each of which 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/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 CCR4-NOT complex has been shown to have role in transcriptional initiation, elongation, and mRNA degradation with the primary role of CCR4 and CAF1 in the deadenylation and degradation of mRNA. Previous work in our lab has identified a

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physical connection between the CCR4-NOT complex and SPT5. The role of SPT5 in mRNA degradation was examined. Mutation in *SPT5* or an *spt4* deletion slowed the rate of mRNA degradation, a phenotype associated with defects in the CCR4 and CAF1 mRNA deadenylase complex. Moreover, like *ccr4* and *caf1* deletion, *spt5* and *spt4* defects affected the rate of deadenylation, but not that of decapping or 5'-3' degradation of mRNA. Re-examination of SPT5 location in the yeast cell confirmed that SPT5 is primarily nuclear but some SPT5 was also found to locate to the cytoplasm. These results support a role for SPT5/SPT4 in mRNA degradation through inhibition of CCR4 deadenylase activity.

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GENERAL INTRODUCTION

Gene expression is a complex biochemical process that is governed by the concerted action of a diverse collection of regulatory factors. These process can be divided into synthesis of pre-mature mRNA, modification of pre-mRNA, export of mature mRNA from the nucleus to the cytoplasm, translation of mRNA into protein and, finally, degradation of mRNA. At this time, many genome sequencing projects have been completed and the number of genes for several organisms have been quantitated. *E.coli*, for instance, has more than 5000 genes; Yeast has more than 6000 genes. Drosophila has about 13,500 genes and our human cells are estimated to contain about 30,000 genes. The response of cells to physiological and environmental stimuli requires tight regulation of their gene expression, for the multicellular organism, the remarkable diversity in cell specialization is achieved by precisely controlling when and to what extent genes are expressed. In humans, many diseases result from mutations that affect various aspects of mRNA metabolism, including pre-mRNA processing, export, stability, and translational control. Understanding how gene expression is regulated is therefore very important to comprehending biological processes.

The Regulation of Gene Transcription

The first step of gene expression is the synthesis of mRNA, a process termed transcription. Most eucaryotic genes are transcribed by RNA polymerase II (RNA pol II), also known as RNA polymerase B (RPB). Yeast RNA pol II is composed of 12 subunits

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encoded by the *RPB1* to *RPB12* genes (Kolodziej et al., 1990). The two largest RNA pol II subunits, RPB1 (~200 kDa) and RPB2 (~150 kDa), are the most highly conserved subunits of this complex and encode proteins that are orthologs of bacterial RNA polymerases.

The first event of transcription is the binding of RNA pol II to the promoter region of a gene. Eucaryotic promoters include two independent regulatory DNA sequences, named core elements and regulatory elements. Core elements define the site for recruiting RNA pol II and some general transcription factors. Regulatory elements are gene-specific sequences that are located upstream of the core promoter and control the rate of transcription initiation. General transcription factors, including TBP, TFIIB, TFIIE, TFIIF, and TFIIH, are required for RNA pol II to bind to core promoter elements and initiate transcription accurately (Roeder, 1996). The first transcription factor to associate with the promoter region during transcription initiation is TBP which recognizes the TATA element in the core promoter. After TBP and its associated factors bind, TFIIB will associate with TBP to stabilize the TATA-TBP interaction and recruit RNA pol II and other general transcription factors (Orphanides et al., 1996; Roeder, 1996).

Many factors play roles in the regulation of transcription initiation. Some proteins can directly bind to the DNA regulatory elements to control the rate of transcription initiation. Other proteins regulate initiation positively or negatively by interaction either with general transcription factors (TFIIA, TBP-Associated Factors (TAFs), CCR4/NOT etc..) or with nucleosomes (SWI/SNF, Histone Acetyltransferase (HAT), SPT5/SPT4 etc.).

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Following transcription initiation, RNA pol II moves away from the promoter region and dissociates from some of the general transcription factors after synthesizing a 10 to 15 nucleotide-long transcript. This is called promoter escape. Biochemical studies have shown that during early elongation and promoter escape the transcripts less than approximately 9 nucleotide-long are unstable and RNA polymerase is prone to aborting transcription (Holstege et al., 1997; Keene and Luse, 1999). Transcription factor TFIIF can decrease the frequency of abortive transcription, and TFIIE and TFIIH can also help RNA pol II to overcome some transcription difficulties during promoter escape (Dvir et al., 1997; Kugel and Goodrich, 1998; Kumar et al., 1998; Yan et al., 1999). After RNA pol II leaves the promoter region, mRNA will continue to be synthesized along the DNA template. This step is called elongation. During transcriptional elongation, addition of the next nucleotide to a growing RNA chain by RNA pol II requires that the 3'-OH terminus of the nascent transcript be precisely aligned with the DNA template and with the catalytic site of pol II for nucleotide addition. Pausing can occur when RNA pol II undergoes modest and reversible backsliding on the DNA template. RNA pol II retains the ability to restart synthesis without the aid of additional factors. Arrest occurs with essentially irreversible backsliding of the RNA pol II. Ancillary factors are required to release RNA pol II from arrest, one of which is elongation factor IIS that enables pol II to be reactivated from transcriptional arrest by activation of nascent RNA cleavage (Wind and Reines, 2000).

Recently, a number of factors have been identified as either components of the elongating polymerase or as possible modulators of the process of elongation. For instance, DSIF and its yeast orthologs SPT4 and SPT5 possibly regulate elongation

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through interactions with hypophosphorylated RNA polymerase (Hartzog et al., 1998; Wada et al., 1998b; Wada et al., 1998c; Yamaguchi et al., 1999a). RPB1, -2, -5, -6 and –9 are components of the RNA polymerase II enzyme and make contact with the DNA that would be important to the elongation process (Cramer et al., 2000; Hemming et al., 2000; Powell and Reines, 1996). In addition, a vast amount of genetic evidence has implicated these and other factors such as the recombination important protein HPR1 (Chavez and Aguilera, 1997; Chavez et al., 2001) and components of the CCR4-NOT complex (Denis et al., 2001) in controlling elongation.

The last phase of transcription is termination/3' end formation. Upon reaching the relatively ill-defined termination signals at the end of a gene, the nascent RNA is cleaved and the poly (A) tail is added to the 3' end of RNA. The RNA transcript is released from RNA pol II and RNA pol II is dissociated from the DNA template. Sequence elements in the 3' untranslated region (3'-UTR) of pre-mRNAs specify the site at which adenosines are added (termed poly (A) site). The mammalian 3'-end formation complex selects the poly (A) sites through recognition of the conserved upstream AAUAAA sequence and a degenerate downstream element. In contrast to higher eukaryotes, the yeast *Saccharomyces cerevisiae* uses degenerate and complex signals to direct the 3'-end formation. At least three elements are needed to make up a minimal yeast mRNA 3'-end: the UA-rich efficiency element; the A-rich positioning that directs the position of the cleavage site; and the actual site of polyadenylation (Zhao et al., 1999). RNA pol II transcription termination, in contrast, is a somewhat random process occurring at sites between 200-2000 bp downstream of the poly (A) site, although it is dependent upon the presence of a functional poly (A) signal (Proudfoot, 1989). On the other hand,

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transcriptional pause sites located downstream of the poly (A) site are thought to cause a transient pause to Pol II progression and so enhance poly (A) site recognition (Aranda and Proudfoot, 1999; Birse et al., 1997; Yonaha and Proudfoot, 1999; Yonaha and Proudfoot, 2000).

The Regulation of mRNA Degradation

The stability of mRNA is also an important determinant in gene expression. Regulation of the rate of mRNA degradation is a major factor in determining the abundance of cellular mRNA. In eucaryotic cells, the decay rates of individual mRNAs vary by more than two orders of magnitude. Moreover, there are numerous examples of mRNA decay rates being regulated in response to various stimuli.

The mature mRNA bears a 5' cap structure and 3' poly (A) tail, both of which protect the mRNA from degradation. Recent work has demonstrated that at least four related, yet distinct, pathways allow polyadenylated mRNAs to be degraded in eucaryotic cells (Beelman and Parker, 1995). In most cases the degradation of the transcript begins with the shortening of the poly (A) tail at the 3' end of the mRNA. A principal mRNA-degradation pathway is initiated by deadenylation (shortening of the poly (A) tail at the 3'-end of mRNA), which is followed by decapping (removal of the 5' cap structure). The rest of mRNA is then digested 5' to 3' by exonucleases.

Many of the components required for RNA 3'-end processing have now been identified and appear to be conserved between yeast and mammals. In yeast, a poly (A)-specific ribonucleases (PAN) was identified based on its requirement for the poly (A) binding protein (PAB1) for activity (Lowell et al., 1992; Sachs and Deardorff, 1992).

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PAN is composed of at least two subunits, PAN2 and PAN3, and is the first deadenylase for which the genes encoding the enzymatic activity have been identified (Boeck et al., 1996; Brown et al., 1996). Recently, the CCR4-NOT complex has been identified as the major cytoplasmic deadenylase complex (Chen et al., 2002; Daugeron et al., 2001; Tucker et al., 2002; Tucker et al., 2001). It is suggested that PAN2/PAN3 exonuclease may play a role in initial trimming of the nuclear poly (A) tail (Brown and Sachs, 1998). After the nascent poly (A) tail of the mRNA is trimmed by the PAN2/PAN3 nuclease, it is subsequently passed to the CCR4-NOT complex for the majority of cytoplasmic deadenylation (Tucker et al., 2001).

The second step of mRNA degradation is decapping. Genetic and biochemical studies have shown that the DCP1 protein is the major mRNA decapping enzyme in yeast (Beelman et al., 1996). Several experiments suggest possible ways in which the activity of the decapping enzyme may be modulated. First, DCP1 can be posttranslationally modified, which will influence its activity. Second, the polypeptide DCP2 has been identified as a positive regulator of the decapping enzyme and suggests that a direct or indirect interaction of DCP1 with DCP2 is required for production of active decapping enzyme (Dunckley and Parker, 1999). Finally, the protein chaperone HSP70 may function as a global regulator of DCP1 activity (Zhang et al., 1999).

The requirement of deadenylation before decapping indicates that the poly (A) tail also acts as an inhibitor of decapping(Decker and Parker, 1993; Muhlrad et al., 1994; Muhlrad et al., 1995). Several observations imply that the ability of the poly (A) tail to inhibit decapping is mediated through the poly (A) binding protein (PAB1) (Caponigro and Parker, 1995; Morrissey et al., 1999; Sachs et al., 1987). One model is that the

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process of deadenylation can lead to a loss of PAB1 binding at the 3' end of the mRNA and the subsequent activation of decapping.

Cleavage of capped mRNAs by DCP1 yields the products m^7GDP and a 5'-monophosphate mRNA. Production of a transcript with a 5'-monophosphate mRNA is functionally significant resulting in the rapid degradation by the 5' to 3' exonuclease Xrn1p, which degrades mRNA following decapping.

The regulation of gene expression can also happen during pre-mRNA processing, mRNA transport from nucleus to cytoplasm, and the processes by which information is transferred from RNA to protein (translation). My project involved understanding how gene transcription and mRNA degradation are regulated, especially in the identification of factors and mechanisms utilized in the control of eucaryotic transcriptional elongation and mRNA deadenylation.

I: Investigate the Role of Elongation Factors in Transcriptional Elongation *in vivo*

Recently, in yeast and other organisms, a number of factors have been identified as either components of the elongation 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 such as RPB2, SPT5/SPT4, HPR1, and TFIIS.

During the process of elongation, RNA pol II can encounter a variety of transcriptional blocks including DNA sequences, DNA bound proteins, DNA binding drugs, and covalent modifications due to DNA damage. As a result of the RNA pol II structure being recently determined, it is clear that RPB2 may play multiple roles during

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elongation (Cramer et al., 2000). For instance, N-terminal regions of RPB1, RPB6, and C-terminal regions of RPB2 can form a clamp that can be used to bind the DNA templete near the active site during elongation. RPB1 and RPB2 can also form a funnel for substrate entry, backtracking, and elongation factor access. In vitro evidence confirms a key role for RPB2 in elongation. Mutations in the *RPB2* gene result in sensitivity to the drug 6-azauracil (6AU), which reduces intracellular NTP concentrations and thus is expected to decrease the rate of transcript elongation. *RPB2* mutations also increase transcriptional arrest at known mammalian pause sites in vitro (Powell and Reines, 1996). In particular, the mutation in region H of *RPB2* (*rpb2-10*), a domain highly conserved among all organisms that is associated with nucleotide binding (Scafe et al., 1990), has the most severe effect in elongation. Moreover, TFIIS is also required by RNA pol II to overcome the arrest in elongation that occurs at such sites in vitro (Powell and Reines, 1996) and the *rpb2-10* allele in combination with a *dst1* (encode TFIIS) deletion cause severe defects in the synthesis of most mRNA in vivo (Lennon et al., 1998), indicating that in vivo *rpb2-10* is actually causing pausing/arrest of RNA pol II during elongation that requires TFIIS to suppress and overcome.

Suppressor of Ty (*SPT*) genes were originally identified through a genetic screen for mutations in the yeast *Saccharomyces cerevisiae* that restore gene expression disrupted by the insertion of the transposon Ty (Malone et al., 1993; Swanson et al., 1991b; Winston and Carlson, 1992; Winston et al., 1984). The *SPT* genes have been identified as encoding either histones or proteins that affect chromatin function (Winston, 1992). *SPT4* and *SPT5*, as members of *SPT* class of genes, were implicated in transcriptional regulation by affecting chromatin function (Swanson and Winston, 1992).

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SPT4 and SPT5 form a complex in yeast and their higher eucaryotic orthologs are components of the DSIF complex. DSIF and SPT5/SPT4 have been found to be capable of both stimulating and repressing pol II elongation. DSIF complex is known to be important to repressing elongation in vitro and appears to do so by acting together with negative elongation factor (NELF) and positive transcription elongation factor (P-TEF) b to regulate the phosphorylation of the C-terminal domain (CTD) of the pol II largest subunit (Wada et al., 1998b; Wada et al., 1998c; Yamaguchi et al., 1999a; Yamaguchi et al., 1999c). Studies on HIV transcription have revealed the possible mechanism underlying the stimulatory activity of DSIF. Successful transcription from the HIV LTR promoter requires the viral transactivator TAT. The absence of TAT results in a strong block to elongation at around +50 nt from the transcriptional start site. The SPT5 subunit of DSIF has been identified as one of the factors that are required for Tat-dependent transcription and depleting SPT5 in a TAT dependent system promotes pausing and transcriptional termination, indicating a positive role for SPT5 in elongation (Bourgeois et al., 2002; Wu-Baer et al., 1998). Moreover, various genetic analyses have also implicated SPT5 and SPT4 in controlling elongation in vivo. In yeast *S. cerevisiae*, some *spt4* and *spt5* defects elicit a 6-azauracil (6AU) sensitive phenotype (Hartzog et al., 1998). In addition, certain *spt5* alleles can be suppressed by mutations in two of the largest subunits of pol II and by elongation factor TFIIS (Hartzog et al., 1998).

HPR1 was originally identified as playing a role in mitotic intrachromosomal excision recombination (Aguilera and Klein, 1988). Recently, HPR1 has been found to be the component of an RNA pol II holoenzyme complex involved in elongation and the component of a multi-subunit complex containing other putative elongation factors

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(Chavez and Aguilera, 1997; Chavez et al., 2000). Moreover, the absence of *HPR1* causes impairment of transcription elongation through the *lacZ* gene. The intensity of such a transcriptional impairment depends on the transcribed DNA sequence, and *HPR1* is preferentially required for transcription of either long or G+C-rich DNA sequences in *Saccharomyces cerevisiae* (Chavez and Aguilera, 1997; Chavez et al., 2001).

Although these proteins were identified as elongation factors by using genetic assays and in vitro biochemical studies, their effects on transcriptional elongation in vivo is not clear. In the yeast *Saccharomyces cerevisiae*, the principal limitation to characterizing the action of factors involved in elongation has been the lack of identification of specific genes whose expression is affected by defects in these elongation factors and the lack of the suitable in vivo elongation assay. In this thesis, I identified several bona fide yeast genes containing naturally occurring internal poly (A) sites whose elongation is impaired with *rpb2-10*, *spt5-4* and *dst1* defects. An in vivo transcriptional elongation assay was established and the effect of these elongation factors on elongation in vivo was thoroughly examined.

II: Investigate Roles of SPT5/SPT4 in mRNA Degradation And Their Relationships With The CCR4-NOT Complex

The evolutionarily conserved proteins CCR4/CAF1 belong to the CCR4-NOT complex. The CCR4-NOT complex exists in two forms, 1.9 MDa and 1.0 MDa in size (Liu et al., 1997). The smaller complex contains CCR4, CAF1, the five NOT proteins (NOT1-5), CAF40 and CAF130 (Chen et al., 2001; Liu et al., 1998). CCR4 and CAF1 bind a central section of NOT1 whereas the NOT2-5 proteins bind the C-terminal region

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of NOT1. CAF40 and CAF130, whose precise biological functions are not known, bind other areas of NOT1 (Bai et al., 1999; Chen et al., 2001). The organization of the CCR4-NOT complex, with a distinct CCR4/CAF1 module and a NOT2-NOT5 module, recapitulates the functional differentiation of these two groups of proteins. CCR4 and CAF1, as the major cytoplasmic deadenylase complex, act to control the degradation of mRNA (Chen et al., 2002; Daugeron et al., 2001; Tucker et al., 2002; Tucker et al., 2001). NOT4 protein has been found to display ubiquitin-protein ligase activity, which indicate a role for NOT4 in protein degradation (Albert et al., 2002). NOT1-NOT5 proteins physically interact with TFIID and are presumed to restrict TFIID access at certain promoters (Badarinarayana et al., 2000; Deluen et al., 2002; Lemaire and Collart, 2000). Genetic studies also indicate a role for CCR4 in transcriptional elongation (Denis et al., 2001). Moreover, whole genome cDNA microarray analysis using defects of the components of the CCR4-NOT complex showed that, while the CCR4-NOT facters particularly affected genes involved in stress response, protein degradation, pheromone response, DNA repair, and carbon metabolism as previously suggested, a number of newly identified groups of genes appear regulated by CCR4-NOT components (Cui, Shrivastav and Denis, unpublished results). Consistent with their multiple functions, the CCR4-NOT proteins have been localized to both nuclear and cytoplasmic compartments (Tucker et al., 2002; Tucker et al., 2001).

The most well understood biochemical function for the CCR4-NOT complex is its role as the cytoplasmic mRNA deadenylase. Several pieces of evidence indicate that CCR4 is the critical enzymatic component of the major cytoplasmic deadenylase. First, both *ccr4* and *caf1* deletions reduce the rate of mRNA deadenylation (Tucker et al.,

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2001). Second, purified CCR4 displayed deadenylase activity but CAF1 solely did not have this activity (Chen et al., 2002; Tucker et al., 2001). Third, overexpression of CCR4 can suppress the deadenylation defects of a *caf1* strain, but CAF1 overeexpression can not complement a *ccr4* defect (Tucker et al., 2002). Finally, mutations in the key, catalytic residues of CCR4 can abolish both its in vivo function and its in vitro deadenylase activity (Chen et al., 2002; Tucker et al., 2002). Although other components of CCR4-NOT complex do not have deadenylase activity, several observations indicate that CCR4-NOT complex as a whole functions as the cytoplasmic mRNA deadenylase. First, CCR4 and CAF1 are in the same complex with the NOT proteins (Liu et al., 1998). Second, all components of CCR4-NOT complex are present in the cytoplasm (Tucker et al., 2002; Tucker et al., 2001). Moreover, deletion of *NOTs* and *CAF1* genes can also result in deadenylation defects for certain mRNAs. While deleting the *NOTs* does not result in as severe effects as *ccr4* observed with (Tucker et al., 2002), deleting *caf1* gives very similar phenotype and mRNA degradation defects observed with *ccr4*. These results suggest that CAF1 is critical for CCR4 deadenylase activity.

