

SPT5 affects the rate of mRNA degradation and physically interacts with CCR4 but does not control mRNA deadenylation*

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ABSTRACT

The CCR4-NOT complex has been shown to have multiple roles in mRNA metabolism, including that of transcriptional elongation, mRNA transport, and nuclear exosome function, but the primary function of CCR4 and CAF1 is in the deadenylation and degradation of cytoplasmic mRNA. As previous genetic analysis supported an interaction between SPT5, known to be involved in transcriptional elongation, and that of CCR4, the physical association of SPT5 with CCR4 was examined. A two-hybrid screen utilizing the deadenylase domain of CCR4 as a bait identified SPT5 as a potential interacting protein. SPT5 at its physiological concentration was shown to immunoprecipitate CCR4 and CAF1, and in vitro purified SPT5 specifically could bind to CAF1 and the deadenylase domain of CCR4. We additionally demonstrated that mutations in *SPT5* or an *spt4* deletion slowed the rate of mRNA degradation, a phenotype associated with defects in the CCR4 mRNA deadenylase complex. Yet, unlike *ccr4* and *caf1* deletions, *spt5* and *spt4* defects displayed little effect on the rate of deadenylation. They also did not affect decapping or 5' - 3' degradation of mRNA. These results suggest that the interactions between SPT5/SPT4 and the CCR4-NOT complex are probably the consequences of effects involving nuclear events and do not involve the primary role of CCR4 in mRNA deadenylation and turnover.

Keywords: mRNA Degradation; CCR4-NOT Complex; SPT5/SPT4; Deadenylation

1. INTRODUCTION

The levels of eukaryotic 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 therefore an important control point in determining the abundance of cellular mRNA. A principal mRNA decay pathway in eukaryotic cells is initiated by shortening of the mRNA poly(A) tail at the 3' end (deadenylation), followed by removal of the 5' cap structure (decapping) and subsequent 5' to 3' exonuclease digestion [1-3]. Deadenylation is a key rate-limiting step in the decay of mRNA in that it is the initial step for mRNA degradation. The rate of mRNA deadenylation also plays the most important role in affecting the overall mRNA turnover rate [4], and the poly(A) tail can stimulate the translation of mRNA [5]. Regulation of poly(A) tail length can therefore play multiple roles in controlling protein expression.

In yeast, the evolutionarily conserved CCR4 protein, as part of the CCR4-NOT complex, has been identified as the major cytoplasmic deadenylase [6-8]. Deletion of both CCR4 and PAN2, a poly(A) nuclease that processes the poly(A) tails of mRNA [9], completely blocks deadenylation *in vivo* [8], indicating that these two deadenylases comprise the vast majority of deadenylation function in yeast. Yet, 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 [6-8,10,11], these factors also function in repressing the initiation of transcription through TFIID contacts [12,13], in transcriptional elongation [14,15], in aspects of transcription-coupled DNA repair [16], in regulating nuclear exosome function [17], in controlling mRNA export [18], and in the activation of transcription [19-22]. Consistent with their multiple functions, the CCR4-NOT proteins have been localized to both nuclear and cytoplasmic compartments [7,8].

The CCR4-NOT complex consists of two principal forms, 1.9×10^6 daltons (1.9 MDa) and 1.0 MDa in size [21,23]. The core 1.0 MDa complex has been purified and shown to consist of CCR4, CAF1, the five NOT pro-

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teins (NOT1-5), CAF40, CAF130, and BTT1 [24,25]. The 1.9 MDa complex consists of this core and possibly several other proteins [21,26]. Within the 1.0 MDa complex the arrangement of factors has been delineated. NOT1 is the central protein in the complex [23,27]. CAF1 binds to the middle region of NOT1 and is absolutely required for linking CCR4 to the NOT1-5 proteins [21,23]. The NOT2, -4, and -5 proteins, in contrast, bind to the C-terminal region of NOT1. CAF130, BTT1, and CAF40 are located off of NOT1 in regions separate from CAF1/CCR4 and from NOT2/NOT4/NOT5 [24,25]. The multiple sizes of the CCR4-NOT complex, the numerous proteins present in it, and the distinct architecture of components within the 1.0 MDa complex are consistent with the complex, through its various interactions, playing multiple roles within the cell.

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 is absolutely required for CCR4 enzyme function, and a C-terminal exonuclease III like domain that comprises the CCR4 deadenylase function [6,7,26,28-30].

