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

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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-
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 deacylenase 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 sprt-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% bactopeptone) 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 Asays, 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 phosphorimagere 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 deacylenase 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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGY188</td>
<td>MATa ura3 his3 trp1 LexAop-LEU2</td>
</tr>
<tr>
<td>EGY188-1a</td>
<td>isogenic to EGY188 except ccr4::URA3</td>
</tr>
<tr>
<td>EGY191</td>
<td>MATa ura3 his3 trp1 LexAop-LEU2</td>
</tr>
<tr>
<td>FY1635</td>
<td>MATa his4-912δlys2-128δleu2Δ1 ura3-52 sptr5-242</td>
</tr>
<tr>
<td>FY300</td>
<td>MATa his4-912δlys2-128δleu2Δ1 ura3-52 sptr5-194</td>
</tr>
<tr>
<td>FY1668</td>
<td>MATa his4-912δlys2-128δsptr5-4</td>
</tr>
<tr>
<td>FY1642</td>
<td>MATa his4-912δlys2-128δleu2Δ1 ura3-52</td>
</tr>
<tr>
<td>GHY180</td>
<td>MATa ura3-52 leu2Δ1 his 4-912δlys2-128δsptr5-8</td>
</tr>
<tr>
<td>FY276</td>
<td>MATa leu2Δ1 ura3::TRP1 his4-9128 lys2-128δsptr5-8</td>
</tr>
<tr>
<td>FY1635-1a</td>
<td>isogenic to FY1635 except ccr4::URA3</td>
</tr>
<tr>
<td>FY1668-1a</td>
<td>isogenic to FY1668 except ccr4::URA3 and ura3: HIS3</td>
</tr>
<tr>
<td>FY1642-1a</td>
<td>isogenic to FY1642 except ccr4::URA3</td>
</tr>
<tr>
<td>FY276-ut-1a</td>
<td>isogenic to FY276 except ccr4::URA3 and ura3: TRP1</td>
</tr>
<tr>
<td>FY1642-cl</td>
<td>isogenic to FY1642 except caf1::LEU2</td>
</tr>
<tr>
<td>L615</td>
<td>Mata his4-912δlys2-128δura3-52 ade2-1 trp5 can1-100 sptr5-25</td>
</tr>
</tbody>
</table>

LexA-CCR4 (495-837) (31 U/mg β-galactosidase activity for LexA-CCR4 with B42-SPT5 (SPT5 fused to the E. coli transactivation 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 re-examine 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 immunoprecitate-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 physiologic 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.

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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 nor4 (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 GAL1 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 glutathine 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 MFα2pG RNA relative to the total

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

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

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 \textit{MF}A2pG RNA present [46]. The \textit{MF}A2pG 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, \textit{spt}5-4 had no obvious effect on the abundance of the poly(G) mRNA fragment relative to the full-length \textit{MF}A2pG mRNA (Figure 5, lane 2 compared to lane 1). Careful analysis of the data indicated that \textit{spt}5-4 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 \textit{spt}5-4 background is about 1-3 nucleotides longer in length as compared to that found in wild-type. Similar results were obtained with \textit{spt}5-194 and \textit{spt}4 (data not shown). In comparison, \textit{ccr}4 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 \textit{spt}5/\textit{spt}4 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 \textit{GAL1} mRNA. Expression of \textit{GAL1} was rapidly induced for 15 minutes by adding galactose to a raffinose growth culture and then repressed with glucose to mid-log stage. Total RNA were extracted after shift of 2% galactose for 4 hours. \textit{MF}A2pG transcripts were detected following Northern analysis [8]. The upper band represents the full-length \textit{MF}A2pG transcript and the lower band corresponds to the poly(G) to 3' end RNA fragment. The fully deadenylated \textit{MF}A2pG RNAs were identified by RNase H treatment after hybridization to oligo d(T) (not shown) [8].

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 \textit{spt}5-242 strain, which had no effect on \textit{ADH2} or \textit{GAL1} mRNA decay rate, the oligo(A) tail was formed in 10 to 15 minutes (Figure 6, left panel). In the \textit{spt}5-4 and \textit{spt}4 deletion strains, the oligo(A) tail form of \textit{GAL1} mRNA routinely appeared in 15 minutes and some was visible at ten minutes (Figure 6, right panel). The \textit{spt}5-4 and \textit{spt}4 alleles had similar effects on the deadenylation of the long \textit{GAL1} mRNA (data not shown). \textit{ccr}4 effects on the rate of \textit{GAL1} deadenylation, however, have been shown to be much more severe in which the oligo (A)
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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REFERENCES


Evidence that Spt4, Spt5, and Spt6 control transcriptional regulatory complex.

Caffe, and Caf16 reveal a functional connection between the Ccr4-notch complex.


Caffe, and Caf16 reveal a functional connection between the Ccr4-notch complex.


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