Several factors could affect CCR4 deadenylase activity. First, CCR4 deadenylase activity depends on its RNA substrates. CCR4 prefers RNA with at least 2A residues at its 3' end, and reacts better with RNA substrates that are at least 17 nucleotides in length. In addition, longer RNA substrates (45 nucleotides) convert CCR4 from a slow distributive enzyme to a fast processive enzyme (Viswanathan et al., 2003). Second, poly (A) binding protein (PAB1) has also been shown to inhibit CCR4 deadenylase activity in vitro (Tucker et al., 2001).

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Recently, a physical interaction between SPT5 and CCR4-NOT complex was identified in our lab. First, we found that immunoprecipitation of SPT5 can co-immunoprecipite multiple CCR4-NOT components. Two-hybrid analysis also confirms an interaction between SPT5 and the C-terminal region of CCR4. Second, we have demonstrated in vitro that purified CAF1, CCR4-C-terminus, and NOT1 can each bind purified SPT5. Third, CCR4 and CAF1, like SPT5, are known to associate with RPB1, the largest subunit of RNA polymerase II (Chang et al., 1999). Moreover, different *spt5* alleles display either suppression or exacerbation of *ccr4* defects, suggesting that not only there is a physical interaction between SPT5 and CCR4-NOT complex, SPT5 also functionally interact with CCR4-NOT complex.

In this thesis, I examined the effect of SPT5/SPT4 on mRNA degradation and whether it performs this effect through its physical and functional interaction with the CCR4-NOT complex.

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CHAPTER I

IDENTIFICATION OF GENES WHOSE FULL-LENGTH EXPRESSION IS AFFECTED BY DEFECTS IN THE TRANSCRIPTIONAL ELONGATION FACTORS RPB2, TFIIS AND SPT5: IN VIVO EVIDENCE THAT ELONGATIONAL DEFECTS ENHANCE UPSTREAM POLY (A) SITE UTILIZATION

Abstract

While a number of proteins are involved in elongational processes, the mechanism of action of most of these factors remains unclear primarily because of the lack of suitable in vivo model systems. We have identified in yeast several genes, each of which contain internal poly (A) sites, whose full-length mRNA formation is reduced by mutations in RNA polymerase II subunit RPB2 (*rpb2-10* that slows the enzyme's elongation rate), elongation factor SPT5 (*spt5-4*), or TFIIS (*dst1*). Genes containing cryptic poly (A) sites (as found in the *E.coli lacZ* gene), internally inserted defective *ADH2* poly (A) signaling sequences, or naturally occurring internal poly (A) sites (*RNA14, CBP1,* and *AEP2*) are subject to decreased full-length mRNA formation and enhanced internal poly (A) site utilization as a result of the *rpb2-10*, *spt5-4* and *dst1* alleles. Full-length expression through yeast genes lacking internal poly (A) sites was unaffected by these alleles. Importantly, the *rpb2-7* allele, which does not cause RNA pol II arrest, had little or no effect on the in vivo full-length expression of genes containing internal poly (A) sites. *rpb2-10* and *spt5-4* also promoted the utilization of upstream poly (A) sites for genes that contain multiple 3' poly (A) signaling sequences, suggesting a role for elongation in differential poly (A) site choice. Our data suggest that defects such

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as *rpb2-10, spt5-4* or *dst1* cause increased transcriptional pausing or arrest that results in increased deposition of poly (A) cleavage/adenylation factors and hence utilization of internal or upstream poly (A) sites. Transcriptional pausing/arrest can, therefore, be amplified and visualized in vivo if a gene contains internal poly (A) sites, allowing biochemical and genetic study of the elongation process.

Introduction

The regulation of the expression of eucaryotic genes 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 (Uptain et al 1997; Conaway et al 2000). It is expected, therefore, that numerous biological controls would be in place to ensure that elongation occurs to the extent and degree it should 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 post-transcriptional processes such as mRNA capping, splicing, polyadenylation/cleavage, and transport (Calvo and Manley 2001; Strasser et al 2002; Proudfoot et al 2002). Elongation can therefore be viewed as the center through which the whole quality control of mRNA formation can be integrated (Maquat and Carmichael 2001; Orphanides and Reinberg 2002; Proudfoot et al 2002).

Recently, in yeast and other organisms, a number of factors have been identified 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

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structure being recently determined, it is clear that the RPB2 subunit of RNA pol II may play multiple roles during elongation (Cramer et al 2000). *In vitro* evidence confirms a key role for RPB2 in that RNA pol II containing either the *rpb2-10* or *rpb2-4* proteins fails to elongate well *in vitro* (Powell and Reines 1996). In particular, the *rpb2-10* protein causes arrest at known mammalian pause sites and requires elongation factor TFIIS for overcoming the arrest that occurs at such sites. Moreover, the *rpb2-10* allele in combination with a *dst1* (encoding TFIIS) deletion causes severe defects in the synthesis of most mRNA in vivo (Lennon et al 1998) and reduces the induction of a number of other genes (Wind-Rotolo and Reines, 2001). These observations suggest that in vivo *rpb2-10* is actually causing promiscuous arrest of RNA pol II that requires TFIIS to suppress and overcome.

Another important factor involved in elongation are yeast proteins SPT5/SPT4 whose higher eucaryotic orthologs are components of the DSIF complex. SPT5/4 have been found to be both activators and repressors of elongation. The DSIF complex is known to be important to repressing elongation *in vitro* and appears to do so by its interaction with the hypophosphorylated form of the RNA polymerase II CTD (Wada et al 1998a,b; Yamaguchi et al 1999a,b). Drosophila SPT5 in the control of *hsp70* is known to be recruited to the transcribing polymerase, although it also may have a role in forming the paused RNA pol II complex at *hsp70* (Andrulis et al 2000; Kaplan et al 2000). Also, depleting SPT5 in a TAT dependent system promotes pausing and transcriptional termination, indicating that SPT5 can have positive roles on elongation in addition to previously described negative roles (Bourgeois et al 2002). Various genetic analyses have implicated SPT5 and SPT4 in controlling elongation *in vivo* (Hartzog et al

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1998) and initiation (Swanson and Winston 1992). In addition, certain *spt5* alleles can be suppressed by *rpb2-10*, or by the presumed slowing of elongation (Hartzog et al 1998). The physical association of the SPT5/4 complex with RNA pol II further confirms its importance to RNA pol II function (Hartzog et al 1998).

In the yeast *Saccharomyces cerevisiae* the principal limitation to characterizing the action of factors involved in elongation has been the lack of identification of specific genes whose expression is affected by defects in these elongation factors. For instance, no particular gene whose elongation is affected by these factors has been identified (Lennon et al 1998; Shaw and Reines 2000; (Wind-Rotolo and Reines, 2001). Similarly, whole genome microarray analysis with *dst1* and *rpb9* alleles did not yield particular genes controlled at the level of elongation (Hemming et al 2000). Because many putative elongation factors also can play roles in affecting initiation (SPT5/4, PAF1 complex, TFIIE/IIF/IIH, RPB subunits, TFIIS, CCR4-NOTs) (Swanson et al 1991; Swanson and Winston 1992; Tan et al 1995; Conaway et al 2000; Shaw and Reines 2000; Collart and Struhl 1993; Denis et al 2001; Denis and Malvar 1990), it is paramount to identify genes in yeast regulated at the level of elongation.

The *lacZ* gene, albeit an *E.coli* gene, has been found to be regulated at the level of elongation when expressed in yeast (Chavez and Aguilera 1997). The *hpr1* deletion reduces transcription through *lacZ* apparently because of both its high G-C content and its extreme length (Chavez et al 2001). We have consequently examined whether other defects in elongation factors also affected expression through *lacZ*. Our results show that defects in SPT5 and RNA polymerase subunit RPB2 impair transcription through the *E. coli lacZ* gene but by a different mechanism than found for *hpr1*. We show that

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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 with *rpb2-10*, *spt5-4* and *dst1* defects. Our model is that *spt5-4*, *rpb2-10* and *dst1* alleles cause pausing or arrest during elongation, resulting in increased deposition of poly (A) cleavage/adenylation factors at these internal poly (A) sites. These results are consistent with other studies linking downstream pause sites to poly (A) site utilization (Aranda and Proudfoot, 1999; Birse et al., 1997; Yonaha and Proudfoot, 1999; Yonaha and Proudfoot, 2000) and imply that elongational pausing/arrest can be studied biochemically and genetically in vivo using genes containing internal poly (A) sites.

Material And Methods

Yeast Strains, Growth Conditions And Enzyme Assays

Yeast strains are listed in Table 1. Yeast were grown on YEP medium (1% yeast extract/2% bactopeptone), minimal medium or CAA-U$^-$ medium (Liu et al 1998) supplemented with an appropriate carbon source as indicated in the Figures. β-galactosidase activities were determined as described previously (Liu et al 1998).

RNA Analyses

Quantitative S1 nuclease protection assays were conducted as described (Collart and Struhl 1993) 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.

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Total RNA and mRNA were purified and Northern blots were conducted as described previously (Cook and Denis, 1993; Denis et al., 1983). Oligonucleotides were radiolabelled at their 5' end with T4 polynucleotide kinase as described (Chen et al., 2002).

For the analysis of the polyadenylated species of *GAL1* mRNA, *GAL1* gene expression was induced for 15 minutes by shifting yeast from raffinose-containing medium to galactose-containing medium. The newly synthesized *GAL1* mRNA were detected using an RNase H assay in which a 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 (Tucker et al., 2001). The resultant *GAL1* 3' polyadenylated species were detected by Northern analysis using a probe that was complementary to the 3' end of *GAL1* (5'-GCCCAATGCTGGTTTAGAGACGATGAT AGCATTTTCTAGCTCAGCATCAGTGATCTTAGGG-3').

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Table 1

Yeast Strains

Strain	Genotype
FY1642	*MATa his4-912δ lys2-128δ leu2Δ1 ura3-52 SPT5-FLAG*
FY1642-1a	isogenic to FY1642 except *ccr4::URA3*
FY1668-uH	*MATa his4-912δ lys2-128δ spt5-4 ura3::HIS3*
FY1668-uH	isogenic to FY1668-uH except *ccr4::URA3*
FY1635	*MATα his4-912δ lys2-128δ leu2Δ1 ura3-52 spt5-242*
L615	*MATa his4-912δ lys2-128δ ura3-52 ade2-1 trp5 can1-100 spt5-25*
FY300	*MATa his4-912δ lys2-128δ leu2Δ1 ura3-52 spt5-194*
GHY180	*MATα ura3-52 leu2Δ1 his 4-912δ lys2-128δ spt4Δ2::HIS3*
FY276-uT	*MATa leu2Δ1 ura3::TRP1 his4-9128 lys2-128δ spt5-8*
AYW3-1B1	*MATa leu2 trp1 ura3 his3*
AYW3-3D1	*MATa leu2 his3 ura3 leu2-k::ADE2-URA3-leu2-k hpr1Δ3::HIS3*
Z96	*MATa ura3-52 leu2-3,112 his3Δ200 rpbΔ297::HIS3* [*pRP214* (*LEU2 RPB2*)]
Z96-1a	isogenic to Z96 except *ccr4::URA3*
Z100	isogenic to Z96 except [*pRP2-4L* (*LEU2 rpb2-4*)]
Z100-1a	isogenic to FY1642 except *ccr4::URA3*
Z106	isogenic to Z96 except [*pRP2-10L* (*LEU2 rpb2-10*)]
Z106-1a	isogenic to FY1642 except *ccr4::URA3*
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*
EGY188	*MATa ura3 his3 trp1* $LexA_{op}$*-LEU2*
EGY188-1a	Isogenic to EGY188 except *ccr4::URA3*

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Table 2. Oligonucleotide probe:

Probe name	Probe number	Sequence	Location
ADH2 probe	5' probe	5'-GTATTACGATATAGTTAATAG TTGATAGTTGATTGTATGCTTTTT GTAGCTTGATATTCTATTTACCA AGAAGAAAC-3'	-80 ~ -4
	3' probe	5'-GGCATACTTGATAATGAAAAC TATAAATCGTAAAGACATAAG-3'	+1126 ~ +1167
NOT1 probe	5' probe	5'-GCGGCCTGTTTTCCTTTGATG TTTCTAAATTAGATGCTGTGTTCA AATCACGGGTCC-3'	+18 ~ +71
	3' probe	5'-GGCGGATTGGTCATCTTGTTC ACTGGTTGTGTATTTTTTGG-3'	+6245 ~ +6285
YAT1 probe	5' probe	5'-GGCGCTCGTCCTGCAGGGGTT CCACGCGTGCCAGGTAGCGG-3'	+51 ~ +91
	3' probe	5'-GCGGTCGATCTCCAGCAGCGA CTTTTCCATGAGCGACGCAAACC GAGCAGTCTGGCG-3'	+1876 ~ +1932
RNA14 probe	5' probe	5'-GGCTCTGCGACTTTGTCCGCA GAGGGATATAGTAAATCAGGAG TCGTAGAG-3'	+5 ~ +59
	3' probe	5'-GCGCATCGAGTAAATTTGTAT TAAAATATTGACGTTTTGG-3'	+1927 ~ +1966
CBP1 probe	5' probe	5'-CCCTGCTGCTGTGGTTGATTCG TCGCAAGGTCCTGGTAGGTACCA TTTTTATAAACCTCTCGG-3'	+32 ~ +94
	3' probe	5'-GCCGTTCATCTTAAGTAACGTT TGACAGCCGACACACCATGC-3'	+1929 ~ +1970
AEP2 probe	5' probe	5'-CGGGGATAGACAGAATGACAT ATTGTGCTATTCGGCAGTACACC AAATTCACGCAGCG-3'	+86 ~ +143
	3' probe	5'-CCCCGGTTTGAAACTCCTTAA AACATCAATTCCAAGCGGG-3'	+1401 ~ +1440
lacZ probe	5' probe	5'-GGATCCGGTCATTATTAATTTA GTGTGTGTATTTGTGTTTGCGTGT CTATAGAAGTATAGTA-3'	-49 ~ +12
	0.5 kb probe	5'-GCGCTCAGGTCAAATTCAGAC GGCAAACGACTGTCCTGGCC-3'	+487 ~ +527
	1.5 kb probe	5'-CGGGAAGGGCTGGTCTTCATC CACGCGCGCGTACATCGGGC-3'	+1502 ~ +1542
	2.3 kb probe	5'-CGCCAATGTCGTTATCCAGCG GTGCACGGGTGAACTGATCGC-3'	+2345 ~ +2386
	3' probe	5'-CCGCGTGCAGCAGATGGCGAT GGCTGGTTTCCATCAGTTGC-3'	+2898 ~ +2938

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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 the expression of the *PHO5-lacZ* or *GAL1-lacZ* gene in yeast although *hpr1* had no effect on endogenous *PHO5* or *GAL1* expression (Chavez and Aguilera 1997). These and other results led to the conclusion that HPR1 was required for efficient transcriptional elongation through the *lacZ* gene (Chavez and Aguilera 1997). We used this observation that the *lacZ* gene might contain specific sequences or structures that interfered with elongation to determine whether defects in other factors known or presumed to play roles in transcriptional elongation also failed to properly express *lacZ.* To conduct this analysis we used three *lacZ* reporter constructs each of which contained a different promoter: *ADH2-lacZ, FKS1-lacZ,* and *GAL1-lacZ* (Liu et al 1998). These promoters were chosen to represent genes that are constitutively expressed (*FKS1*), inducible (*GAL1*), and subject to derepression by non-fermentative growth (*ADH2*). We subsequently analyzed the effect of defects in transcription elongation and initiation factors with each of these three reporters. As 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 to be due to effects on the respective promoters as *spt5-4* does not reduce *GAL1* or *ADH2* expression (data not shown; also see Figure 3). *rpb2-10* is known to reduce *GAL1* steady state mRNA but does so by only 1.4-fold (Wind-Rotolo and Reines, 2001), and it had no effect on *ADH2* derepression (Figure 3). In addition, these reductions in

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lacZ expression by the *spt5-4* and *rpb2-10* alleles were not the result of increased rate of *lacZ* mRNA degradation (data not shown).

Another *rpb2-7* allele, which like *rpb2-4* and *rpb2-10* confers 6AU sensitivity (Powell and Reines 1996), 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 factors ELP1 (Otero et al 1999) or SPT16 (Orphanides et al 1999) did not affect *lacZ* expression (data not shown). We conclude that the *rpb2-10, rpb2-4, spt5-4,* and *spt4* alleles are affecting an apparently post-initiation step in the expression of the *lacZ* gene. Importantly, the rpb2-4- and rpb2-10-containing RNA pol II have been shown in vitro to display increased transcriptional arrest whereas rpb2-7-RNA pol II did not (Powell and Reines 1996).

The effect of a number of factors important to transcriptional initiation was also tested using this assay. Defects in none of the CCR4-NOT complex components (except *not3-2*) displayed consistent decreases in *lacZ* gene expression (Liu et al 1998; Chen et al 2001; data not shown). The *not3-2* effect on *lacZ* expression was found, however, to result from dramatic decreases in *lacZ*-reporter plasmid stability (data not shown). Deleting the RNA pol II holoenzyme components SRB9, -10, -11, SIN4, and GAL11 also displayed no consistent decreased effects on *lacZ* expression (data not shown). Deletion of the SAGA components ADA2 and GCN5 also were found to only affect one or another of the *lacZ* reporters but not all three, suggesting their effects were limited to control of specific promoters (data not shown; Chiang et al 1996; Berger et al 1992).

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Table 3

Effects of elongation defects on *lacZ*-reporter activities

Strain	Relative β-galactosidase activity		
	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	N.D.	290	56
spt5-4	**23**	**2.9**	**4.1**
spt5-8	65	35	56
spt5-242	N.D.	59	51
spt4	**1.5**	**4.9**	**0.50**

Table 3: 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* on 2% ethanol/2% glycerol, and *GAL1-lacZ* on 2% galactose/2% raffinose. All values represent the average of five separate transformants. Standard errors of the mean (SEMs) were less than 20%.

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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 (Collart and Struhl 1993) 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 the full-length *lacZ* mRNA. Previously, it was shown using Northern analysis that an *hpr1* deletion blocked the synthesis of *lacZ* mRNA (Chavez and Aguilèra 1997). In our assay system, *hpr1* similarly displayed an inability to form full-length *lacZ* mRNA regardless of the promoter: in an *hpr1* strain compared to its isogenic parent, six-fold less full-length *lacZ* mRNA (corresponds to 3' end bands) was observed as compared to total *lacZ* RNA (corresponds to 5' end bands) (Figure 1A; data not shown).

As shown in Figures 1B and 1C, 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 as compared to the quantity of total *lacZ* mRNA that was present (Figure 1B, two-to three-fold effects for *rpb2-4* and *rpb2-10* and in Figure 1C, three- to seven-fold for *spt5-4* and *spt4*). In contrast, *rpb2-7* (Figure 1B), *spt5-8, spt5-25* and *spt5-194* (Figure 1C) had no significant or less effect on formation of full-length *lacZ* mRNA. The same results obtained with *FKS1-lacZ* (Figure 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

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Figure 1. S1 nuclease protection analysis of the effect of HPR1, RPB2, SPT4 and SPT5 defects on full-length *lacZ* mRNA formation.
lacZ 5' and 3' end RNA levels were quantified using an S1 nuclease protection assay with probes directed against the 5' end of *lacZ* RNA and 2.9 kb downstream of the *lacZ* initiation site, respectively (see Table 2). Total RNA was extracted and the ratios of 3' to 5' RNA levels were quantified using a Phosphoimager. The values are normalized based on the ratio for wild-type and the average ratio is given below each Figure except as indicated.
(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 average of five determinations and the SEM for *hpr1* was 20%.
(B) *rpb2-4* and *rpb2-10* alleles reduce full-length *lacZ* mRNA formation. Growth conditions with *FKS1-lacZ* and calculations were conducted as described in (A). WT, strain Z96 (lane 1); *rpb2-4*, strain Z100 (lane 2); *rpb2-10*, strain Z106 (lane 3) and *rpb2-7*, strain Z103 (lane 4). The values represent the average 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 were observed for *rpb2-7* with the analysis of *ADH2-lacZ* mRNA expression.
(C) *spt5-4* and *spt4* alleles reduce full-length *lacZ* mRNA formation. Growth conditions with *FKS1-lac*Z and calculations were conducted as described in (A). WT, strain FY1642 (lane 1); *spt5-4*, strain FY1668-uH (lane 2); *spt4*, strain GHY180 (lane 3); *spt5-194*, strain FY300 (lane 4); *spt5-8*, strain FY276-uT (lane 5); and *spt5-25*, strain L615 (lane 6). The *spt5-4* value represents the average of eight determinations with an SEM of 8%. The other values represent single determination, although similar results were obtained for these defects with the analysis of *ADH2-lacZ* mRNA expression.