Previously, genetic analysis had indicated a functional connection between the CCR4-NOT complex and SPT5 [14]. For example, a *ccr4* deletion or overexpression of NOT1 suppressed the cold-sensitive phenotype of the *spt5-242* allele [14]. SPT5 forms a complex with the SPT4 protein [31], called DSIF in mammalian cells [32]. 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 transcriptional elongation [31,33,34]. SPT5/SPT4 has also been found to be involved in pre-mRNA processing and with the exosome [35,36]. These various functions indicate a role for SPT5/SPT4 in pre-mRNA processing

We report here that SPT5/SPT4 can co-immunoprecipitate CCR4 and CAF1. Correspondingly, defects in SPT5 or SPT4 affected the rate of mRNA degradation. However, because these same defects did not display large effects on mRNA deadenylation, the SPT5/SPT4 effects on mRNA degradation and interactions with the CCR4-NOT complex may not be directly related to the primary function of CCR4 in mRNA deadenylation. Instead, these observations suggest that links between SPT5 and CCR4 may result from other interactions that regulate mRNA expression, processing, or transport in the nucleus.

2. MATERIALS AND METHODS

2.1. Yeast Strains and Growth Conditions

Yeast strains are listed in **Table 1**. Growth conditions were on YEP medium (2% yeast extract/1% bacto-peptone) supplemented with the appropriate carbon source as indicated below. Two-hybrid assays were conducted in diploid strain EGY188/EGY191 containing the p34 plasmid (8 *LexA* operators upstream of *lacZ*) following growth on minimal medium [21] lacking uracil, tryptophan, and histidine that was supplemented with 2% galactose/2% raffinose. Standard errors of the means for the assays were less than 20%. The plasmid pCAF1 is YEp13-CAF1-U whose *LEU2* gene is now *URA3*.

2.2. Immunoprecipitations, *in Vitro* Binding Assays, and Western Analysis

Immunoprecipitations with anti-Flag antibody were conducted as previously described [21,37]. Western analysis utilized standard procedures [28]. GST fusion proteins were isolated from *E. coli* as described previously [38] and used to bind Flag purified SPT5-Flag isolated as described [6].

2.3. RNA Analysis

Quantitative S1 nuclease protection assays were conducted as described for determining mRNA degradation rates [39]. 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 [40,41]. Oligonucleotides were radiolabelled at their 5' end with T4 polynucleotide kinase as described [6].

The deadenylation rates and deadenylation end points for *GAL1* and *MFA2pG* mRNA were determined by using a transcriptional pulse-chase and RNase H assay as described previously [1,11,42-44]. For S1 analysis, the *ADH2* probe contained the sequence +1126 to 1167 of *ADH2* and the *ACT1* probe contained the sequence +1039 to +1098 of *ACT1*. For *GAL1*, a 3' *GAL1* probe was used for S1 analysis [42]. A phosphorimager was used to quantitate the relative densities of the *ADH2* or *GAL1* mRNA levels as compared to the control *ACT1*.

3. RESULTS

3.1. SPT5 Can Interact Physically with CCR4

Previously, we had conducted a two-hybrid screen for proteins interacting with the C-terminal deadenylase domain of CCR4 (residues 495-837) and had identified a small piece of SPT5 (residues 137-217) that could interact with

Table 1. Yeast strains.

Strain	Genotype
EGY188	<i>MATa ura3 his3 trp1 LexA_{op}-LEU2</i>
EGY188-1a	isogenic to EGY188 except <i>ccr4::URA3</i>
EGY191	<i>MATα ura3 his3 trp1 LexA_{op}-LEU2</i>
FY1635	<i>MATα his4-912δ lys2-128δ leu2Δ1 ura3-52 spt5-242</i>
FY300	<i>MATa his4-912δ lys2-128δ leu2Δ1 ura3-52 spt5-194</i>
FY1668	<i>MATa his4-912δ lys2-128δ spt5-4</i>
FY1642	<i>MATa his4-912δ lys2-128δ leu2Δ1 ura3-52 SPT5-FLAG</i>
GHY180	<i>MATα ura3-52 leu2Δ1 his 4-912δ lys2-128δ spt4Δ2::HIS3</i>
FY276	<i>MATa leu2Δ1 ura3::TRP1 his4-9128 lys2-128δ spt5-8</i>
FY1635-1a	isogenic to FY1635 except <i>ccr4::URA3</i>
FY1668-1a	isogenic to FY1668 except <i>ccr4::URA3</i> and <i>ura3::HIS3</i>
FY1642-1a	isogenic to FY1642 except <i>ccr4::URA3</i>
FY276-uT-1a	isogenic to FY276 except <i>ccr4::URA3</i> and <i>ura3::TRP1</i>
FY1642-cl	isogenic to FY1642 except <i>caf1::LEU2</i>
L615	<i>Mata his4-912δ lys2-128δ ura3-52 ade2-1 trp5 can1-100 spt5-25</i>