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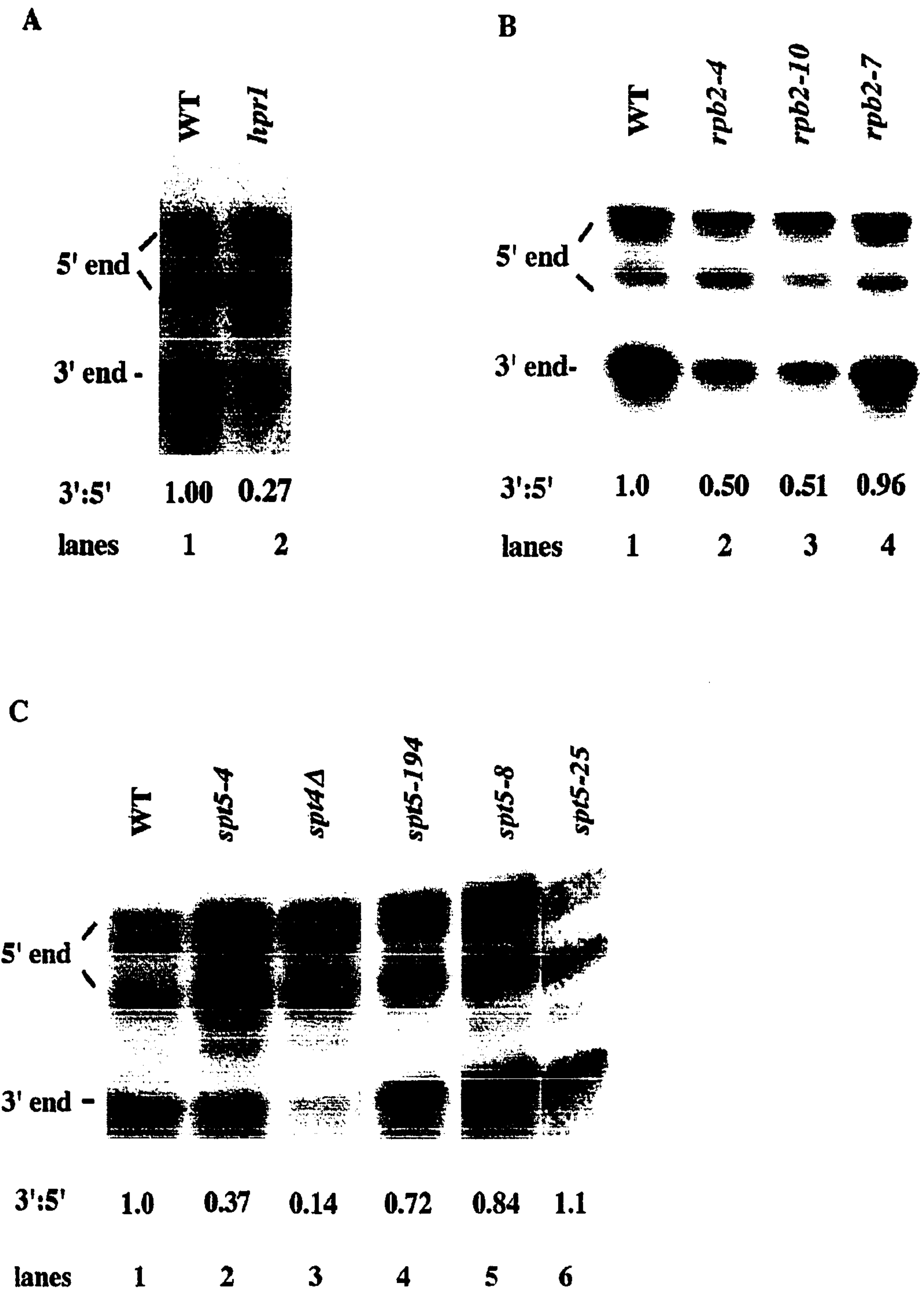
Figure 1:



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formation of full-length *lacZ* mRNA. Importantly, only the *rpb2* alleles which displayed reduced ability to transcribe through elongational blocks in vitro (Powell and Reines 1996) failed to form full-length *lacZ* mRNA in vivo.

It must be mentioned in regards to these S1 analyses that our experiments indicate in agreement with other studies (Luebke et al., 2003; Woolf et al., 1992) that oligonucleotide probes, due to differences in length and sequence, 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. Also, 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 wild-type (for example, see *spt5-4*, Figure 1C). However, as we are interested in comparing the relative ability of different elongation defects to reduce the formation of the full-length mRNA, alterations in the level of initiation of the transcript do 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 elongation through the *lacZ* gene or to increased degradation of the 3' end of the mRNA as compared 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. However, neither the *spt5-4* nor the *rpb2-10*

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allele augmented the rate of degradation of the 3' end of the *lacZ* transcript relative to the 5' end (data not shown).

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 using the S1 nuclease protection assay with probes spaced across the *lacZ* gene. In *spt5-4, rpb2-4* and *rpb2-10* mutation backgrounds, *lacZ* mRNA synthesis appeared unimpeded through the first 500 bp of the *lacZ* gene (Figure 2). However, using 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 (Figure 2).

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Figure 2

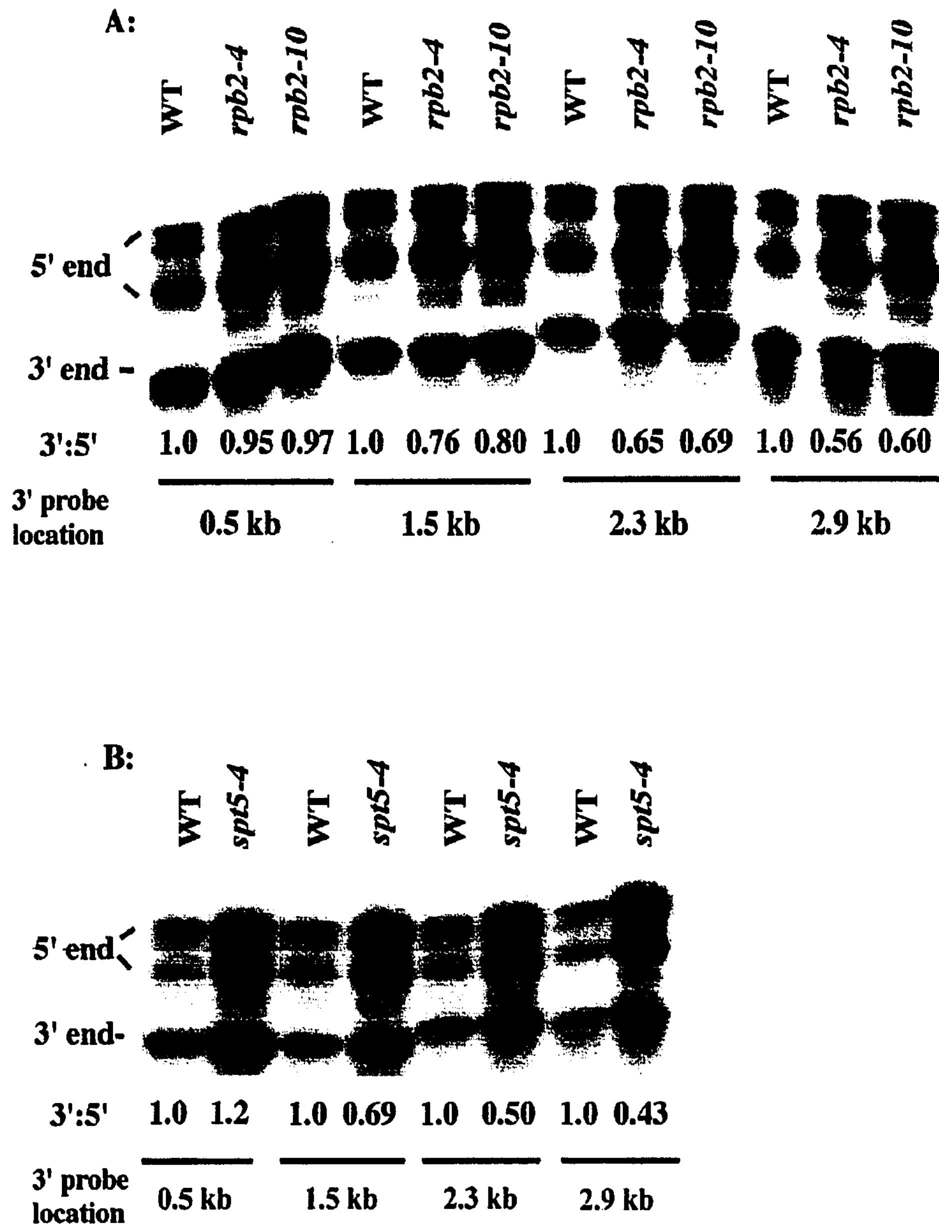

Figure 2. *spt5-4* and *rpb2* alleles affect full-length *lacZ* RNA formation at multiple sites. The 3' *lacZ* probes for conducting the S1 nuclease protection assays are listed in Table 2. In all cases the 5' probe corresponded to sequences at the initiation codon of *lacZ*. Growth conditions, assays and analyses were conducted as described in Figure 1. The *rpb2* (panel A) and *spt5-4* (panel B) strains contained *FKS1-lacZ*. The values represent the data as presented, but two to three other repeats gave the same pattern of effects and similar results were also observed when analyzing *ADH2-lacZ*.

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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 this data is that transcriptional pausing or arrest caused by the *spt5-4, spt4, rpb2-10,* and *rpb 2-4* alleles results in increased deposition of poly (A) site cleavage/adenylation factors at known cryptic poly (A) sites located within the *lacZ* gene (Russnak et al 1995). Alternative hypotheses for these effects at *lacZ* gene have been suggested for how *hpr1* affects *lacZ*; that is, the long length of *lacZ* or its high G-C content contributes to its impaired transcription (Chavez et al 2001). *spt5-4* and *rpb2-10,* however, unlike *hpr1* had no effect on transcription through the native *YAT1* gene which is extremely G-C rich (Figure 3) and which is affected by *hpr1* (Chavez et al 2001). Likewise *spt5-4* and *rpb2-10* did not affect transcription through *NOT1*, a 6.5 kb transcript (Figure 3), suggesting that it is not the length per se of *lacZ* which is impeding expression. Finally, *rpb2-4*, *rpb2-10*, and *spt5-4* did not affect full-length *ADH2* expression (Figure 3) although they all affected *ADH2-lacZ* (Table 3; data not shown).

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 that both *spt5-4* and *rpb2-10* should reduce the formation of full-length *lacZ* mRNA to the same extent they reduced full-length *lacZ* RNA formation when total RNA is analyzed. As shown in Figure 4, *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* are enhancing utilization of cryptic poly (A) sites in *lacZ*

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Figure 3

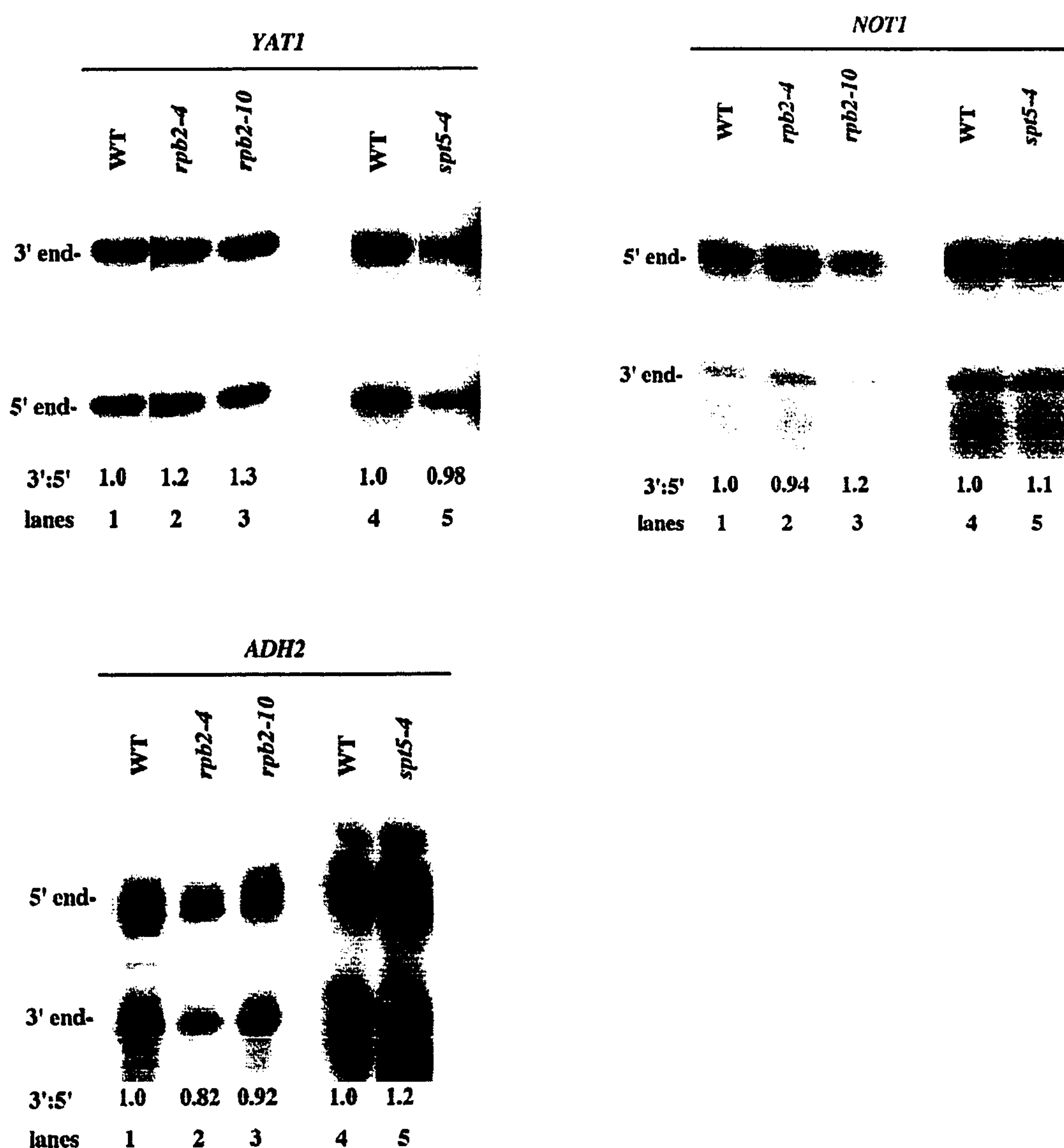

Figure 3. *spt5* and *rpb2* alleles do not affect elongation through the *ADH2*, *NOT1* and *YAT1* gene.
Cells were grown on YEP medium containing 4% glucose (for *NOT1*), 2% galactose/2% raffinose (for *YAT1*) or 2% ethanol (for *ADH2*). 5' and 3' end RNA levels were quantitated as described in Figure 1 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 (Chavez et al., 2001). The values represent the data as presented but repetitions showed no significant differences.

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Figure 4:

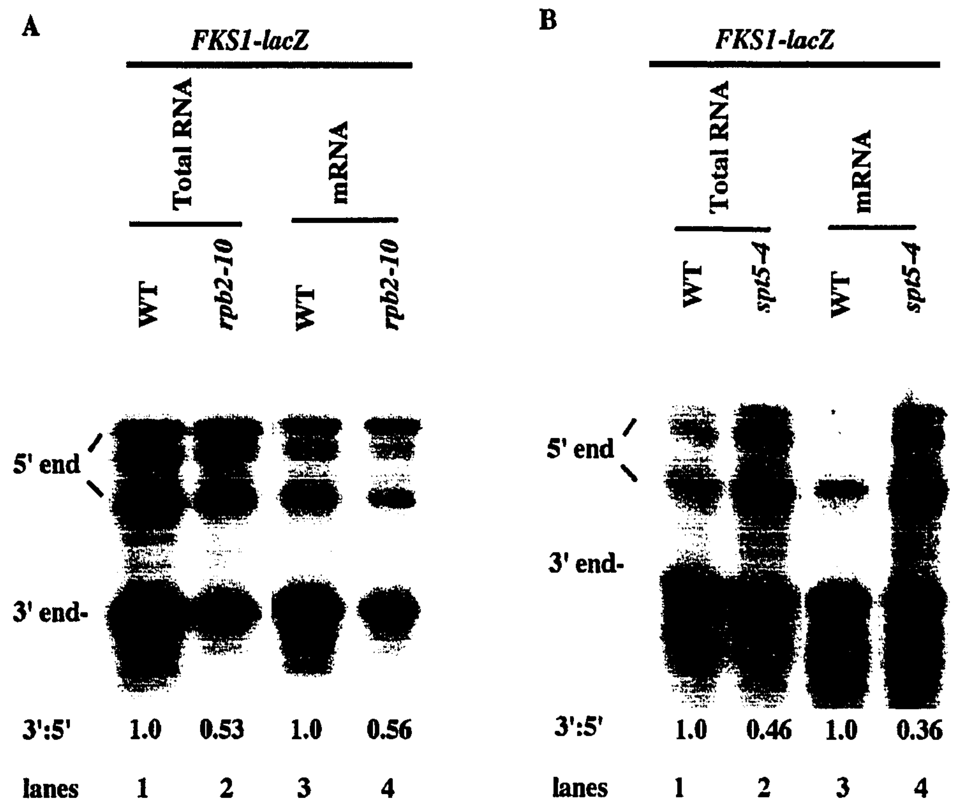

Figure 4. *rpb2-10* and *spt5-4* alleles reduce full-length *lacZ* mRNA formation by increasing partial-length *lacZ* mRNA formation.
Both total RNA (A: lanes 1 and 2; B: lanes 1 and 2) and poly (A) RNA (A: lanes 3 and 4; B: lanes 3 and 4) were extracted and analyzed using an S1 nuclease protection assay. S1 nuclease protection assays, growth conditions, and calculations were conducted as described in Figure 1. The values represent the data as presented.
(A) WT, strain Z96 (lanes 1 and 3) and *rpb2-10*, strain Z106 (lanes 2 and 4).
(B) WT, strain FY1642 (lanes 1 and 3) and *spt5-4*, strain FY1668-uH (lanes 2 and 4)

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and are not simply causing RNA pol II to cease transcription, resulting in shortened RNAs lacking poly (A) tails. Northern analysis, however, was unable to detect discrete shortened RNAs, probably because of multiple diffuse ends and more rapid turnover in the cell of the shortened RNAs (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 use of known defective poly (A) sites found 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 (Hyman et al 1991). For this assay, as shown in Figure 5A, the 3' end of the *ADH2* gene containing its poly (A) signaling sequences has been 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*. 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 using either probe 1 that overlaps the 5' *RP51* junction of the exon and intron (thus measuring mRNA resulting from *ADH2* poly (A) site usage) or probe 2 that 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 above in Figure 2, transcription through the first 500 bp of *lacZ* is unaffected by *spt5-4* or *rpb2-10* alleles.

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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 blocks poly (A) site usage (Figure 5A). As depicted in Figure 5B, in a wild-type strain, with pL101 the *ADH2* poly (A) site is used and very little read-through mRNA is made (upper and lower panel, lane 1). With both pL401 and pL402 (lanes 2 and 3, respectively) poly (A) site usage is weakened at *ADH2* and correspondingly read-through into the *lacZ* gene occurs. These are the same results as previously obtained by Hyman et al 1991.