LexA-CCR4 (495-837) (31 U/mg β -galactosidase activity for LexA-CCR4 with B42-SPT5 (SPT5 fused to the *E. coli* transactivity domain B42) [21] as compared to 3.4 U/mg of activity for LexA-CCR4 with B42 alone). To verify that the above described two-hybrid interaction was the result of an *in vivo* physical association, immunoprecipitation analysis was conducted using full-length SPT5. The full length version of SPT5 when it was fused to B42 was capable of immunoprecipitating LexA-CCR4 (495-837) (**Figure 1**, lane 6). These data indicate that the CCR4 exonuclease domain can contact SPT5. Other comparable LexA fusion proteins, such as LexA-MOB1 or LexA-VpU were not immunoprecipitated by B42-SPT5 (**Figure 1**, lanes 4 and 5) and previous data has shown that CCR4 does not interact with the B42 domain alone [28].

Because these above experiments were conducted with overexpressed versions of SPT5 and CCR4, we subsequently used the SPT5-Flag allele present at a single copy at its natural location in the genome to reconduct these immunoprecipitations [31]. Immunoprecipitating SPT5-Flag with anti-Flag antibody resulted in CCR4 being co-immunoprecipitated (**Figure 2**, lane 7). The protein that was immunoprecipitated was identified as CCR4 as no protein co-immunoprecipitated from a strain deleted for CCR4 (lane 9). Also, a *caf1* deletion eliminated the ability of CCR4 to co-immunoprecipitate with SPT5-Flag (lane 8), indicating that the CCR4 contact to at least the CAF1 component of the CCR4-NOT complex was required for CCR4 to be brought down with SPT5-

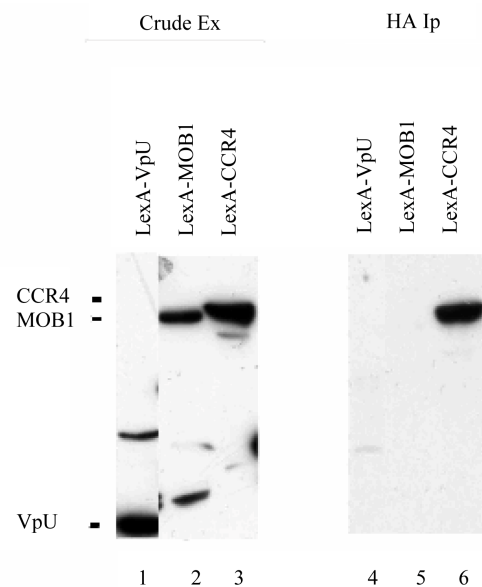


Figure 1. SPT5 co-immunoprecipitates with CCR4-NOT components. Immunoprecipitations were conducted in diploid strain EGY188/EGY191 containing the B42 and LexA fusion proteins as indicated with the HA1 antibody that recognized the HA1 epitope fused to the B42 transactivation domain. Western analysis utilized the anti-LexA antibody. Crude extracts-lanes 1 - 3; HA1 immunoprecipitates-lanes 4 - 6. B42-SPT5 contained full length SPT5; LexA fusions contained full length LexA (1 - 202) fused to complete versions of the indicated protein except for CCR4 (residues 495 - 837 were present).

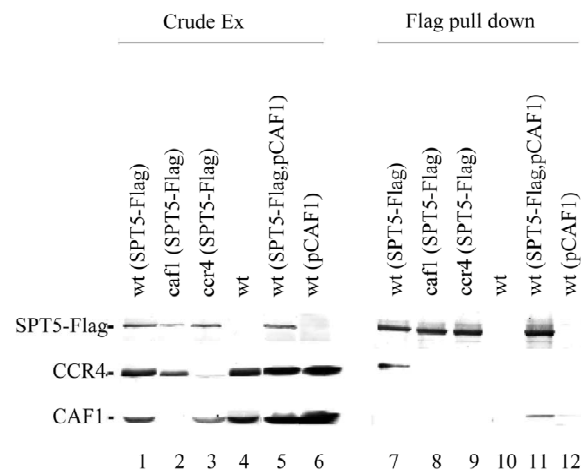


Figure 2. SPT5 immunoprecipitates CCR4 at their physiological concentrations. Immunoprecipitations were conducted with anti-Flag antibody. Crude extracts: lanes 1 - 6; Flag immunoprecipitates lanes 7 - 12. Anti-CCR4, -CAF1, and Flag antibodies were used to identify CCR4, CAF1, and SPT5-Flag, respectively. Lanes 1 and 7: strain FY1642 (wt); lanes 2 and 8: strain FY1642-cl (*caf1*); lanes 3 and 9: FY1642-1a (*ccr4*); lanes 4 and 10: strain FY1639 (no SPT5-Flag); lanes 5 and 11: strain FY1642 with pCAF1 (YEP13-CAF1-U); lanes 6 and 12: strain FY1639 with pCAF1.