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 to that of read-through RNA increased about five-fold in both cases relative to the ratio observed in wild-type (Figure 5B, upper panel, lanes 5 and 6 compared to lanes 2 and 3). In contrast, the *spt5-242* allele which does not affect *lacZ* expression correspondingly does not affect poly (A) site usage in pL401 and pL402 (Figure 5B, upper panel, lanes 8 and 9). The exact same results are obtained for the *rpb2* alleles as observed for *spt5*. *rpb2-10*, which reduces *lacZ* expression, increased the ratio of poly (A) site usage RNA to that of read-through RNA by about three- and five-fold relative to the wild-type ratio in plasmids pL401 and pL402, respectively (Figure 5B, lower panel, lanes 5 and 6, compared to lanes 2 and 3). *rpb2-7* which does not affect *lacZ* also does not affect pL401 and pL402. These data provide strong support for the model that the difficulties in transcribing through the *lacZ* gene arise as a result of increased cryptic poly (A) site usage.

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One alternative explanation for these results with the *ADH2* poly (A) usage assay is that *spt5-4* and *rpb2-10* are decreasing the rate of degradation of the short mRNA that terminates in the intron as compared to the read-through mRNA. This result is unlikely in that as observed in lane 4, both upper and lower panels of Figure 5B, *spt5-4* and *rpb2-10* do not correspondingly augment the levels of the short mRNA relative to the long mRNA. We did, however, quantitate 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 (data not shown).

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Figure 5. *rpb2-10* and *spt5-4* enhance utilization of defective *ADH2* poly (A) sites embedded within the *RP51* gene.

(A) The internal *ADH2* poly (A) site assay as previously described (Hyman et al 1991) is depicted. 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/adenylation) is inserted in 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'-GCCTCCTTTAGTCCATATTAACATA CCATTTTGTTATTGC-3'; see panel B, lane 1). Plasmids pL401 and pL402 contain the same *ADH2* sequence inserted in the intron of *RP51* except each contains mutations in the *ADH2* poly (A) recognition sequences resulting in substantial read-through and the formation of full-length mRNA transcript that is detectable with probe 2 (5'-GCGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGAT CGGTGCGGGCCAGAA-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 hr, and then shifted to CAA-U$^-$ medium with 2% galactose/2% raffinose at 30°C for 4 hr. RNA was subjected to S1 nuclease protection assays as described in Figure 1 using probe 1 and 2 (panel A).

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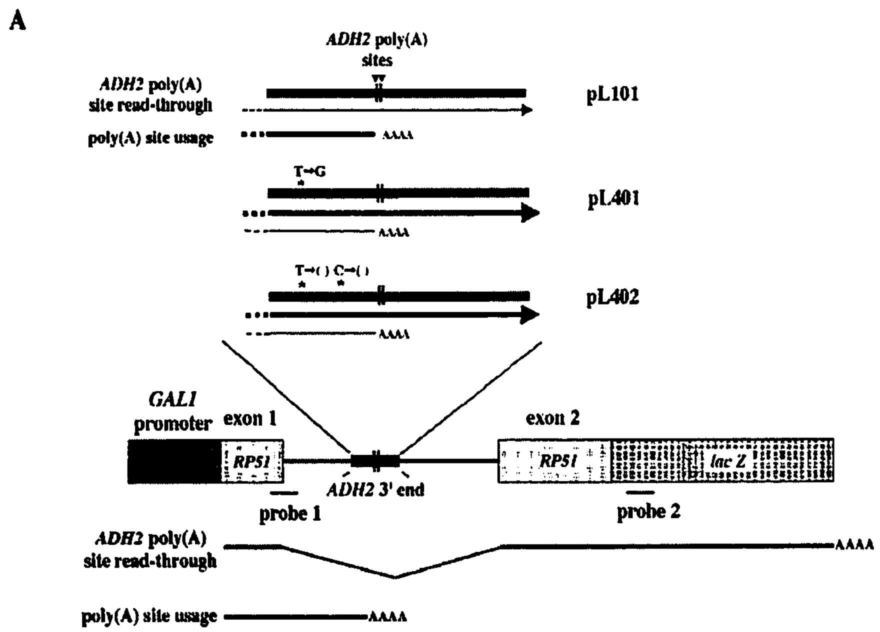
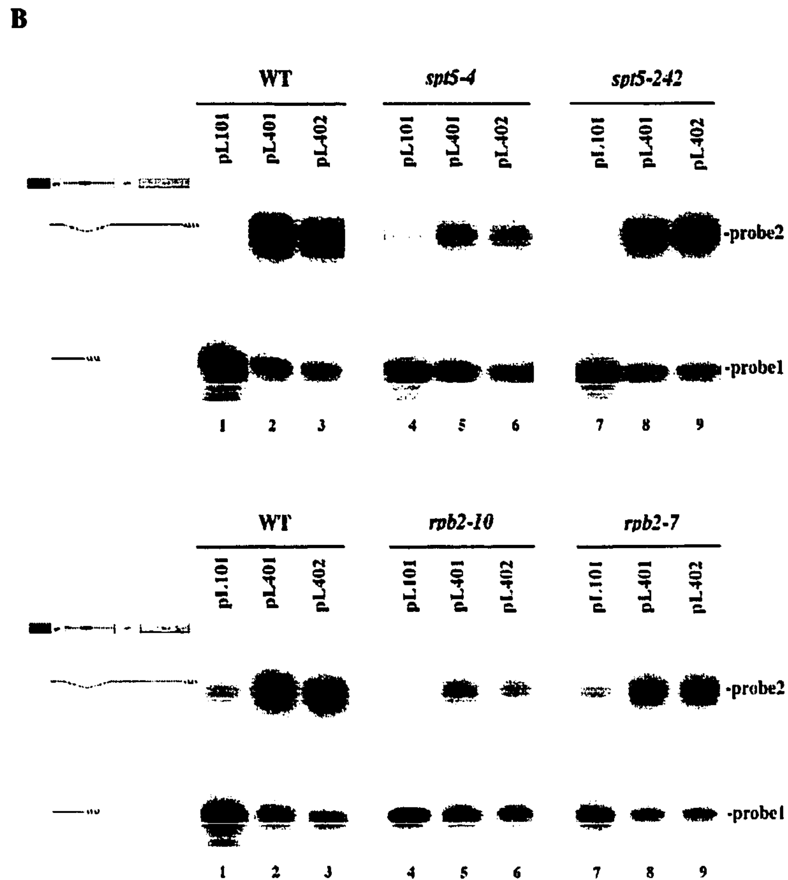
Figure 5

A



B



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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/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 promotes greater deposition of poly (A) site cleavage/adenylation factors at the *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 poly (A) sites are present internal to a gene. In yeast, three genes, *RNA14, AEP2,* and *CBP1,* have been shown to contain internal inefficiently utilized poly (A) sites (Sparks and Dieckmann 1998; Sparks et al 1997). *RNA14* contains two such sites whereas *AEP2* and *CBP1* each contain one. We, therefore, assayed the effect of *rpb2-10* and *rpb2-7* on the expression through *RNA14, AEP2*, and *CBP1* (Figure 6). Using 5' and 3' probes to the *RNA14* mRNA, *rpb2-10* reduced the level of the 3' RNA relative to the amount of 5' RNA (Figure 6, upper panel, lane 5 compared to lane 4). Over seven such experiments the average drop in the 3':5' ratio in an *rpb2-10* mutant was 0.62±0.045 (SEM). The *rpb2-7* allele again displayed much less of an effect (Figure 6, upper panel, lane 6) for 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 was being preferentially affected. As shown in Figure 7, 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 displayed in Figure 6, top panel. It suggests further that the two internal poly (A) sites in

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RNA14 might behave differently or that there are specific sequences across the *RNA14* gene that display different responsiveness to *rpb2-10*. Importantly, *rpb2-10* also reduced *CBP1* full-length formation (Figure 6, lower panel, lane 4) and that of *AEP2* (Figure 6, middle panel, lane 4). 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 affect on *RNA14* full-length expression (Figure 6, upper panel, lane 8) or that of *AEP2* (middle panel), confirming that *hpr1* operates by a different mechanism than *rpb2-10* in affecting elongation. It should be noticed that *rpb2-7* did display some enhanced internal poly (A) site utilization at *RNA14* (Figure 7, lane 3) and at *AEP2* (Figure 6, middle panel, lane 5). While these effects are consistently less than that observed with *rpb2-10*, they do indicate that the *rpb2-7* allele, which confers a 6AU phenotype in vivo (Powell and Reines, 1996), consistent with an elongational defect, can affect elongation under certain in vivo condition.

spt5-4 also resulted in decreased full-length formation for *RNA14* (Figure 6, upper panel, lane 2) with an average fold decrease in the 3':5' ratio of 0.67$\pm$ 0.046 for six experiments. In contrast, *spt5-242* had little apparent effect on full-length *RNA14* expression (Figure 6, upper panel, 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 (Figure 7). *spt5-4*, however, displayed no consistent effect on *CBP1* or *AEP2* expression (Figure 6A, lower and middle panel, respectively), suggesting that either there is sequence specificity to its pausing, its effects are weaker, or that the multiple poly (A) sites within *RNA14* allow greater amplification of its putative pausing.

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

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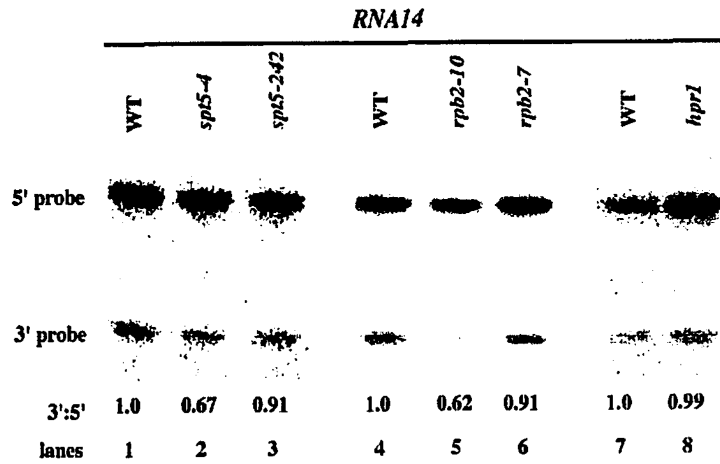
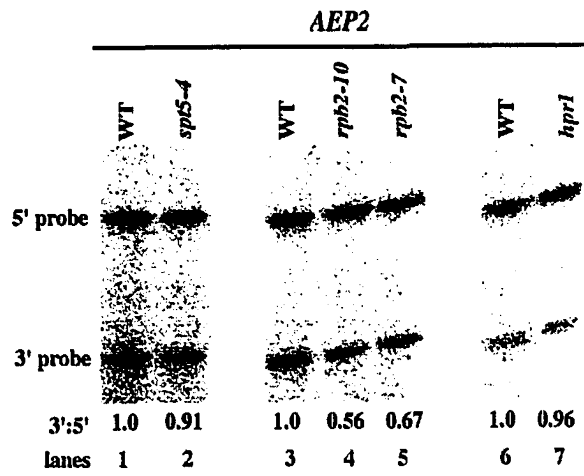
Figure 6





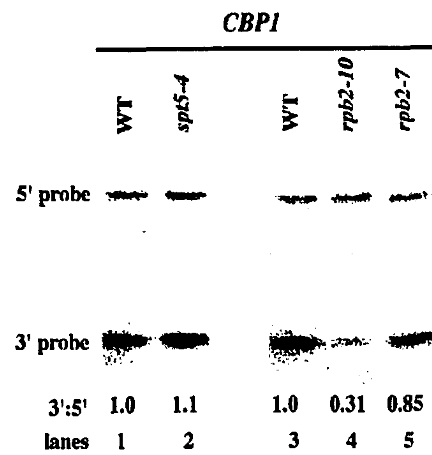


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Figure 7

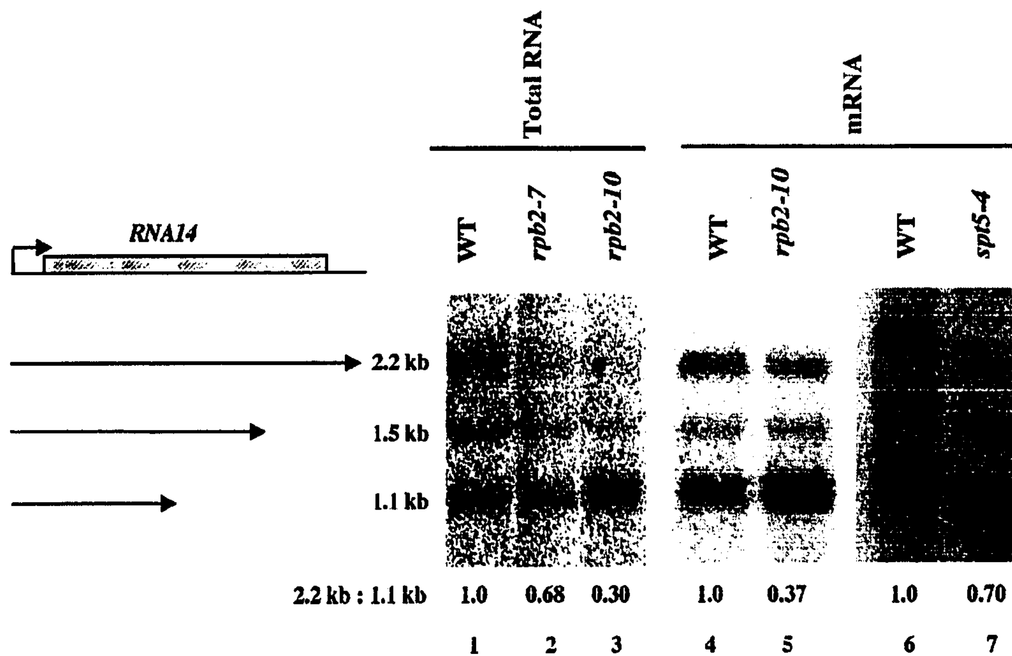

Figure 7. Northern analysis indicates that *spt5-4* and *rpb2-10* cause reduced full-length *RNA14* mRNA formation and increased internal poly (A) site utilization.
Cells were grown as described in Figure 6. Both total RNA (lanes 1 to 3) and poly (A) RNA (lanes 4 to 7) were extracted and subjected to Northern analysis using the 5' probe from *RNA14*. Values represent the data as presented. Repeat experiments gave similar data.

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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 previously shown that a *dst1* deletion blocks *GAL1-lacZ* expression (Kulish and Struhl 2001). We subsequently examined the effect of *dst1* using the defective *ADH2* poly (A) signal inserted into the *RP51* gene. As shown in Figure 8A, lane 4 compared to lane 2, *dst1*, like *rpb2-10* and *spt5-4* (Figure 5B), caused the formation of increased truncated *RP51* RNA (for *dst1* lane 4, about 2.0-fold more shortened transcript relative to read-through transcript as compared to that found in wild-type, 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 Figure 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 two-fold (Figure 8C). Combining a *dst1* deletion with an *rpb2-10* allele did not result, however, in any significantly worse effects than observed with just the *rpb2-10* allele alone (Figure 8C). These observations identify the first native gene in vivo whose elongation is impaired by deletion of TFIIS and confirm the use of the poly (A) amplification elongation assay.

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Figure 8

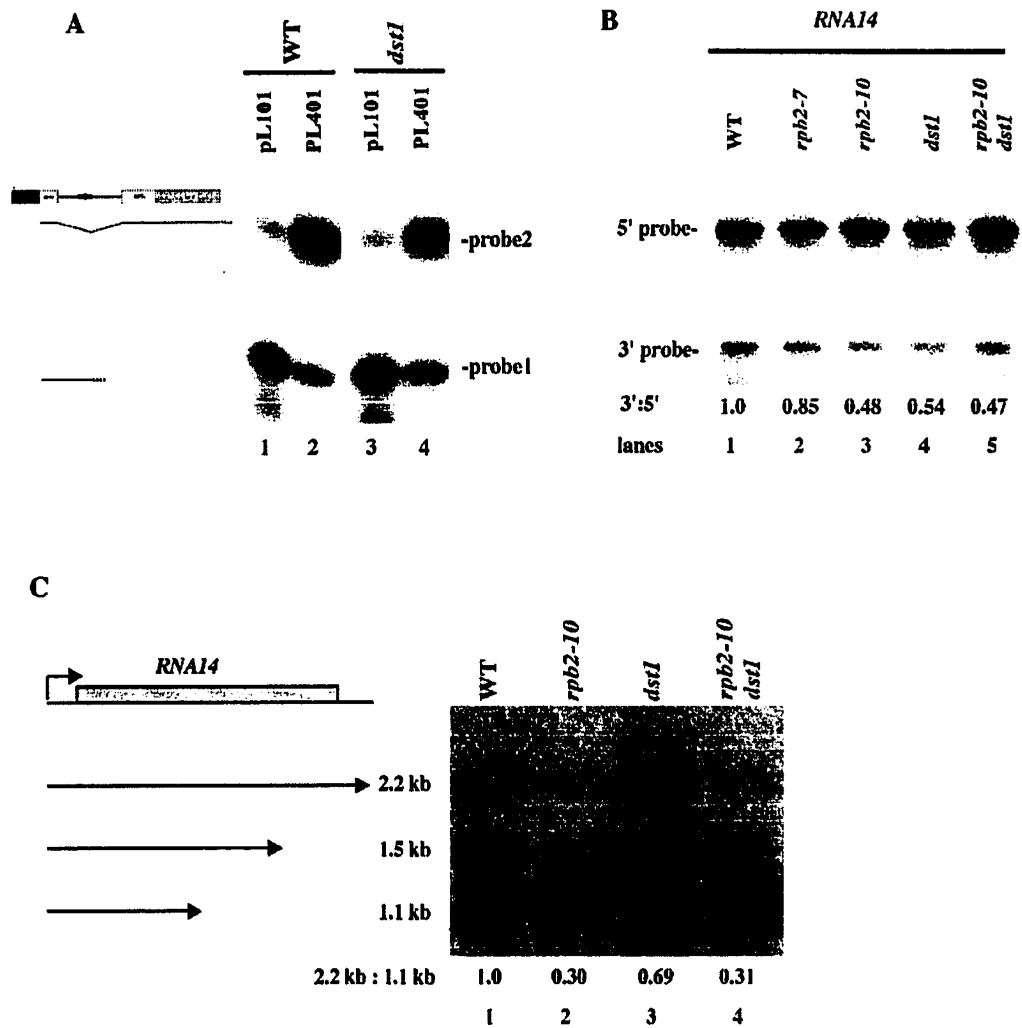

Figure 8. *dst1* has similar effects on full-length RNA formation as *rpb2-10.*
(A) WT, strain Z96 (lanes 1 and 2) and *dst1*, strain DY106-u (lanes 3 and 4) were analyzed for their effects on *ADH2* poly (A) site usage as described in Figure 5B. Plasmids pL101 and pL401 are as in Figure 5.
(B) *RNA14* full-length RNA expression was analyzed as described in Figure 6. Total RNA was extracted from strains Z96 (WT), Z103 (*rpb2-7*), Z106 (*rpb2-10*), DY106-u (*dst1*) and DY108 (*dst1 rpb2-10*). Values represent the data as presented. Repeat experiments gave similar data.
(C) Northern analysis of RNA14 mRNA levels in strain backgrounds described above was conducted as described in Figure 7. Values represent the data as presented. Repeat experiments gave similar data.