Flag. As an additional control, Flag antibody did not co-immunoprecipitate CCR4 from a strain lacking SPT5-Flag (lane 10). These results confirm that CCR4 at its physiological concentration can associate with SPT5.

Although no CAF1 protein appeared to co-immunoprecipitate with SPT5-Flag (**Figure 2**, lane 7), we were concerned that we might have missed observing

CAF1 in the immunoprecipitate if the titer of the anti-CAF1 antibody were insufficient to detect the low levels of CAF1 that might be associated with SPT5. To ensure our ability to detect CAF1 we expressed CAF1 on a high copy plasmid under the control of its own promoter. The abundance of CAF1 protein made when CAF1 is expressed from this high copy plasmid was about 3-to 5-fold more than found in a wild-type CAF1 strain (**Figure 2**, compare lanes 1 to 5 or 4 to 6). When SPT5-Flag was immunoprecipitated with anti-Flag antibody from a strain carrying plasmid expressing CAF1, CAF1 was co-immunoprecipitated (**Figure 2**, lane 11). Although we observed some CAF1 being immunoprecipitated with Flag antibody from a strain lacking SPT5-Flag, the amount was reproducibly less than observed when SPT5-Flag was present (compare lanes 11 to 12, **Figure 2**). While the amount of CAF1 immunoprecipitating with SPT5 is low, these results indicate that CAF1, like CCR4, can associate with SPT5. It is worth nothing, however, that overexpression of CAF1 interfered with SPT5 co-immunoprecipitating CCR4. Since nearly all of the CCR4 found in the cell is present in the CCR4-NOT complexes, the inability of SPT5 to apparently co-immunoprecipitate other components of the complex along with CCR4 may imply that immunoprecipitating SPT5 helps separate CCR4 from the remainder of the complex or makes CCR4 less stably associated with other components of the complex.

We also conducted the reverse experiment. When CCR4 or CAF1 was immunoprecipitated, no SPT5-Flag protein was found to co-immunoprecipitate with the CCR4-NOT complex components (data not shown). This result is expected, however, as it has been previously established that immunoprecipitating the CCR4-NOT complex, when expressed at its physiological concentration, does not immunoprecipitate any proteins other than those which have been identified in the core 1.0 MDa CCR4-NOT complex [21,23,24].

3.2. SPT5 Does Not Associate in CCR4-NOT Complexes

The above data indicates that SPT5 can physically interact with CCR4 and CAF1. SPT5 is unlikely, however, to be a component of the 1 MDa CCR4-NOT complex since neither SPT5 nor SPT4 were detected in the purified 1 MDa complex [24]. It remained possible that SPT5 could be a component of the 1.9 MDa CCR4-NOT complex. To

address this issue we analyzed the migration of SPT5 in crude extracts following Superose 6 gel chromatography. The majority of SPT5 was found to migrate at about 0.7 MDa (**Figure 3(a)**), albeit a small amount was found to migrate at 1.9 MDa. We examined whether the SPT5 migrating at 1.9 MDa was affected by deleting CCR4-NOT factors. SPT5 migration at 1.9 MDa was not affected by *ccr4* (**Figure 3(a)**, bottom panel), or other deletions in CCR4-NOT components such as *caf40*, *caf1*, or *not4* (data not shown), suggesting that SPT5 is not part of the 1.9 MDa CCR4-NOT complex.

3.3. SPT5 Binds CCR4 and CAF1 *in Vitro*

Our results indicate that SPT5 can interact with CCR4 and CAF1 *in vivo* but it is unlikely to be tightly associated with the CCR4-NOT complex. We conducted *in vitro* binding assays to examine more thoroughly the SPT5 interaction with CCR4 and CAF1. These *in vitro* binding assays utilized SPT5-Flag that was expressed in yeast and GST-CAF1 and GST-CCR4 (495-837) isolated from *E.coli* (**Figure 3(b)**, top panel). As shown in **Figure 3B**, bottom panel, SPT5-Flag could be specifically retained by GST-CAF1 and GST-CCR4 (lanes 3 and 5, respectively). GST alone or other comparable GST fusions such as that of GST-CTD (C-terminal domain of RNA pol II) or GST-NOT2 were unable to retain SPT5-Flag (**Figure 3(b)**, lanes 1, 2, and 5, respectively).