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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 (Edwalds-Gilbert et al., 1997). 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 (Graber et al., 2002; Miyajima et al., 1984, see Figure 9, top panel). Newly synthesized *GAL1* polyadenylated mRNA were created by inducing *GAL1* expression for 15 minutes in galactose-containing medium followed by repression of *GAL1* mRNA synthesis with the addition of glucose. The 3' ends of *GAL1* mRNA were detected using an RNase H assay (Tucker et al., 2001) and a DNA probe that was complementary to sequences present in both species (Figure 9, top panel). Two polyadenylated species migrating at about 380 and 275 nt were identified (Figure 9, bottom panel) that corresponded to poly (A) sites at about 160 bp and 50 bp, respectively, downstream of the *GAL1* stop codon. Each mRNA species contained 80 nt of poly (A) as determined by a deadenylation assay (data not shown; Tucker et al. 2001). As shown in Figure 9, lanes 1 and 4, the downstream site is preferred by about two-fold over the upstream site in the wild-type strain. In a *rpb2-10* background, the use of the *GAL1* upstream poly (A) site was increased by about 2-fold (Figure 9, lane 3, *rpb2-10*, compared to lane 1, wild-type), whereas the *rpb2-7* allele had no effect on *GAL1* poly (A) site utilization (Figure 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 2.0-fold, respectively (Figure 9, lanes 5 and 6). The *spt5-242* allele which does not affect internal

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Figure 9

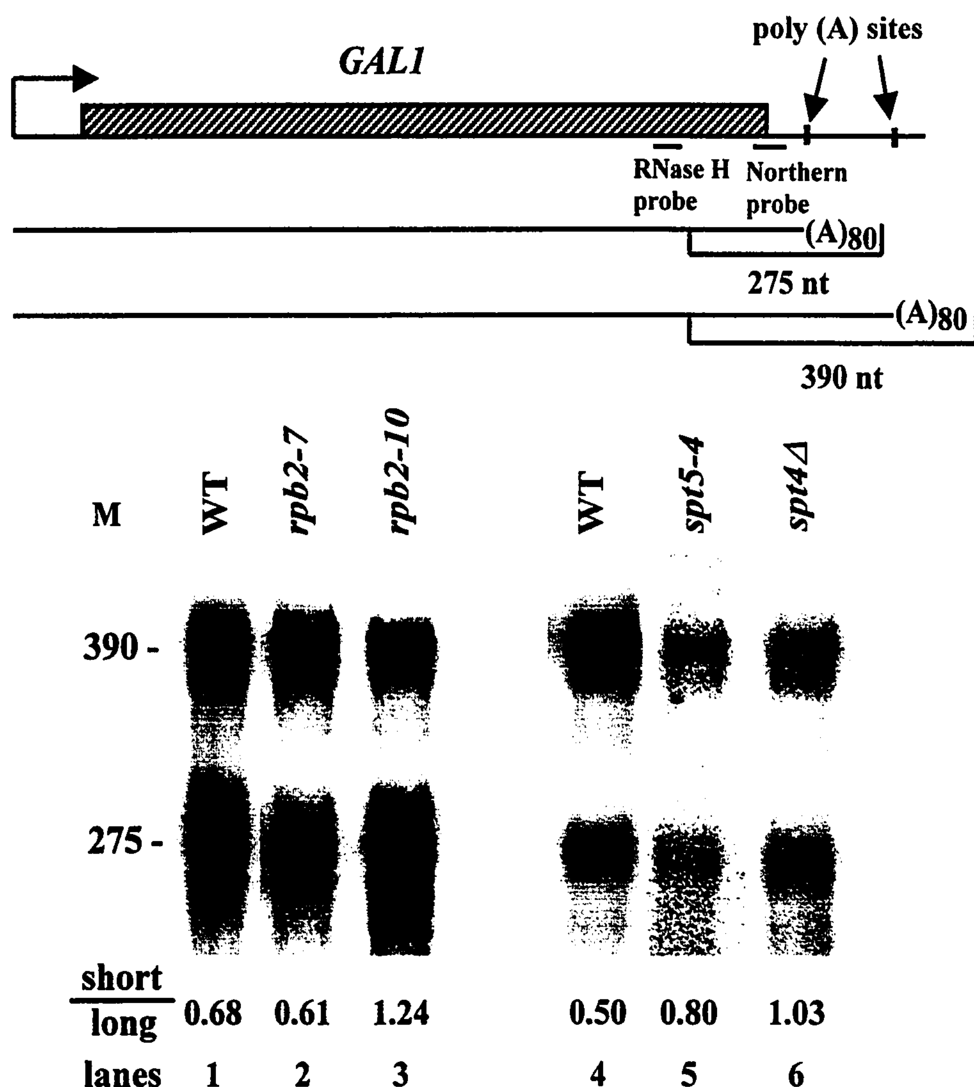

Figure 9. *rpb2-10*, *spt5-4* and *spt4* affect 3' end poly (A) site choice at *GAL1*.
Top panel - Diagram of *GAL1* gene RNA. The two poly (A) sites located 50 bp and 160 bp downstream of the stop codon are indicated as are the RNase H probe and the Northern probe. Bottom panel – Northern analysis of *GAL1* mRNA.
Yeast were grown on galactose-containing medium for 15 minutes followed by glucose addition upon which total RNA was extracted. Newly synthesized *GAL1* polyadenylated mRNA species were detected using an RNase H assay (Tucker et al., 2001). *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 (migrating at about 275 nt) 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 ratio of the 275 nt species to that of the 390 nt species for two to five determinations with SEMs less than 20%.

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poly (A) site utilization (see above) had no effect on augmenting the *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 the that of the long *GAL1* mRNA (data not shown). 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. The experiments represent the first demonstration in vivo of genes whose transcriptional elongation is regulated by these factors as previous studies, including microarray analyses, were unsuccessful in clearly identifying such genes (Hemming et al., 2000; Shaw and Reines, 2000; Wind-Rotolo and Reines, 2001). 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 their containing internal poly (A) sites. These observations should aid the study of elongation in vivo and allow the development of genetic assays for the identification of novel elongation factors and new relationships among known factors.

It has been shown previously in vitro and in vivo that transcriptional pausing downstream to a poly (A) site promotes polyadenylation/cleavage of the RNA both in yeast and mammalian cells (Birse et al. 1997; Aranda and Proudfoot 1999; Yonaha and

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Proudfoot 1999). These observations are consistent with several observations for the requirement of RNA polymerase II in 3' processing (Hirose and Manley 1998; McCracken et al 1997(Proudfoot et al., 2002). We have established in this report that defects in the SPT5, SPT4, RPB2 and TFIIS, all factors involved in transcriptional elongation, affect the mRNA formation *in vivo* of genes containing internal poly (A) sites. We postulate, therefore, that transcriptional pausing caused by defects in elongation factors enhances the internal poly (A) site usage and consequently increases truncated mRNA formation relative to 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 *lacZ* RNA formation, a gene known to contain cryptic poly (A) sites (Russnak et al., 1995). 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* RNA were polyadenylated. Third, these SPT5 and RPB2 defects did not affect the full-length mRNA formation for genes, such as *YAT1* or *NOT1*, containing high G-C content or excessive length which 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 (Chavez et al 2001). 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. 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) sites usage whereas the *rpb2-7* allele which does not block in vitro

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elongation did not display the same effects. Fifth, we showed that full-length RNA formation of several yeast genes with known internal poly (A) sites displayed decreased full-length mRNA expression with *rpb2-10* and to a lesser extent with *spt5-4* and *dst1*. 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 is occurring throughout the gene and that deposition of poly (A) site cleavage factors becomes favored at the first available poly (A) signal. Our results also imply that at normal genes where *rpb2-10* is not having an apparent effect on 3' end formation, such as *ADH2, NOT1, YAT1*, or the first 1.0 kb of *lacZ*, *rpb2-10* induced RNA pol II pausing is probably still occurring 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 "amplified" and 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 is introduced into a gene (Wind-Rotolo and Reines, 2000). Relatedly, *dst1* did not affect RNA pol II occupancy at the *lacZ* gene (Kulish and Struhl, 2001), 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 positively or negatively (Swanson and Winston, 1992; Wind-Rotolo and Reines, 2001) must therefore be interpreted carefully as to whether they are due to transcriptional initiation or elongation

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defects. That is, although we did not observe with these defects reductions in full-length mRNA formation for long genes, such as *NOT1*, or genes with a very high G-C content, such as *YAT1*, this 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 non-fermentative 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* (Sparks and Dieckmann 1998). These effects of non-fermentative growth on *RNA14*, *CBP1*, and *AEP2* expression are similar to that 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 (Hoopes et al., 2000). Non-fermentative growth and other stress conditions may be impairing elongation and thereby altering 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 non-fermentative 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* (Sparks and Dieckmann 1998; Sparks et al 1997) and differential 3' end poly (A) usage at *GAL1*, *spt5-4* only clearly affected 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* contain one internal poly (A) site, *RNA14*

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contains two internal poly(A) sites, which may allow a greater amplification of the effect caused by pausing/arrest. *RNA14* and the 3' end of *GAL1* may also contain specific sequences that can result in more pausing. Alternatively *spt5-4* allele may have a weaker effect on elongation as compared to *rpb2-10.*

It could be argued that the *spt5, spt4, rpb2* and *dst1* alleles are affecting full-length RNA formation, not by blocking RNA elongation, but by directly enhancing the polyadenylation/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 (Powell and Reines 1996). 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* again displayed much stronger effects. In addition, while SPT5 has been indicated to play a role in transcription initiation, elongation, and mRNA capping (Swanson and Winston 1992, Swanson et al 1991, Wada et al 1998, Hartzog et al 1998; Yamaguchi et al 1999, Pei and Shuman 2002), it has not been identified as affecting polyadenylation/cleavage directly. Finally, known defects in poly (A) cleavage/adenylation factors reduce poly (A) site utilization and have not been shown to enhance upstream poly (A) site use as presented herein. 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 (Edwalds-Gilbert et al., 1997). It remains possible therefore that the *rpb2-10*, *spt5-*

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4, and *dst1* defects could exert indirect effects on the activities of poly (A) cleavage/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 (see review by Edwalds-Gilbert et al. 1997). Using EST data, more than 5000 human and 1000 mouse genes have been identified with two or more poly (A) sites (Beaudoing and Gautheret 2001). Therefore, regulation of poly (A) site choice is an important method for regulating 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 1000 yeast genes could contain internal poly (A) sites (Graber et al 2002). It is obvious, therefore, that the proper regulation of pausing/arrest in vivo will be extremely important to maintaining both formation and fidelity of full-length mRNA.

Not only do our results confirm an in vivo connection between transcription elongation and polyadenylation/cleavage, but also they provide a method to examine transcriptional elongation in vivo. The amplified 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, elucidating their mechanisms in

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transcriptional elongation, and for analyzing the types of DNA sites that affect transcriptional elongation in vivo.

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CHAPTER II

SPT5 AFFECTS RATE OF mRNA DEGRADATION THROUGH PHYSICAL INTERACTION WITH AND INHIBITION OF CCR4 DEADENYLASE ACTIVITY

Introduction

The levels of eucaryotic mRNA can be regulated at many steps, from its initial synthesis to its eventual degradation in the cytoplasm. Regulation of the rate of mRNA decay is an important control point in determining the abundance of cellular mRNA. The mature mRNA bears a 5' cap structure and 3' poly (A) tail that protect the mRNA from degradation. A principal mRNA-decay pathway in eucaryotic cells is initiated by shortening of the poly (A) tail at the 3' end (deadenylation) which is then followed by removal of the 5' cap structure (decapping) and subsequent 5' to 3' exonuclease digestion (Decker and Parker, 1993; Hsu and Stevens, 1993; Muhlrad et al., 1994; Muhlrad et al., 1995; Muhlrad and Parker, 1992).

Deadenylation is a key rate-limiting step in the decay of mRNA in that it is the initial step for mRNA degradation and the mRNA deadenylation rate can directly affect the overall mRNA turnover rate (Cao and Parker, 2001; Couttet et al., 1997; Decker and Parker, 1993; Muhlrad et al., 1994; Muhlrad et al., 1995). It has also been shown that the poly (A) tail is capable of stimulating the translation of mRNA (Sachs et al., 1997; Wells et al., 1998) and inhibiting decapping in an eIF4E-independent manner (Wilusz et al., 2001). Therefore, understanding the mechanism by which mRNA deadenylation occurs

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and its regulation is very important for our understanding of the regulation of mRNA and protein levels.

In yeast, the evolutionarily conserved CCR4 protein, as part of the CCR4-NOT complex, has been identified as the major cytoplasmic deadenylase (Chen et al., 2002; Tucker et al., 2002; Tucker et al., 2001). While PAN2/PAN3, as poly (A) nuclease processes the poly (A) tails of mRNA in the nucleus (Brown and Sachs, 1998; Brown et al., 1996), CCR4 performs its deadenylase function in the cytoplasm (Tucker et al., 2001). Biochemical and bioinformatic studies have shown that CCR4 protein contains three major functional domains: an N-terminal activation domain that may interact with the transcriptional machinery, a central leucine-rich repeat (LRR) domain that binds CAF1 and several other putative components of the 1.9 MDa CCR4–NOT complex, and a C-terminal exonuclease III like domain that comprises the apparent CCR4 deadenylase function (Chen et al., 2002; Draper et al., 1994; Draper et al., 1995; Liu et al., 2001; Liu et al., 1997; Tucker et al., 2002).

The CCR4-NOT complex acts at several junctures in the formation of mRNA. Not only can the CCR4-NOT proteins, as the cytoplasmic deadenylase complex, act to control the degradation of mRNA (Chen et al., 2002; Daugeron et al., 2001; Tucker et al., 2002; Tucker et al., 2001), these factors also function in repressing the initiation of transcription through TFIID contacts (Badarinarayana et al., 2000; Deluen et al., 2002; Lemaire and Collart, 2000), in transcriptional elongation (Denis et al., 2001), and in the activation of transcription (Denis, 1984; Liu et al., 1998; Sakai et al., 1992). Consistent with their multiple functions, the CCR4-NOT proteins have been localized to both nuclear and cytoplasmic compartments (Tucker et al., 2002; Tucker et al., 2001).

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Therefore, an important issue is how the regulation of each of these functions occurs at the appropriate location and time.

In this study, we identified a physical and functional connection between the CCR4-NOT complex and SPT5. SPT5 forms a complex with the SPT4 protein (Hartzog et al., 1998), called DSIF in mammalian cells (Wada et al., 1998a). SPT5/SPT4, like CCR4-NOT complex, is conserved throughout eukaryotes and has been found to be involved in multiple steps of mRNA processing. SPT5/SPT4 works in conjunction with both the positive transcription elongation factor b (P-TEFb) and RNA pol II to control transctiptional elongation (Hartzog et al., 1998; Kim et al., 1999; Yamaguchi et al., 1999b). SPT5/SPT4 has also been found to interact with the mRNA capping enzyme and cap methyltransferase. Moreover, SPT5/SPT4 defects lead to accumulation of unspliced pre-mRNA (Lindstrom et al., 2003; Wen and Shatkin, 1999). These indicate a role for SPT5/SPT4 in pre-mRNA processing. Relatedly, in Drosophila, SPT5 has been found to associate with RNA processing exosome (Andrulis et al., 2002).

Yueh-Chin Chiang has reported that immunoprecipitation of SPT5 co-immunoprecipites multiple CCR4-NOT components and that in vitro, purified CAF1, and the CCR4-C-terminus can each bind purified SPT5. We subsequently determined that several *spt5* alleles and an *spt4* deletion can reduce the degradation rate of *ADH2* and *GAL1* mRNA, and that the same alleles can decrease *GAL1* mRNA deadenylation rate. While the majority of SPT5 localizes to the nucleus, some SPT5 is present in the cytoplasm. Finally, we showed that SPT5 can inhibit CCR4 deadenylase activity in vitro. These observations indicate that SPT5 can affect mRNA degradation through its

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interaction with and inhibition of CCR4 deadenylase activity. These data also imply that SPT5 may be important in regulating CCR4 deadenylase activity in the nucleus.

Materials and Methods

Yeast Strains And Growth Conditions

Yeast strains are listed in Table 1. Strains containing *spt5* or *spt4* alleles are all isogenic to FY1642 (wild-type) except as indicated (Hartzog et al., 1998). Yeast were grown on YEP medium (1% yeast extract/2% bactopeptone), minimal medium or CAA-U^- medium (Liu et al 1998) supplemented with an appropriate carbon source as indicated in the Figures. The plasmid pCAF1 is YEp13-CAF1-U whose *LEU2* gene has been converted to *URA3*.

Immunofluorescence Assay

The localization of Flag-Tagged SPT5 in the cells was determined by indirect fluorescent immunostaining. FY1639 and FY1642 were grown to midlog stage and analyzed by standard methods using anti-Flag antibody at a dilution of 1:200 and FITC-conjugated goat anti-mouse IgG at 1:400 dilution.

RNA Analysis

Quantitative S1 nuclease protection assays were conducted as described (Collart and Struhl, 1993). 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 were purified as described previously (Cook and Denis, 1993; Denis et al.,

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1983). Oligonucleotides were radiolabelled at their 5' end with T4 polynucleotide kinase as described (Chen et al., 2002).

The polyadenylated species of *GAL1* and *MFA2pG* mRNA were analyzed by using transcriptional pulse-chase and RNase H assay as described previously (Decker and Parker, 1993; Muhlrad and Parker, 1992). A 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 (Muhlrad and Parker, 1992). The resultant *GAL1* 3' polyadenylated species were detected by Northern analysis using a probe that was complementary to the 3' end of *GAL1* (5'-GCCCAATGCTGGTTTAGAGACGATGATAGCATTTTCTAGCTCAGCATCAGTGATCTTAGGG-3'). *MFA2pG* transcript was detected directly by Northern analysis using a probe as described previously (Decker and Parker, 1993).

Results

spt5-4, *spt5-194* And *spt4* Deletion Can Reduce the Rate of mRNA Degradation.

As SPT5 specifically interacts with the C-terminal region of CCR4 that comprises the CCR4 deadenylase function (Chen et al., 2002) and with CAF1, we hypothesized that SPT5 might be regulating the rate of mRNA degradation through its contacts to CCR4-NOT components. We subsequently examined the effect of several *spt5* alleles and an *spt4* deletion on the rate of *ADH2* mRNA decay. CCR4 has been shown previously to affect *ADH2* mRNA degradation rate (Chen et al., 2002).

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We observed that *ADH2* mRNA was stabilized approximately 2- to 3-fold in *spt5-194*, *spt5-4*, and *spt4* deletion strain backgrounds as compared to wild type (Figure 10). In the same strain background, a *ccr4* deletion also resulted in a 2.5-fold stabilization of *ADH2* mRNA. Combining *ccr4* with *spt5-4*, *spt5-194*, or *spt4* did not further slow the rate of *ADH2* mRNA degradation. In contrast, in *spt5-25* and *spt5-242* strain backgrounds, the rate of *ADH2* mRNA decay was not affected (Figure 10, left panel), *ccr4*, however, in combination with *spt5-242* or *spt5-25* still decreased the rate of *ADH2* mRNA degradation by 2-fold (data not shown). We also observed that *spt5-4*, like *ccr4*, slowed the rate of *GAL1* mRNA degradation by two-fold whereas *spt5-242* did not (data not shown). These results indicate that certain alleles of *spt5* or deletion of *spt4* result in a slowing mRNA decay. The observation that effect of *ccr4* and these *spt5/spt4* alleles on mRNA degradation is not additive suggests that CCR4 and SPT5/SPT4 are functioning in the same pathway in regards to mRNA degradation.

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Figure 10. *spt5* and *spt4* alleles affect *ADH2* mRNA degradation rate.
Wild-type and mutant strains (see Table 1) were pregrown on YEP medium containing 2% ethanol prior to shifting to medium containing 4% glucose at zero time and the taking of RNA samples at the times indicated. The *ADH2* mRNA levels were determined by an S1 nuclease protection assay using probe corresponding to sequences 1126 bp downstream of the *ADH2* translation start codon (5'-GGCATACTTGATAATGAAAACTATAAATCGTAAAGACATAAG-3'). The *ACT1* RNA was used to standardize loadings for RNA samples at the different time points indicated. ACT1 probe is complementary to the sequences 1039 bp downstream of the *ACT1* translation start codon (5'-CCACTTTCGTCGTATTCTTGTTTTGAGATCCAC ATTTGTTGGAAGGTAGTCAAAGAAGC-3').

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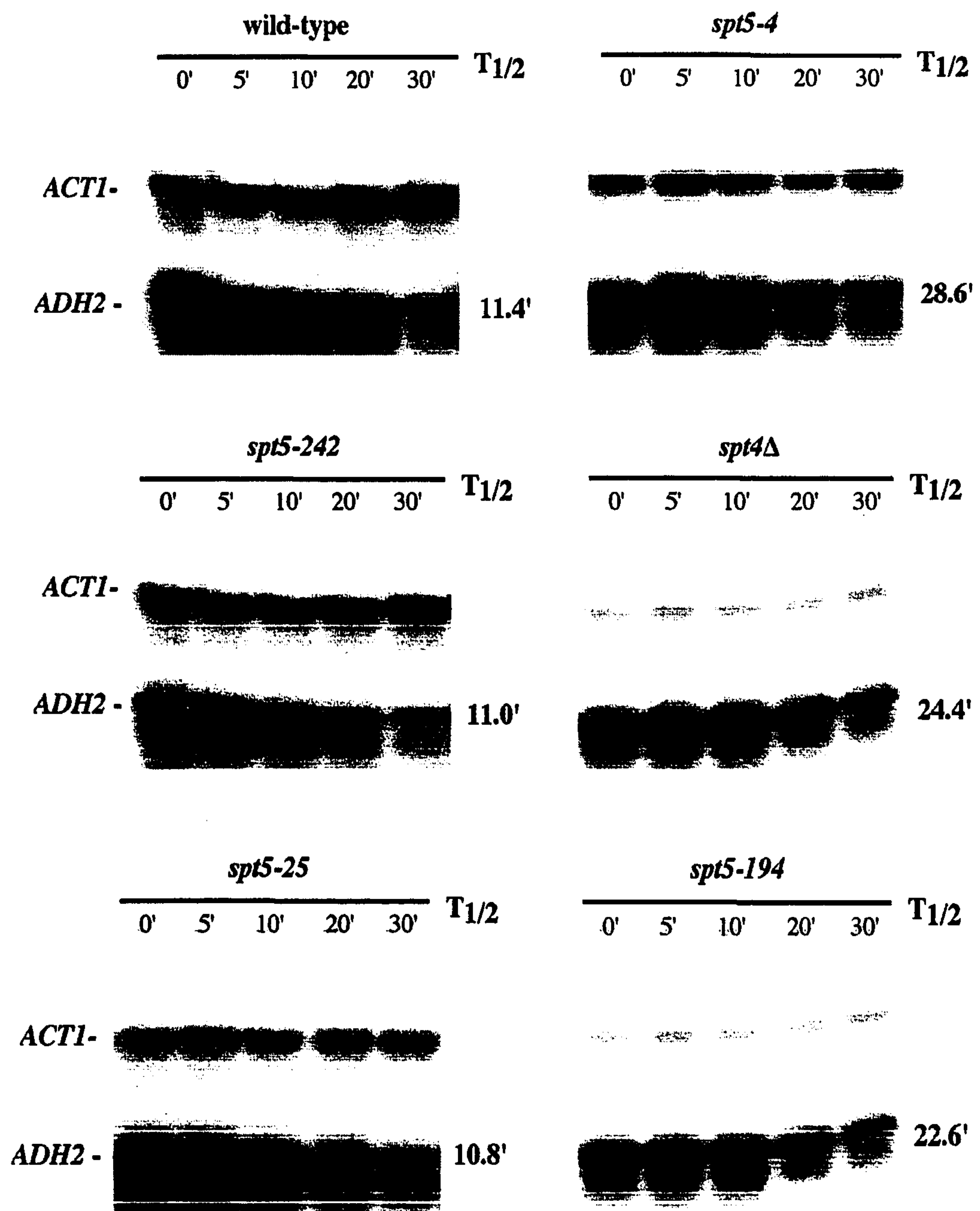
Figure 10



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SPT5 And *SPT4* Mutations Affect mRNA Deadenylation But Not Decapping

The effect of SPT5/SPT4 on mRNA degradation could be occurring by altering the rate of deadenylation, decapping or 5' to 3' degradation of RNA transcript (Beelman and Parker, 1995). A simple test to determine whether defects in mRNA decay occurs at the decapping or later step is to analyze the production of degradation products of the MFA2pG RNA relative to the total level of MFA2pG present (Tharun and Parker, 1999). MFA2pG gene contains a poly (G) tract insertion in the 3' UTR, which inhibit exonuclease activity (Decker and Parker, 1993). Therefore, a poly (G) to 3' end RNA fragment will accumulate when the decapped mRNA is degraded from the 5' to 3'direction. However, in the absence of any decapping and 5' to 3' mRNA digestion, the formation of the poly (G) to 3' end RNA fragment will be blocked. As shown in Figure 5A, like *ccr4* (Figure 11A, lane 3), *spt5-4* has no obvious effect on the formation of poly (G) to 3' end mRNA fragment (Figure 11A, lane 2 compared to lane 1). Since these results indicate that the *spt5/spt4* alleles are not affecting decapping or subsequent 5' RNA degradation, we tested whether these alleles affected the rate of mRNA deadenylation, the same step that CCR4 and CAF1 control. Changes in the mRNA deadenylation rate was followed using a transcriptional pulse chase analysis (Decker and Parker, 1993) with the *GAL1* mRNA. Expression of *GAL1* was rapidly induced for 15 minutes by adding galactose to raffinose growth culture and then repressed by adding glucose. The deadenylation of this pool of newly synthesized *GAL1* mRNA was monitored at different time points by Northern analysis.

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Figure 11. *spt5* and *spt4* alleles affect mRNA deadenylation but not decapping
(A). Wild-type (FY1642), *spt5-4* (FY1668-uH), and *ccr4* (FY1642-1a) strains containing plasmid RP??? were grown in CAA-U$^-$ medium with 4% glucose to midlog stage. Total RNA was extracted after shifting to 2% galactose for 4 hours. *MFA2pG* transcripts were detected by using transcriptional pulse-chase assay (Decker and Parker, 1993). The upper band represents the full-length *MFA2pG* transcript and the lower band corresponds to the poly (G) to 3' end RNA fragment.
(B). Wild-type (FY1642), *spt5-4* (FY1668-uH), *spt5-242* (FY1635), and *spt4* (GHY180) strains were shifted from raffinose-containing YEP medium to galactose-containing YEP medium for 15 minutes to induce *GAL1* gene expression. After transcriptional repression by the addition of glucose, total RNA were extracted at different time point as indicated and RNase H digestion was conducted and Northern analysis was used to detect the *GAL1* specific mRNA.

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Figure 11

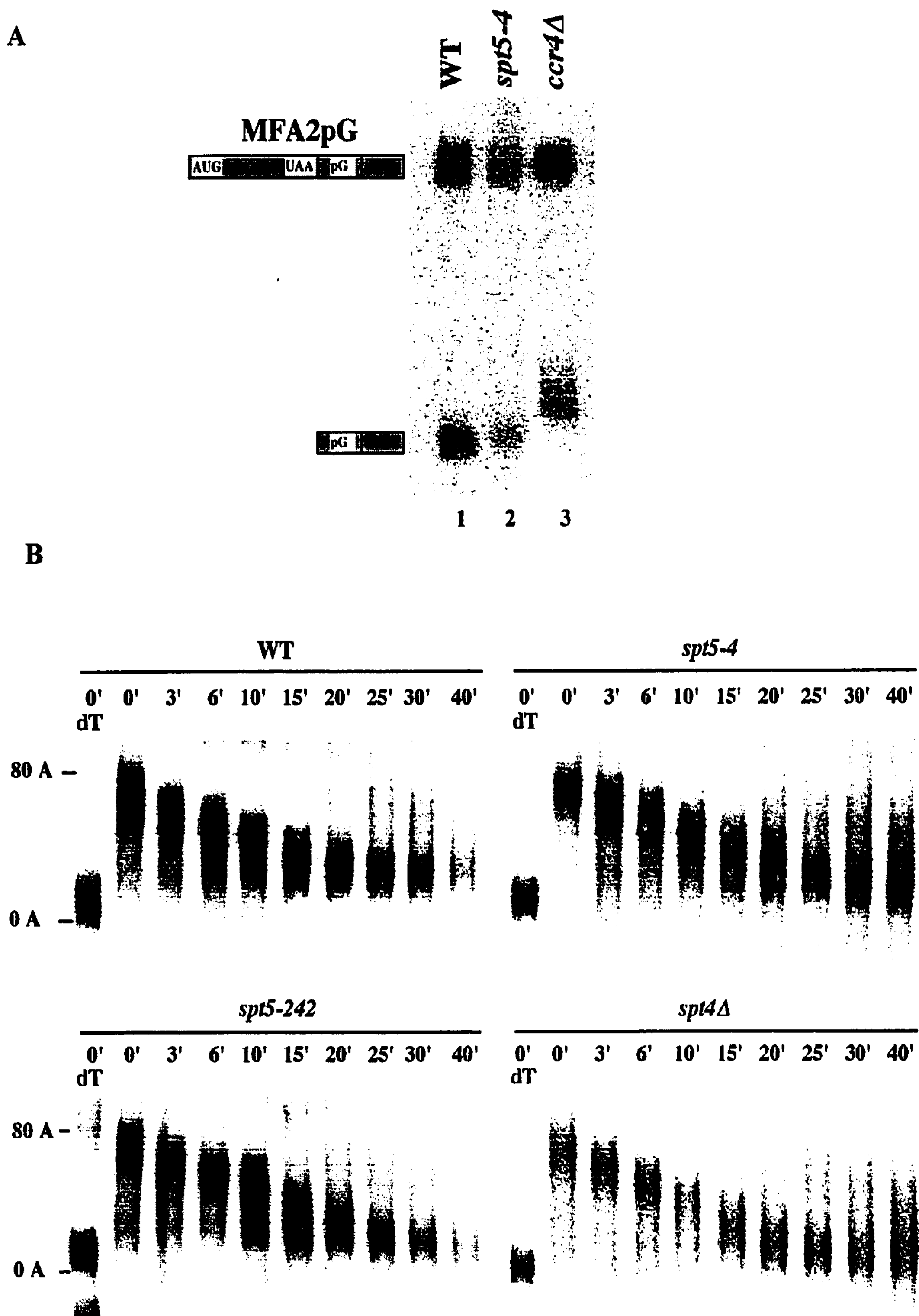

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Table 4 deadenylation rate of *GAL1* mRNA in wild-type, *spt5*, and *spt4* strains

strain	deadenylation rate (nucleotide/minute)
wild-type	2.9 ± 0.28
spt5-242	3.3 ± 0.42
spt5-4	2.1 ± 0.12
spt4 deletion	2.1 ± 0.14

As shown in Figure 11B, we observed that, in a wild-type strain, the poly (A) tail is degraded to an oligo (A) tail length of approximately 8-13 A's by around 10-15 minutes, followed by slow deadenylation of the oligo (A) tail. It has been shown previously that decapping and 5' to 3' degradation of the transcript occurs once the oligo (A) tail form appears (Couttet et al., 1997; Decker and Parker, 1993; Muhlrad et al., 1994). The rate of *GAL1* mRNA deadenylation was found to about 2.9 A's per minute. Similarly in the *spt5-242* strain which had no effect on *ADH2* or *GAL1* mRNA decay rate, the poly (A) tail was shortened at a rate of approximately 3.3 nucleotides per minutes (Table 4), with a portion of *GAL1* mRNA with oligo (A) tail observed also in 10 to 15 minutes (Figure 11B, left panel). In contrast, in *spt5-4* and *spt4* deletion strains, deadenylation rate of *GAL1* mRNA was calculated to be about 2.1 nucleotides per minutes (Table 4), and the oligo (A) tail form of *GAL1* mRNA routinely appeared in 20 to 25 minutes (Figure 5B, right panel).It should be noted that the total RNA intensity decreased after about 20 minutes in the wild-type and *spt5-242* strain (Figure 11B, left panel). However, we did not see an obvious reduction in the intensity of the *GAL1* mRNA even after 40 minutes in *spt5-4* and *spt4* deletion strain (Figure 11B, right panel), supporting the view that the deadenylation, decapping, and 5' to 3' mRNA digestion of

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the *GAL1* mRNA was significantly delayed in *spt5-4* and *spt4* background because of the slower rate of deadenylation. These observations indicate that the deadenylation rate of *GAL1* mRNA can be reduced by *spt5-4* and *spt4* but not by *spt5-242* and that SPT5/SPT4 affects mRNA turnover through their effects on mRNA deadenylation.

SPT5 is Present in The Cytoplasm

The above experiments indicated a role for SPT5 in mRNA turnover. Previous research has shown that SPT5/SPT4 play a role in RNA metabolism that occurs in the nucleus (Hartzog et al., 1998; Lindstrom et al., 2003; Pei and Shuman, 2002; Swanson et al., 1991a; Wada et al., 1998a; Wen and Shatkin, 1999; Wu-Baer et al., 1998). CCR4-NOT components, in contrast, are found in both nuclear and cytoplasmic compartments (Tucker et al., 2002; Tucker et al., 2001) and the primary function of CCR4 in mRNA degradation is presumed to be cytoplasmic. If SPT5 is involved in mRNA degradation through its interaction with CCR4-NOT components, it might be expected that some SPT5 should be present in the cytoplasm. To reexamine the subcellular localization of SPT5, we used a strain with a Flag tag on the C-terminus of the chromosomal *SPT5* gene, and an immunofluorescence assay was conducted using a FITC-conjugated secondary antibody. As shown in Figure 12, the majority of SPT5 is localized to the nucleus, consistent with previous observations. Careful analysis of the data indicates, however, that some SPT5 is present in the cytoplasm (Figure 12). These results support the hypothesis that SPT5 is involved in mRNA degradation through its interaction in the cytoplasm with CCR4. These data do not exclude the possible role that SPT5 may still regulate CCR4 deadenylase activity in the nucleus, especially as CCR4 has been shown

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to play roles in transcriptional initiation and elongation (Badarinarayana et al., 2000; Deluen et al., 2002; Denis et al., 2001; Lemaire and Collart, 2000).

Figure 12

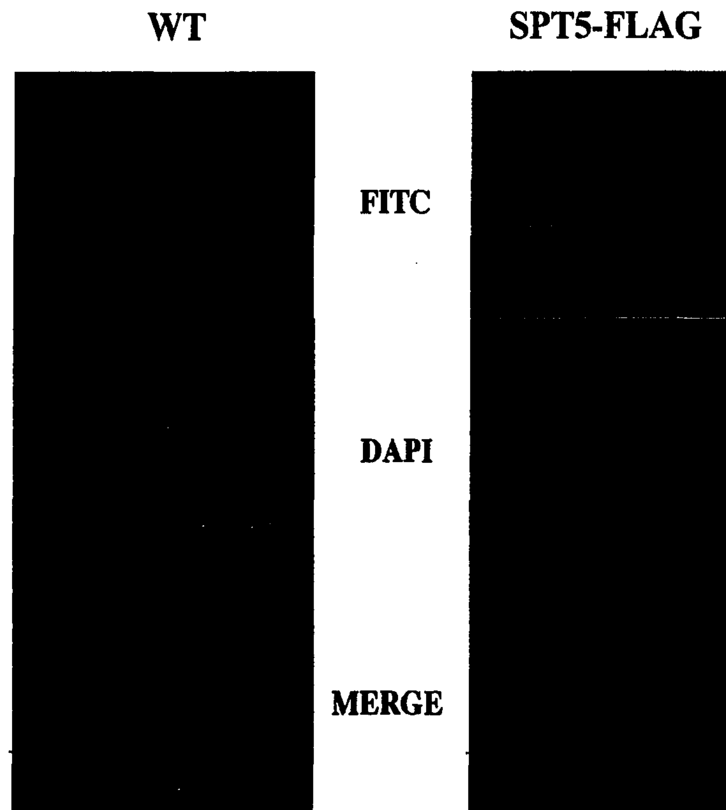

Figure 12. SPT5 is localized to both the nucleus and the cytoplasm.
Strains expressing Flag-Tagged SPT5 (FY1642) and wild-type control (FY1639) were incubated with anti-Flag antibody and stained with FITC-conjugated anti-mouse antibody and DAPI as indicated.

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Discussion

The SPT4/SPT5 complex has been shown to be important in multiple steps of mRNA processing (Hartzog et al., 1998; Lindstrom et al., 2003; Pei and Shuman, 2002; Swanson et al., 1991a; Wen and Shatkin, 1999; Wu-Baer et al., 1998; Yamaguchi et al., 1999b). We show herein that this complex in yeast physically associates in vivo with components of the CCR4-NOT complex. The CCR4-NOT complex, in turn, has known or suggested roles in transcriptional initiation, elongation, and mRNA degradation (Chen et al., 2002; Daugeron et al., 2001; Denis, 1984; Denis et al., 2001; Tucker et al., 2002; Tucker et al., 2001; Viswanathan et al., 2003). Several pieces of evidence provided by Yueh-Chin Chiang support a close physical association of SPT5 with components of the CCR4-NOT complex. First, in the two-hybrid assay SPT5 displayed interactions with CCR4-NOT complex components NOT1, NOT2, CCR4, CAF40, and CAF130. Second, each of these two-hybrid interactions was shown to occur by co-immunoprecipitation analysis. Third, SPT5 and CCR4, at their physiological concentration, were found to specifically co-immunoprecipitate. Fourth, the CAF1 protein when overexpressed interfered with SPT5-CCR4 interactions. Moreover, deleting *CAF1* revealed additional SPT5 contacts to the CCR4-NOT components, NOT1, NOT4, and CAF40 when they were expressed at their physiological concentrations. These results clearly indicate a close proximity in the cell of CCR4-NOT components with SPT5. This physical proximity agrees with various experiments establishing that the components of CCR4-NOT complex and SPT5 protein can be present both in the nucleus and in the cytoplasm (Collart and Struhl, 1993; Swanson et al., 1991a; Tucker et al., 2002; Tucker et al., 2001).

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In addition to the physical interaction between CCR4-NOT proteins and SPT5, several pieces of evidence also suggest that SPT5 functionally interacts with CCR4-NOT complex. First, genetic assay showed that *spt5-4*, *spt5-25*, *spt5-8*, and *spt5-194* were found to suppress defects in *ccr4* (C. L. Denis, pers. Comm.). Second, *spt5-4*, *spt5-194*, and *spt4* can slow down mRNA degradation rate. These alleles when combined with *ccr4*, however, did not further reduce the rate of mRNA decay. Third, these alleles which slow down mRNA decay also reduced the rate of mRNA deadenylation but had no effect on mRNA decapping and 5' to 3' digestion. The *spt5* alleles that did not affect mRNA decay, however, also had no effect on mRNA deadenylation. Fourth, SPT5 is also present in the cytoplasm. Finally, in vitro deadenylase activity assay showed that SPT5 can partially inhibit CCR4 deadenylase activity but SPT5 alone does not have deadenylase activity.

It should be noted that the spt5-4 and spt5-194 proteins are defective for binding SPT4 (G. Hartzog, pers. Comm.). Other *spt5* alleles not defective for binding SPT4, such as *spt5-242* or *spt5-25*, has no effect on mRNA degradation and deadenylation, indicating that SPT5/SPT4 interaction is important in its effect on mRNA degradation and deadenylation. There are two models explaining how SPT5/SPT4 could affect the mRNA deadenylation rate. First, the interaction between SPT4 and SPT5 can interfere with the negative effect of SPT5 on CCR4 deadenylase activity. Therefore, after the SPT5/SPT4 complex is disrupted either by mutating the *SPT5* gene (*spt5-4*, and *spt5-194*) or by deleting the *SPT4* gene, SPT5 will be released to inhibit CCR4 deadenylase activity. Another model is that the SPT5/SPT4 interaction can affect SPT5 localization. SPT4 may retain SPT5 in the nucleus by binding SPT5. Disrupting interaction between SPT5 and

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SPT4 will result in more SPT5 present in the cytoplasm, and therefore, result in inhibition of CCR4 deadenylase activity. We observed that SPT4 is more predominantly localized in the nucleus as compared to SPT5. However, deleting *SPT4* did not appear to affect the subcellular localization of SPT5 (data not shown).