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

As the primary role for CCR4 is in the control of mRNA degradation, we subsequently examined the effect of several *spt5* alleles and an *spt4* deletion on the rate of mRNA decay. We observed that *ADH2* mRNA was stabilized approximately by a factor of 2-fold in *spt5-194*, *spt5-4*, and *spt4* deletion strain backgrounds as compared to the wild type strain (**Figure 4**; **Table 2**). In the same strain background, a *ccr4* deletion also resulted in a 2-fold stabilization of *ADH2* mRNA (**Table 2**), indicating that the magnitude of the *spt5/4* effects was equivalent to deleting *ccr4* the major cytoplasmic deadenylase in yeast [8]. Combining *ccr4* with *spt5-4* did not further slow the rate of *ADH2* mRNA degradation (**Table 2**). In contrast, in *spt5-25*, *spt5-8*, and *spt5-242* strain backgrounds, the rate of *ADH2* mRNA decay was not affected (**Figure 4**, left panel; also **Table 2**). *ccr4*, however, in combination with *spt5-242* or *spt5-8* still decreased the rate of *ADH2* mRNA degradation by 2-fold (**Table 2**). We also observed that *spt5-4*, like *ccr4* and *caf1* [8,43,44], slowed the rate of *GALI* mRNA degradation (*spt5-4*: 20 min T1/2 compared to wild type: 12 min T1/2). These results indicate that certain alleles of *spt5* or deletion of *spt4* result in slowing the rate of mRNA decay. Since the

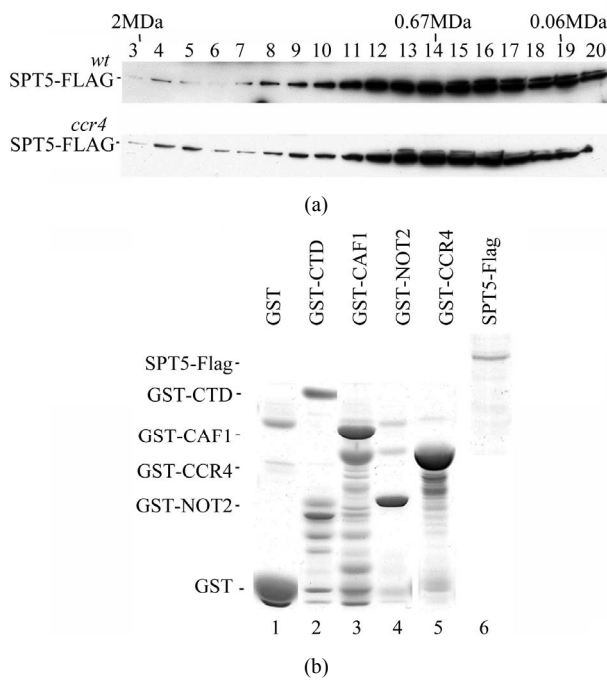


Figure 3. (a) Deletion of *CCR4* does not affect SPT5 migration in a 1.9 MDa complex. Superose 6 gel chromatography was conducted on crude extracts as previously described [23]. Anti-Flag antibody was utilized to detect SPT5-Flag by Western analysis. WT-strain FY1642; *ccr4*-strain FY1642-1a. Molecular weight markers were Blue dextran –2 MDa; Thyroglobulin –0.67 MDa; and bovine serum albumin –0.06 MDa. (b) *CCR4* and *CAF1* can bind SPT5 *in vitro*. Purified GST-*CCR4* (495-837) and GST-*CAF1* (top panel) were used to bind extracts containing Flag purified SPT5-Flag (top panel). GST-pull down experiments were conducted as previously described (38). Top panel—Coomassie stained profiles of GST and Flag fusion proteins; bottom panel—Western analysis of SPT5-Flag retention on glutathione agarose columns containing GST fusion proteins as indicated. All GST fusions contained full-length proteins except for *CCR4* (495-837). GST-*CTD* refers to the C-terminal domain of RNA pol II (52). Input SPT5-Flag represents one-twenty fifth of the amount used to bind the GST fusion proteins. About 12% of the SPT5-Flag was retained by GST-*CAF1* and 2% was retained by GST-*CCR4*.

spt5-4 and *spt5-194* alleles, in contrast to the *spt5-242* allele, have been implicated in disrupting SPT5-SPT4 interactions (G. Hartzog, pers. comm.), the integrity of the SPT5-SPT4 contact may be important to mRNA degradation.