Although *spt5-4*, *spt5-194* and *spt4* can reduce the rate of *GAL1* mRNA deadenylation, these effects are not as significant as that observed with *ccr4*. In addition, these alleles did not display as prominent defect the deadenylation end point as did *ccr4* and *caf1* strains (Figure 11A; Tucker et al., 2001). Since these *spt5* alleles and *spt4* have similar effect on reducing the rate of mRNA degradation as *ccr4* did, but less effects on mRNA deadenylation, SPT5/SPT4 might also be affecting mRNA degradation through other mechanisms. The requirement of deadenylation before decapping indicates that the poly (A) tail also acts as an inhibitor of decapping(Decker and Parker, 1993; Muhlrad et al., 1994; Muhlrad et al., 1995). Several observations suggest that the ability of the poly (A) tail to inhibit decapping is mediated through the poly (A) binding protein (PAB1) (Caponigro and Parker, 1995; Morrissey et al., 1999; Sachs et al., 1987). *ccr4* can affect not only the rate of deadenylation, but also the extent of deadenylation, therefore inhibiting the subsequent decapping and 5' to 3' deadenylation (Tucker et al., 2001). We observed that, in *spt5-4* and *spt4* strains, the poly (A) tail is degraded to an oligo (A) tail length of approximately 6-10A's by around 20-25 minutes, whereas obvious *GAL1* mRNA intensity reduction was not observed even after 40 minutes (Figure 5B, right panel). This observation indicates that *spt5-4* and *spt4* not only slow the rate of deadenylation, but they might also block the poly (A) tail mediated decapping signal.

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CHAPTER III

OTHER RESULTS

Elongation Results

ccr4 Enhances the Ability of RNA pol II to Form the Full-length *lacZ* mRNA

Genetic analyses have implicated the CCR4-NOT complex in playing possible roles in transcription elongation (Denis et al 2001). To investigate whether CCR4 affected elongation in vivo, an S1 nuclease protection assay was employed to analyze the ability of RNA pol II to form full-length *lacZ* mRNA in presence of a *ccr4* deletion. We found, as shown in Figure 13, that in contract to other elongation factor defects, *ccr4* displayed enhanced full-length *lacZ* 3' RNA formation (one and a half to two-fold effects for *ccr4*, Figure 13A, lanes 1 and 2, and Figure 13B, lanes 1 and 2). A *caf1* deletion gave similar results to *ccr4* (Figure 13A, lane 3).

To determine whether *ccr4* affects RNA pol II transcript through *lacZ* gene by affecting cryptic poly (A) sites usage within *lacZ* gene, both total RNA and poly (A) RNA were used to conduct S1 nuclease protection assay (Figure 13B). My results showed that a *ccr4* deletion displayed similar effects on full-length *lacZ* RNA formation when both total and poly (A) RNA were used (1.9-fold effects for total RNA, Figure 13B, lanes 1 and 2; 1.8-fold effects for poly (A) RNA, Figure 13B, lanes 3 and 4). This result suggested that the *ccr4* deletion may enhance the ability of RNA pol II to form full-length *lacZ* RNA by affecting *lacZ* cryptic poly (A) sites usage.

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Figure 13

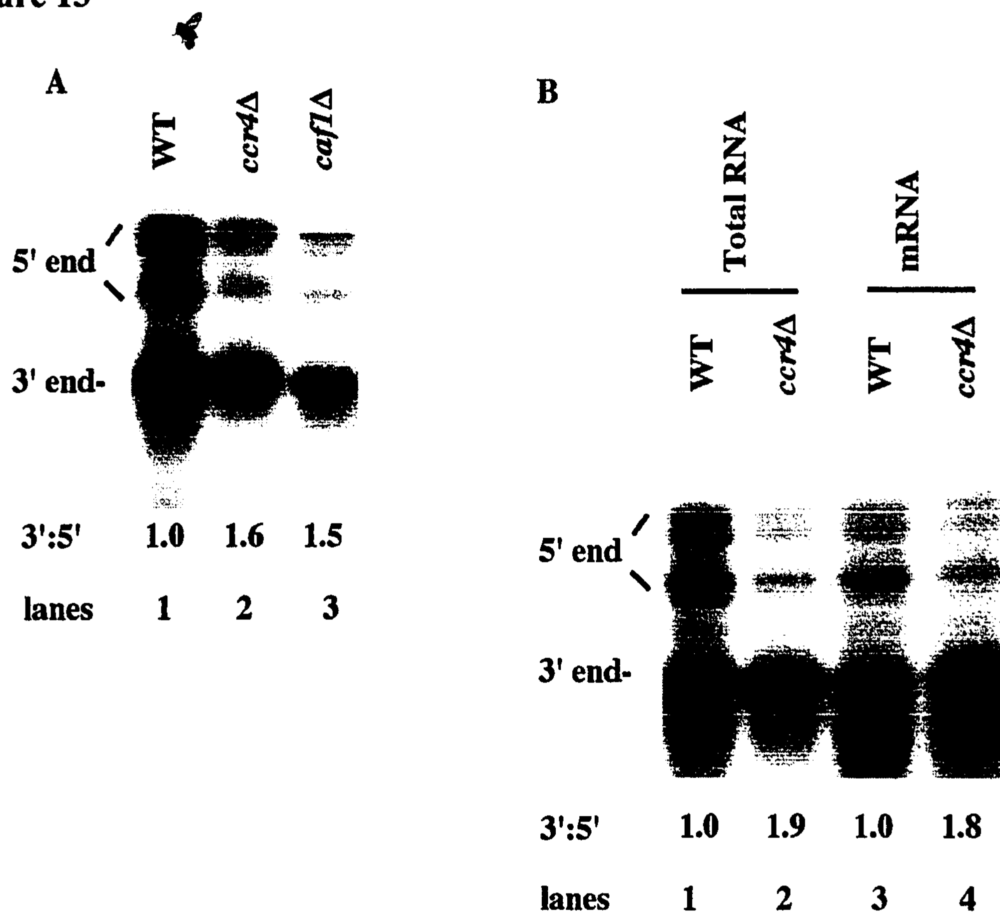

Figure 13. *ccr4* and *caf1* alleles enhance full-length *lacZ* mRNA formation.
(A) S1 nuclease protection analysis of total RNA extracted from the WT strain EGY188 (lane 1), *ccr4* strain EGY188-1a (lane 2) and *caf1* strain EGY188-c1 (lane 3). S1 nuclease protection assays, growth conditions, and calculations were conducted as described in Figure 1.
(B) Both total RNA (lane 1 and 2) and poly (A) RNA (lane 3 and 4) were extracted from WT strain EGY188 (lane 1 and 3) and *ccr4* strain EGY188-1a (lane 2 and 4) and used for S1 nuclease protection assays. S1 nuclease protection assays, growth conditions, and calculations were conducted as described in Figure 1.

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<u>*ccr4* Does Not Enhance the Ability of RNA pol II to Form Full-length *lacZ* RNA By Suppressing Transcriptional Termination/cleavage.</u>

ccr4 may affect *lacZ* cryptic poly (A) sites usage directly or indirectly. It can indirectly reduce cryptic poly (A) site usage through its effect on elongation. It can also reduce cryptic poly (A) site usage by suppressing transcription termination/cleavage. To determine which possibility is the case, the effect of *ccr4* on transcriptional termination/cleavage was examined. S1 nuclease protection assays were carried out in strains carrying plasmid pGCYC1 which expresses *Gal1-CYC1*. Probes against the *CYC1* coding region (+139) and against a site that was 350 bp downstream of the *CYC1* polyadenylation site (+853) were used to examine the abundance of the different *CYC1* transcript species. In the wild-type strain, the normal *CYC1* polyadenylation site (+503) was used and no *CYC1* transcript beyond +853 was detected (Figure 14, lanes 1, 5 and 8). With *rna14* and *rna15* mutations which can affect transcription termination (Birse et al 1998), normal transcription termination was suppressed and more long *CYC1* transcripts beyond +853 were formed after switching growth from the permissive (25°C, Figure 14, lanes 6 and 7) to the restrictive condition (37°C, Figure 14, lanes 3 and 4). The *pap1* mutation, which has no effect on transcription termination (Figure 14, lane 2), and the *ccr4* deletion can not suppress transcription termination (Figure 14, lane 9). These results suggest that *ccr4* does not enhance the ability of RNA pol II to form full-length *lacZ* RNA by suppressing transcriptional termination.

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Figure 14

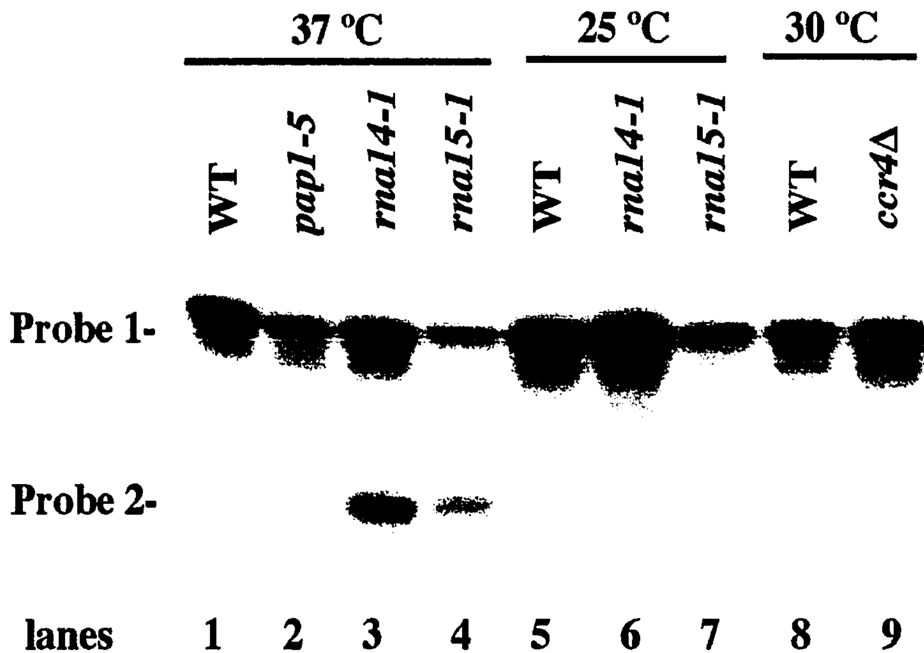

Figure 14. *ccr4* does not suppress transcriptional termination of the *CYC1* gene. S1 nuclease protection analysis of total RNA extracted from WT strain W303 (lane 1 and 5) and EGY188 (lane 8), *pap1-5* strain LM98 (lane 2), *rna14-1* strain LM91 (lane 3 and 6), *rna15-1* strain LM88 (lane 4 and 7) and *ccr4* strain EGY188-1a (lane 9) containing plasmid pGCYC1 which expresses *Gal-CYC1*. For lanes 1 to 7, strains were pregrown in 4% glucose medium at 25°C for 20 hr, and then shifted to 2% galactose/2% raffinose at nonpermissive temperature (37°C) for 4 hr (lanes 1 to 4) or at permissive temperature (25°C) for 4 hr (lanes 5-7). For lanes 8 and 9, strains were pregrown in 4% glucose medium at 30°C for 20 hr, and then shifted to 2% galactose/2% raffinose at 30°C for 4 hr. Probe 1 is directed against the *CYC1* coding region (+139) and probe 2 is directed against a region 350 bp downstream of the *CYC1* polyadenylation site (+853).

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The Effect of *ccr4* on full-length *lacZ* mRNA Formation Is Not Due To Increased mRNA 3' End Degradation.

Since both CCR4 and CAF1 have been shown to be part of the cytoplasmic mRNA deadenylase complex (Tucker et al 2001), it was possible that the above effects on full-length *lacZ* 3' RNA formation were resulting from differential effects on the degradation of the 3' and 5' ends of *lacZ* mRNA. To address this 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 expected, *ccr4* decreased the degradation rates of *lacZ* mRNA by 2- to 3-fold (Figure 15, 6 min. half life for WT and 16 min. half life for *ccr4*), but it did so for both 5' and 3' ends of the *lacZ* mRNA (Figure 15). These results indicate that the enhanced ability to form full-length *lacZ* mRNA by *ccr4* results from an enhanced ability to elongate through the *lacZ* gene.

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Figure 15

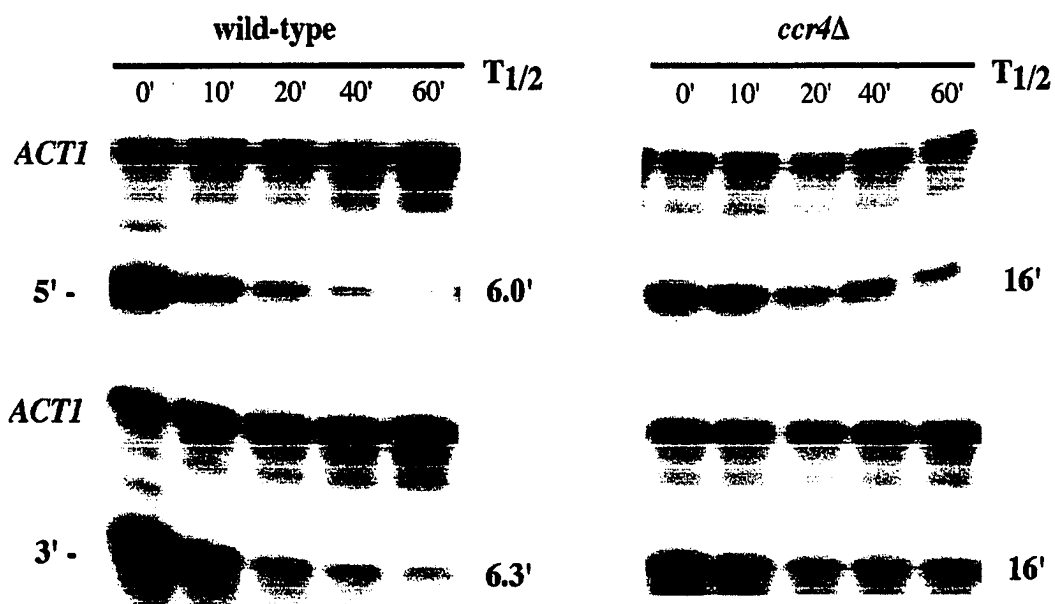

Figure 15. *ccr4* decreases *lacZ* mRNA decay rate but does not decrease degradation of the 3' end of the *lacZ* mRNA relative to that of the 5' end.
Wild-type and *ccr4* strains (see Table 1) transformed with the *GAL1-lacZ* plasmid were pregrown on 2% galactose/2% raffinose prior to shifting to medium containing 4% glucose at zero time and taking of RNA samples at the times indicated. The 5' *lacZ* probe corresponded to sequences 0.5 kb downstream of the initiation site whereas the 3' *lacZ* probe was located 2.9 kb downstream. The *ACT1* RNA was used to standardize loadings for RNA samples at the different time points indicated.

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<u>While *rpb2-4* And *rpb2-10* Alleles Can Suppress a *ccr4* Defect, *ccr4* And *spt5-4* Have a Mixed Effect On Transcription Elongation.</u>

To further investigate the role of CCR4 on transcription elongation, we examined the effect of *ccr4* on *lacZ* mRNA elongation in *rpb2* and *spt5* backgrounds.

As previously shown, *rpb2-4*, *rpb2-10* and *spt5-4* reduced the ability of RNA pol II to form full-length *lacZ* RNA (Figure 16, lanes 2, 3 and 9) while *ccr4* increased it (Figure 16, lane 4 and 8). While *ccr4* caused a 2.2-fold increase in full-length *lacZ* mRNA (Figure 16, lane 4 compared to lane 1), it allowed only 1.4- and 1.6-fold effect, respectively, in full-length *lacZ* mRNA formation in *rpb2-4* and *rpb2-10* backgrounds (Figure 16, lanes 5 and 6 compared to lanes 2 and 3). This result indicates that *rpb2-4* and *rpb2-10* alleles can partially suppress the effect of *ccr4* on *lacZ* elongation and that CCR4 acts upstream of RPB2 to affect elongation. In contrast, the *ccr4* allele, while causing a 1.7-fold increase in full-length *lacZ* formation in a wild-type background (Figure 16 lane 8 compared to lane7), caused a 3.8-fold increase in an *spt5-4* background (Figure 16, lane 10 compared to lane 9). In addition, *spt5-4* still has an effect on *lacZ* elongation in the *ccr4* background (Figure 16, lane 10 compared to lane 8), although not as much as it does in wild-type background (Figure 16, lane 9 compared to lane 7). This mixed effect of *ccr4* and *spt5-4* alleles on *lacZ* elongation indicates that CCR4 may act downstream or in conjunction with SPT5 to affect elongation.

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Figure 16

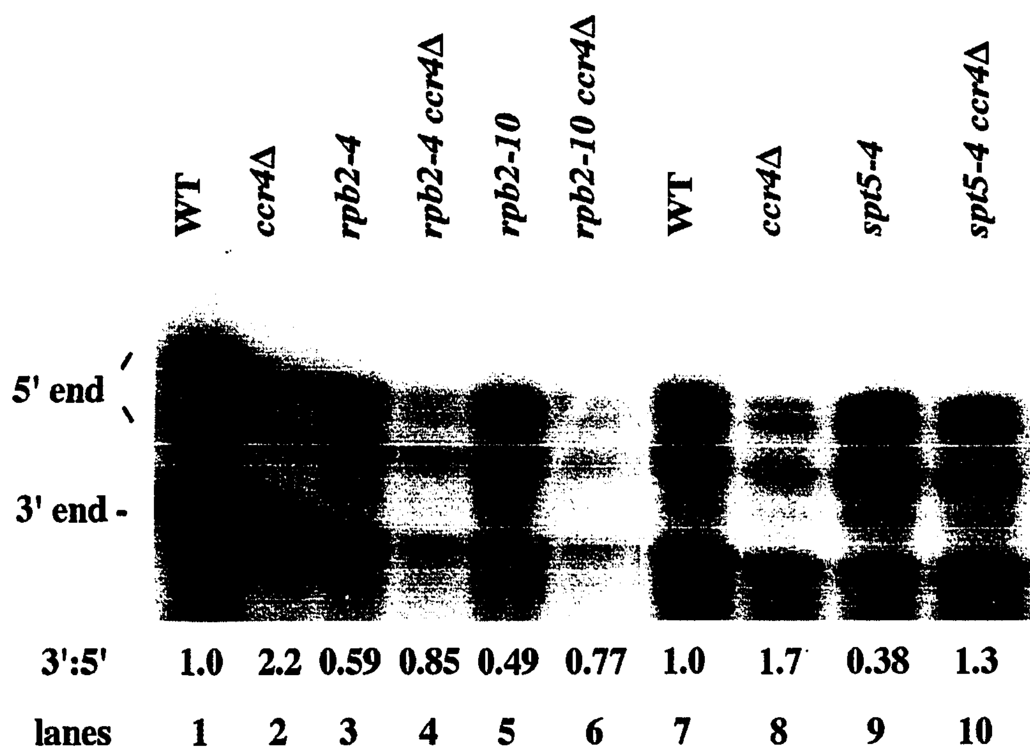

Figure 16. *ccr4* specifically suppresses the *spt5-4* defect on elongation but not that of *rpb2-4* or *rpb2-10.*
S1 nuclease protection analysis of total RNA extracted from *ccr4* strain Z96-1a (lane2), *rpb2-4* strain Z100 (lane 3), *rpb2-4 ccr4* strain Z100-1a (lane4), *rpb2-10* strain Z106 (lane 5), *rpb2-10 ccr4* strain Z106-1a (lane 6) and the isogenic WT strain Z96 (lane10), *ccr4* strain FY1642-1a (lane 8), *spt5-4* strain FY1668-uH (lane 9), *spt5-4 ccr4* strain FY1668-uH-1a (lane 10) and the isogenic WT strain FY1642 (lane 7). S1 nuclease protection assays, growth conditions, and calculations were conducted as described in Figure 1.