3.5. *spt5* and *spt4* Mutations Do Not Affect mRNA Decapping or Deadenylation

The role of SPT5/SPT4 on mRNA degradation could be occurring by effects on the deadenylation, decapping or 5' to 3' degradation of the RNA transcript [45]. A simple test to determine whether defects in mRNA decay occur at the decapping or later step is to analyze the degradation products of the *MFA2pG* RNA relative to the total

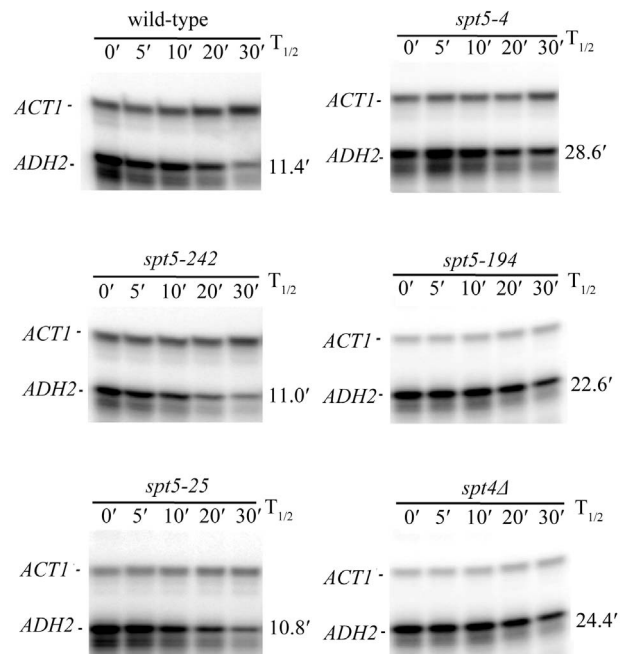


Figure 4. *spt5* and *spt4* alleles affect *ADH2* mRNA degradation rates. Wild-type and mutant strains (see **Table 1**) were pre-grown on YEP medium containing 2% ethanol/2% glycerol prior to shifting to medium containing 4% glucose at zero time and the taking of RNA samples at the times indicated. The *ADH2* and *ACT1* mRNA levels were determined by S1 nuclease protection assays. The *ACT1* RNA was used to standardize loadings for RNA samples at the different time points indicated. $T_{1/2}$ values are for the data shown.

Table 2. Effect of *spt5/spt4* alleles on *ADH2* mRNA degradation rates.

Strain background	mRNA half-life (min)
WT	12
<i>ccr4</i>	23
<i>spt5-4</i>	26
<i>spt5-194</i>	27
<i>spt4</i>	21
<i>spt5-8</i>	12
<i>spt5-25</i>	14
<i>spt5-242</i>	11
<i>spt5-4 ccr4</i>	25
<i>spt5-8 ccr4</i>	31
<i>spt5-242 ccr4</i>	25

ADH2 mRNA half-lives were determined following pre-growth of yeast on YEP medium supplemented with 4% glucose, shifting to medium containing 2% ethanol/2% glycerol for 3 hr, and repression of *ADH2* mRNA synthesis at time zero by the switching to fresh medium containing 8% glucose [6]. RNA half lives were determined using *ACT1* as a standard as described [6]. Values are the average of three to eight determinations. Standard errors of the mean (SEM) were less than 15% except for *spt5-194* whose SEM was 25%. *spt5-242* data represents a single determination.

level of *MFA2pG* RNA present [46]. The *MFA2pG* gene contains a poly(G) tract insertion in its 3' UTR that inhibits exonuclease activity [1]. 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) 3' end RNA fragment will be blocked. As shown in **Figure 5**, *spt5-4* had no obvious effect on the abundance of the poly(G) mRNA fragment relative to the full-length *MFA2pG* mRNA (**Figure 5**, lane 2 compared to lane 1). Careful analysis of the data indicated that *spt5-4* resulted in the poly(G) fragment to be slightly longer than wild-type indicative of a very short poly(A) tail remaining on the poly(G) fragment. Over the course of several experiments, it appears that the oligo poly(A) tail remaining in an *spt5-4* background is about 1-3 nucleotides longer in length as compared to that found in wild-type. Similar results were obtained with *spt5-194* and *spt4* (data not shown). In comparison, *ccr4* also does not block decapping or 5' to 3' mRNA degradation (**Figure 5**, lane 3) and leaves a terminal 20 - 25 poly(A) tail on the fragment [8]. These results indicate that the *spt5/spt4* alleles are not affecting decapping or subsequent 5' RNA degradation.

We subsequently tested whether these alleles affected the rate of mRNA deadenylation, the same step that CCR4 and CAF1 control. Changes in the mRNA deadenylation rate were followed using a transcriptional pulse chase analysis [1] with the *GAL1* mRNA. Expression of *GAL1* was rapidly induced for 15 minutes by adding galactose to a 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. *GAL1* mRNA contains two polyadenylation sites [43,44,47] and the RNA corresponding to the shorter RNA is displayed in **Figure 6**. Similar results were obtained with the longer RNA (data not shown).

As shown in **Figure 6**, we observed that, in a wild-type strain, by following the shortest poly(A) tail fragments present at a particular time [1], the poly(A) tail was degraded to an oligo(A) tail length of approximately 8 - 13 A's by around 10 minutes, and by 15 minutes a significant portion of the mRNA were in the oligo(A) form. It has been shown previously that decapping and 5' to 3' degradation of the transcript occurs once the oligo(A) tail form (8 - 13 A's) appears [1,2,48]. Similarly in the *spt5-242* strain, which had no effect on *ADH2* or *GAL1* mRNA decay rate, the oligo(A) tail was formed in 10 to 15 minutes (**Figure 6**, left panel). In the *spt5-4* and *spt4* deletion strains, the oligo(A) tail form of *GAL1* mRNA routinely appeared in 15 minutes and some was visible at ten minutes (**Figure 6**, right panel). The *spt5-4* and *spt4* alleles had similar effects on the deadenylation of the long *GAL1* mRNA (data not shown). *ccr4* effects

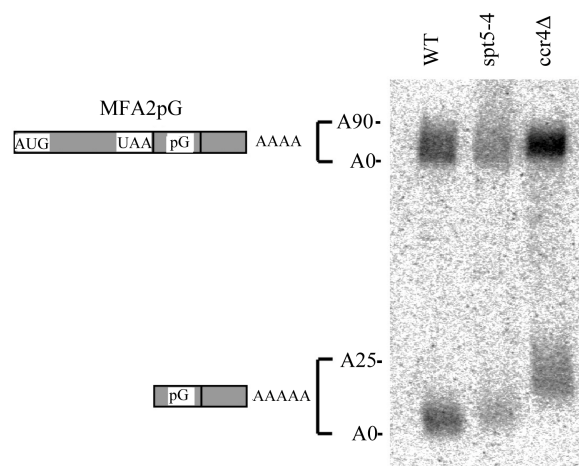


Figure 5. *spt5* and *spt4* alleles do not affect mRNA degradative steps after deadenylation. Wild-type (FY1642), *spt5-4* (FY1668-uH), and *ccr4* (FY1642-1a) strains containing plasmid RP485 (*MFA2pG*) [1] were grown in CAA-U medium [21] with 4% glucose to mid-log stage. Total RNA were extracted after shifting to 2% galactose for 4 hours. *MFA2pG* transcripts were detected following Northern analysis [8]. The upper band represents the full-length *MFA2pG* transcript and the lower band corresponds to the poly(G) to 3' end RNA fragment. The fully deadenylated *MFA2pG* RNAs were identified by RNase H treatment after hybridization to oligo d(T) (not shown) [8].

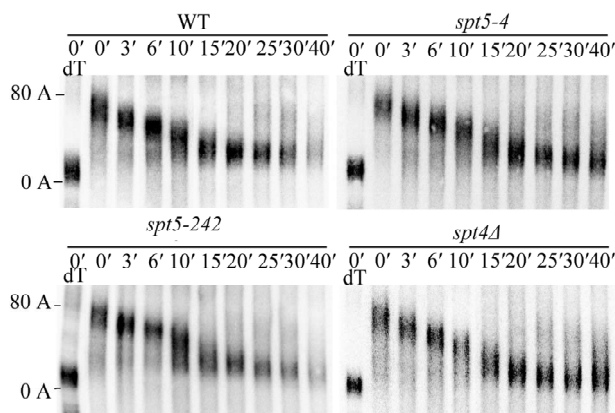


Figure 6. The effect of *spt5/spt4* alleles on the rate of *GAL1* mRNA deadenylation. Wild-type (FY1642), *spt5-4* (FY1668-uH), *spt5-242* (FY1635), and *spt4* (GHY180) strains was shifted from raffinose-containing YEP medium to galactose-containing YEP medium for 15 minutes to induce *GAL1* gene expression. After transcriptional repression by the addition of glucose, total RNA were extracted at different time points as indicated. *GAL1* mRNA was detected by Northern analysis following RNase H treatment of the RNA after hybridization to a 3' *GAL1* RNA probe (Materials and Methods) [42]. The *GAL1* mRNA shown is the short *GAL1* RNA which is 110 nt shorter at its 3' end than the long *GAL1* mRNA [47]. dT-RNase treatment of the RNA following hybridization to oligo d(T) to identify the fully deadenylated species.

on the rate of *GAL1* deadenylation, however, have been shown to be much more severe in which the oligo (A)

did not appear until after at least 40 minutes [8,43,44]. These observations indicate that the deadenylation rate of *GAL1* mRNA is not significantly reduced by the *spt5-4* and *spt4* defects, although they do slow the rate of mRNA degradation to an extent comparable to that observed for *ccr4*. It should be noted that we also examined the ability of the *spt5-4* and *spt4* defect on the ability of SPT5-Flag to associate with CCR4. However, neither of these defects had any effect on the ability of CCR4 to associate with SPT5 (data not shown), suggesting that the effect of these alleles on mRNA degradation was not simply due to their inability to bind CCR4.