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<u>Growth Condition of the Yeast Strain can Affect Full-length Formation of the *lacZ* RNA.</u>

Several *spt5* alleles have been shown to be cold sensitive (Hartzog et al., 1998). It has also been shown that an Artificial Arrest (ARTAR) site does not appear to function under optimal growth condition (Kulish and Struhl, 2001). These data indicate that the growth condition of the cells might affect RNA pol II transcript through the genes. Therefore, the effect of different growth conditions on full-length *lacZ* mRNA formation was examined in wild-type and elongation defective strains background. The yeast were grown in the medium containing 4% glucose, 2% galactose/raffinose, 2% ethanol, or 4% glucose with 200ug/ml 6-azauracil respectively. In the wild-type strain background, as shown in Figure 17, less optimal growth condition (galactose/raffinose or ethanol) can increase full-length *lacZ* RNA formation (Figure 17A, lanes 2, 3, compared to lane 1; 17B, lane 2, compared to lane 1), but the drug 6-AU has no effect on full-length *lacZ* RNA formation. In contrast, in the *spt5-4* strain background, although the ability of RNA pol II to form full-length *lacZ* RNA was decreased compared to wild-type, the growth conditions have no significant effect on full-length *lacZ* formation (Figure 17A, lanes 5 to 8). The exact same result was obtained in an *rpb2-4* strain background (Figure 17B, lanes 3 and 4).

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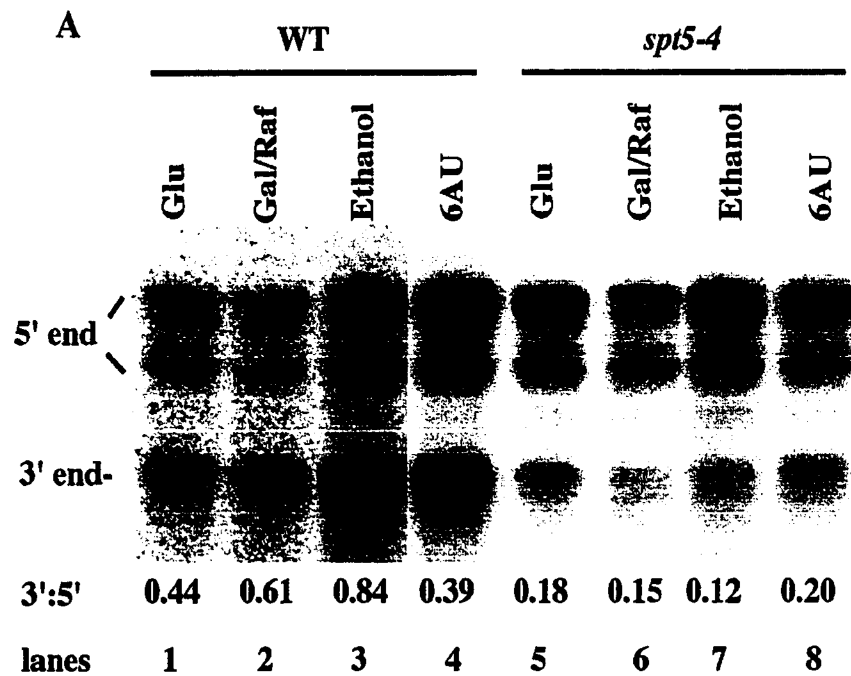
Figure 17

A



B

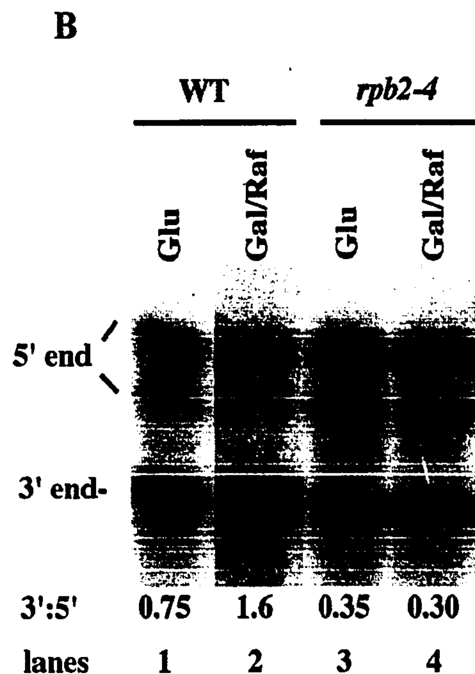


Figure 17. The effect of the growth condition of cells on full-length *lacZ* RNA formation

S1 nuclease protection analysis of total RNA extracted from *spt5-4* strain FY1668-uH (A, lanes 5 to 8) and the isogenic WT strain FY1642 (A, lanes 1 to 4); *rpb2-4* strain Z100 (B, lanes 3 and 4) and the isogenic WT strain Z96 (B, lanes 1 and 2). The strains were pregrown over night in the medium containing 4% glucose prior to shifting to growth condition as indicated in the Figure for 4 hours. S1 nuclease protection assays and calculations were conducted as described in Figure 1.

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Degradation Results

SPT5 Can Affect *MFA2pG* RNA Decay

We showed in Chapter II that SPT5 can affect the degradation rate of *ADH2* and *GAL1* mRNA probably through its physical and functional interaction with CCR4. The effect of CCR4 on *MFA2pG* RNA degradation has been thoroughly investigated previously (Tucker et al., 2001). To further confirm the functional connection between CCR4 and SPT5, the effect of SPT5 on *MFA2pG* RNA decay was examined.

I examined the decay rate of *MFA2pG* RNA in wild-type and *spt5-4* strain backgrounds. *MFA2pG* is under the control of *GAL1* promoter, allowing the measure of its decay rate by adding glucose to repress transcription. I observed that the *MFA2pG* RNA was stabilized about 2-fold in the *spt5-4* strain compared to wild-type (Figure 18). *spt5-4* also reduced the degradation rate of *ADH2* and *GAL1* mRNA as shown in Chapter II, indicating that SPT5 is required for the turnover of a number of yeast mRNA.

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Figure 18

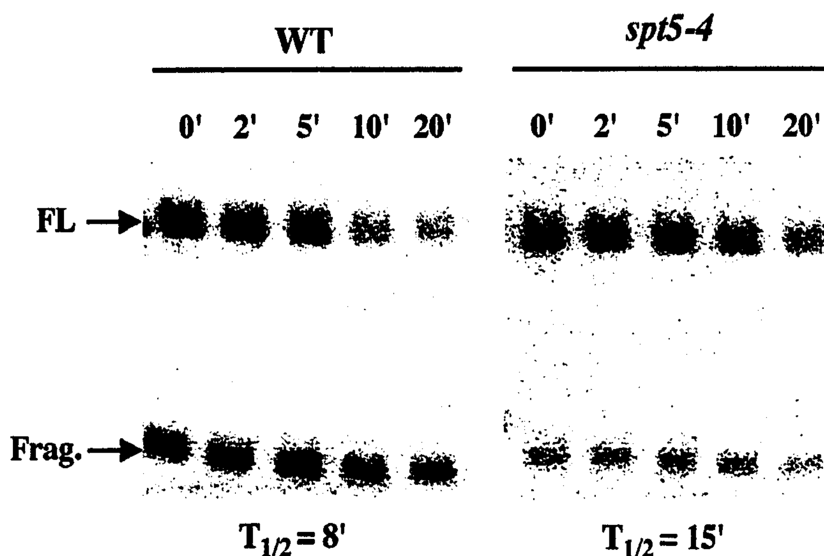

Figure 18. *spt5-4* can reduce the decay rate of the *MFA2pG* mRNA. Transcriptional shutoff analysis of *MFA2pG* mRNA from wild-type (FY1642) and *spt5-4* (FY1668-uH) strains. The full-length (FL) mRNA and decay fragment (Frag.) are indicated at the left. Time points are minutes after the addition of glucose.

SPT5/SPT4, Similar As CCR4/CAF1, Can Affect the Endpoint of *MFA2pG* mRNA Deadenylation

It has been shown previously that not only can CCR4, as major cytoplasmic deadenylase, affect *MFA2pG* mRNA deadenylation rate, it also affect the length of poly (A) tail of *MFA2pG* mRNA at late time point (Tucker et al., 2001). Therefore, to understand the relationship between CCR4 and SPT5, the effect of SPT5/SPT4 on the endpoint of *MFA2pG* mRNA deadenylation was examined.

The distribution of poly (A) tail length was measured for *MFA2pG* under steady state conditions from wild-type, *ccr4Δ*, *spt5* and *spt4* alleles. As shown in Figure 19, if samples were first treated with RNase H and oligo (dT), the poly (A) tail of *MFA2pG*

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mRNA from different strains will be removed. The remaining transcripts have the same length (Figure 19, lanes 1 to 6). Wild-type *MFA2pG* mRNA has a steady state poly (A) distribution from 75 to about 10-12 adenosine residues (Figure 19, lane 7; Tucker et al., 2001). In *ccr4Δ* strain, *MFA2pG* mRNA has the longest poly (A) tail (Figure 19, lane 8). *spt5-242* allele, which has no effect on *ADH2* mRNA degradation and *GAL1* mRNA deadenylation, did not affect on poly (A) distribution (Figure 19, lane 9). In contrast, in the *spt5-4*, *spt5-194*, and *spt4Δ* strains, in which *ADH2* mRNA decay rate and *GAL1* mRNA deadenylation rate were reduced, the poly (A) tail of *MFA2pG* is slightly longer than observed in wild-type strain, but is shorter than observed in *ccr4Δ* strain (Figure 19, lanes 10 to 12, compared to lanes 6 and 7).

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Figure 19

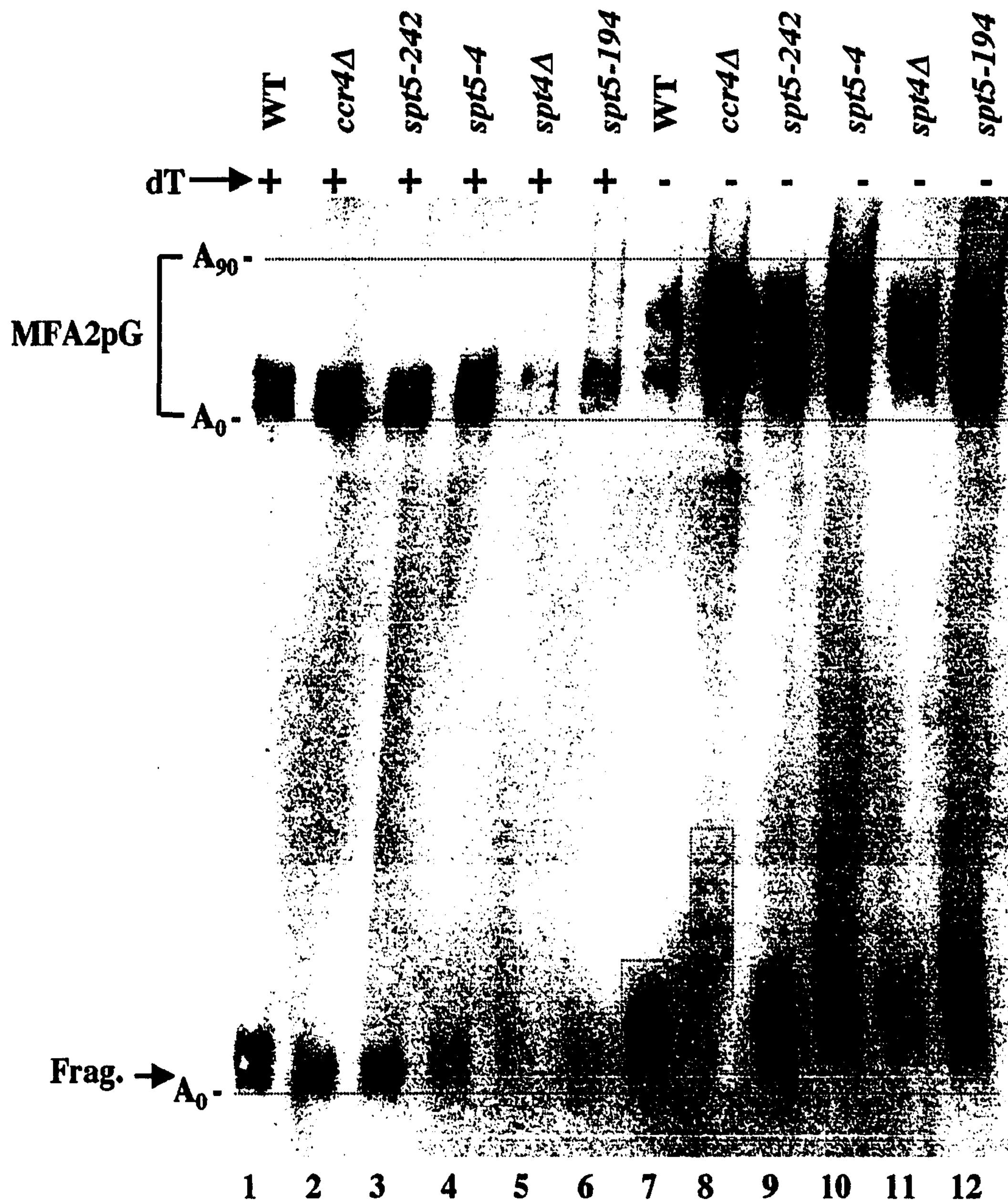

Figure 19. SPT5 and SPT4 can affect *MFA2pG* mRNA deadenylation endpoint. The deadenylation end point for *MFA2pG* transcript was measured in the wild type (FY1642), *ccr4Δ* (FY1642-1a), *spt5-242* (FY1635), *spt5-4* (FY1668-uH), *spt4Δ* (GHY180), and *spt5-194* (FY300) strains. The strains were pregrown over night in the medium containing 4% glucose prior to shifting to 2% galactose for 4 hours. Steady state mRNA were detected by Northern analysis with or without removal of poly (A) tails with RNase H and oligo (dT) as indicated in the Figure.

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GENERAL DISCUSSION

The Regulation of Gene Expression Is a Continuous Process

Gene expression is a pathway with multiple steps. The regulation of gene expression can occur at each step during this process. The different steps in this pathway have previously been treated and studied as independent events. In recent years, a growing number of genetic studies have revealed functional links between the protein factors that carry out the different steps in the gene expression pathway. Many factors have also been found to perform their regulatory function in more than one steps in this process. SPT5 has been identified as a protein that can regulate transcriptional initiation, elongation, and pre-mRNA processing (Andrulis et al., 2002; Hartzog et al., 1998; Kim et al., 1999; Lindstrom et al., 2003; Wen and Shatkin, 1999; Yamaguchi et al., 1999b). In Chapter I, we further confirmed the role of SPT5 in the regulation of transcriptional elongation. In addition, out results in Chapter II also suggested that SPT5 can regulate gene expression by affecting mRNA stability, possiblely through its physical and functional interaction with CCR4-NOT complex,confirming multiple roles for SPT5 in the regulation of gene expression. Similarly, the CCR4-NOT complex acts at several steps in the gene expression pathway, including transcriptional initiation, elongation, and mRNA degradation (Badarinarayana et al., 2000; Chen et al., 2002; Daugeron et al., 2001; Deluen et al., 2002; Denis, 1984; Denis et al., 2001; Lemaire and Collart, 2000; Liu et al., 1998; Sakai et al., 1992; Tucker et al., 2002; Tucker et al., 2001). In Chapter III, we further confirmed that CCR4 can affect transcriptional elongation in vivo.

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Moreover, *rpb2* mutations can suppress this effect. Combined with the data that CCR4 can associate with RNA pol II (Chang et al., 1999), these results suggest that CCR4 might affect transcriptional elongation through its interaction with RNA pol II.

It has also been shown that the regulation of one step of gene expression can subsequently affect another one or more steps of this process. For example, pre-mRNA splicing can promote transcription elongation and is required for efficient export of the resulting mRNA in to the cytoplasm (Reed and Hurt, 2002). In Chapter I, we also showed that increased pausing can enhance upstream poly (A) site utilization, indicating a connection between transcriptional termination/cleavage and elongation. Moreover, elongation defects can increase upstream poly (A) site choice at 3' end of *GAL1*, and therefore, enhance shorter but more stable *GAL1* mRNA formation, suggesting a connection between the transcriptional elongation and termination/cleavage which happened in the nucleus and the mRNA degradation in the cytoplasm.

SPT5 Needs SPT4 To Perform Its Function

Recent studies have demonstrated roles for SPT5 and SPT4, as a complex, in the regulation of transcriptional elongation both in yeast and in humans (Hartzog et al., 1998; Kim et al., 1999; Wada et al., 1998; Yamaguchi et al., 1999a; Yamaguchi et al., 1999c). Although it has been shown that an *spt4* deletion has similar phenotypes as some *spt5* defects (Hartzog et al., 1998) and SPT4 has a positive role in transcriptional elongation (Rondon et al., 2003), SPT5 has been shown to contact with the RNA pol II and is thought to play the major role in regulation of transcriptional elongation. The importance of SPT4 in this process is not clear. In Chapter I, we examined the effect of some *spt5*

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alleles and *spt4* deletion on the full-length *lacZ* RNA formation. It was shown that *spt5-4* and *spt4* deletion can reduce full-length *lacZ* RNA formation. Interestingly, the spt5-4 protein is defective for binding SPT4 (G. Hartzog, pers. comm.), indicating that SPT5 requires SPT4 to play a role in transcriptional elongation. In addition, we also showed in Chapter II that the *spt5-4*, *spt5-194*, and *spt4* alleles slowed the rate of degradation of *ADH2* mRNA and the rate of deadenylation of *GAL1* mRNA. spt5-194 proteins is also defective for binding SPT4 (G. Hartzog, pers. comm.). Other *spt5* alleles not defective for binding SPT4 had no effect on *ADH2* mRNA degradation and *GAL1* mRNA deadenylation. These results suggest that the interaction between SPT5 and SPT4 is also required for their roles in regulation of mRNA degradation. Therefore, SPT4 is indispensable for SPT5 to perform its function in the regulation of gene expression.

The Interaction Between The CCR4-NOT Complex And The SPT5/SPT4 Complex Can Affect Their Roles In The Regulation of Gene Expression

Our lab showed that SPT5 can interact with CCR4-NOT complex and inhibit CCR4 deadenylase activity. While CCR4 is present everywhere in the cell, most of SPT5 is localized to the nucleus. Therefore, an important function of SPT5 might be to inhibit CCR4 deadenylase activity in the nucleus so that the mature mRNA in the nucleus will not be deadenylated before export to the cytoplasm and translate into protein. Both CCR4 and SPT5 have been shown to bind with the RNA Pol II CTD (Chang et al., 1999; Lindstrom and Hartzog, 2001) and we also showed in Chapter III that CCR4 and SPT5 have a mixed effect on full-length *lacZ* RNA formation, indicating that SPT5 can affect the function of CCR4 in regulation of transcriptional elongation and inhibit the CCR4

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deadenylase activity in nucleus. CCR4, in turn, can also affect the function of SPT5 in transcription.

Chromatin immunoprecipitation assay showed that SPT5 can associate with both promoters and open reading frames of transcribed genes in vivo (Pokholok et al., 2002). SPT5/SPT4 has also been found to interact with the mRNA capping enzyme and cap methyltransferase (Lindstrom et al., 2003; Wen and Shatkin, 1999). Moreover, SPT5 has been found to associate with RNA processing exosome in the nucleus (Andrulis et al., 2002). It might be possible that SPT5 associates with RNA directly or by binding with other RNA binding proteins in the nucleus and is exported to the cytoplasm together with mature RNA. Therefore, the stability of mRNA can be regulated by SPT5 through its interaction with and inhibition of CCR4 deadenylase activity.

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