4. DISCUSSION

4.1. SPT5 Interacts Physically with CCR4 and CAF1

The SPT5 protein was shown to physically associate in vivo and in vitro with CCR4 and CAF1. Initially, a portion of the SPT5 protein was identified as interacting with the exonuclease domain of CCR4 using a two-hybrid screen, a result verified by co-immunoprecipitation analysis. We subsequently showed that full-length SPT5 could immunoprecipitate full-length CCR4 when both were expressed at their physiological concentrations. CAF1 could also immunoprecipitate with SPT5, although CAF1 needed to be slightly overexpressed for this effect to be observed. In addition, overexpression of CAF1 interfered with CCR4 interaction with SPT5. These results indicate a close proximity in the cell of CCR4 and CAF1 with SPT5. However, the interaction of CCR4 and CAF1 with SPT5 may not be very tight. The fact that other CCR4-NOT components at their physiological concentrations were not co-immunoprecipitated with SPT5 suggests that the CCR4-SPT5 interaction is not bringing along the whole complex.

Several pieces of evidence indicate that SPT5 is not in the most common complexes associated with CCR4 and CAF1. The purified 1.0 MDa CCR4-NOT complex does not contain SPT5 or SPT4 [24]. Also, immunoprecipitating any of the 1.0 MDa CCR4-NOT protein components does not co-immunoprecipitate any other proteins (data not shown) [49]. SPT5 was shown to migrate in complexes sized at both 1.9 and 0.7 MDa, although the bulk of the SPT5 protein is in the smaller complex. Deleting CCR4, CAF1, CAF40 or NOT4 was found to have no effect on SPT5 migration in either of these complexes. Deleting CAF1 has previously been shown to reduce CCR4 presence in the 1.9 MDa CCR4-NOT complex [21] and deleting CCR4 eliminates the association of CAF16 with the 1.9 MDa complex [26]. It is, therefore, unlikely that SPT5 is part of the 1.9 MDa CCR4-NOT complex unless it were retaining its association in this complex through other as yet undetermined protein interactions.

We conclude, therefore, that the interaction between SPT5 and that of CCR4 and CAF1 is of a weaker and/or more transient nature than the interactions observed for components within the CCR4-NOT complex.

4.2. SPT5 Plays a Role in the Control of mRNA Degradation But Not That of Deadenylation

The SPT5 protein has been ascribed several roles in mRNA metabolism including that of affecting transcriptional initiation, mRNA elongation, and possibly splicing and capping [31,35,50]. Based on our previous data supporting a genetic interaction between SPT5 and CCR4 and the current data supporting their physical interaction, we have examined the role of SPT5/SPT4 in mRNA degradation. Defects in both SPT5 and SPT4 were found to reduce the rate of degradation of mRNA. The magnitude of this effect on the rate of *ADH2* mRNA degradation was equivalent to the effect of deleting CCR4, the major cytoplasmic deadenylase in yeast [8]. However, SPT5 or SPT4 defects that affected the degradation rate did not appreciably affect the rate of deadenylation. Also, SPT5 defects did not result in a dramatic deadenylation end-point defect, as has been observed with either *ccr4* or *caf1* [8]. As observed in the deadenylation of MFA2pG, *spt5-4* resulted in a deadenylation end-point with 1 - 3 A's remaining in contrast to the 20 - 25 A's remaining typically observed in a *ccr4* strain. Similarly, in the deadenylation of *GAL1* mRNA, SPT5 or SPT4 defects resulted in the complete or nearly complete deadenylation of the RNA (**Figure 6**) whereas *ccr4* and *caf1* defects result in the accumulation of only partially deadenylated species [8,11,43,44]. Moreover, defects in either SPT5 or SPT4 had no apparent effects on decapping or 5' exonuclease activity. These data indicate that SPT5/SPT4 are involved in affecting mRNA degradation, but do so by a mechanism that is not clearly related to effects on CCR4 or on cytoplasmic mRNA degradation.

SPT5 hence may associate with CCR4 in the nucleus and affect any number of transcriptional or post-transcriptional roles assigned to these factors. It is also possible, given the variety of roles ascribed to SPT5, that its effects on mRNA degradation are indirect. A key conclusion from these studies is that analysis of mRNA degradation rates alone is insufficient to determine whether particular factors are actually involved in the cytoplasmic control of mRNA turnover.

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