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Identification of *ebs*1, *lsm*6 and *nup*159 as suppressors of *spt*10 effects at *ADH*2 in *Saccharomyces cerevisiae* suggests post-transcriptional defects affect mRNA synthesis[*]

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ABSTRACT

Suppression of the effects of an *spt*10 mutation on *ADH*2 expression is a phenotype shared by a small number of genes whose protein products are either components of the CCR4-NOT complex required for mRNA deadenylation and degradation (*CCR*4, *CAF*1, *NOT*4) or have been shown to interact with the complex (*DBF*2, *SRB*9, *SRB*10). In this work, we conducted a screen for additional suppressors of *spt*10 at *ADH*2 to identify new factors related to CCR4 function. In addition to reisolating *ccr*4 and *caf*1 alleles, three previously unidentified suppressors of *spt*10 were obtained: *ebs*1, *lsm*6, and *nup*159. These three genes are known or presumed to affect mRNA export or degradation. Mutations in *EBS*1, *LSM*6 and *NUP*-159 not only suppressed *spt*10-induced *ADH*2 expression but also, like *ccr*4 and *caf*1 defects, reduced the ability of *ADH*2 to derepress. None of these defects affected the expression of CCR4-NOT complex components or the formation of the CCR4-NOT complex. The reduced *ADH*2 expression was also not the result of increased degradation of *ADH*2 mRNA, as the *lsm*6 and *nup*159 alleles, like that of a *ccr*4 deletion, actually slowed *ADH*2 degradation. Our results indicate that alterations in factors that slow mRNA degradation or affect mRNA transport may also interfere with the synthesis of mRNA and suggest an integration of such events in gene expression.

Keywords: CCR4-NOT Complex; Transcription; mRNA Degradation; mRNA Export

1. INTRODUCTION

The CCR4 protein is involved in several processes controlling the metabolism of mRNA in the cell: mRNA initiation, elongation, and degradation. *CCR*4 was originally identified as a mutation that suppressed the increased *ADH*2 expression on glucose caused by a *spt*10 allele, *cre*1-1 [1]. *SPT*10 is known to function in part by controlling histone gene expression, which in turn can alter chromatin structure and gene expression at sensitive loci [2]. The interaction of *CCR*4 with *SPT*10 is not limited to the *ADH*2 promoter, as *ccr*4 also suppressed *spt*10 at the *his*4-912δ locus [3]. *CCR*4 is also required for maximal expression of *ADH*2 and other nonfermentative genes [1] and appears involved in other processes such as cell wall integrity, amino acid metabolism, cell cycle progression, and DNA repair [4-8]. The effects of CCR4 at *ADH*2 and presumably many of these other processes occur at the transcriptional level implicating CCR4 in the control of mRNA initiation.

CCR4 functions in the context of two CCR4-NOT complexes, 1.0 and 1.9 MDa in size. The 1.0 MDa complex contains CCR4, CAF1, the five NOT proteins (NOT1-NOT5) and three other proteins, CAF40, CAF130 and BTT1 [6,9,10]. In addition to CCR4, mutations in both *CAF*1 and *NOT*4 suppress the effects of an *spt*10 mutation at *ADH*2 and affect the derepression of *ADH*2, suggesting that they also are involved in the activation of transcription [6,11].

The NOT1-5 proteins, however, are clearly involved in the repression of transcription [12,13], initially identified by their binding and restricting TFIID access to non-canonical TATAA sequences [14]. In addition, the CCR4-NOT proteins have been suggested to play a role in mRNA elongation [15]. Recent studies indicate that the CCR4-NOT complex is responsible for restarting transcription after transcriptional arrest has occurred [16].

One defined biochemical function for CCR4 is that it is the catalytic component of the cytoplasmic deadenylase complex [18-20]. CCR4 mRNA poly (A) deadenylation is the first step in targeting the mRNA for degradation and *caf*1 or *not*4 defects also lead to defects in deadenylation [19,20]. A *ccr*4 defect, therefore, in stabi-

lizing mRNA would augment mRNA levels. This effect would be opposite in nature to the *ccr*4 suppression of *spt*10 effects at *ADH*2 or the requirement of CCR4 for *ADH*2 and other genes' syntheses. The CCR4 effect on *ADH*2 RNA accumulation implies that it operates by mechanisms other than or in addition to controlling mRNA degradation.

To gain further insight into the mechanisms of how CCR4 activates gene expression, we have reconducted the screen for factors that can suppress *spt*10-enhanced expression at *ADH*2. The only known suppressors of *spt*10 effects are defects in the CCR4, CAF1 and NOT4 proteins, all components of the CCR4-NOT complex, and the DBF2, SRB9 and SRB10 proteins, which are known to associate with the CCR4-NOT complexes [5, 21]. The SRB9 and SRB10 proteins have been shown to be part of mediator, the transcriptional activator complex [21,22]. Hence, the ability to suppress *spt*10-enchanced *ADH*2 expression reflects a common factor related to CCR4 function, and possibly to a role not directly involving effects on mRNA degradation. Utilizing this screen for suppressors of *spt*10, we have identified three new genes which when mutated are capable of suppressing *spt*10: *NUP*159, *LSM*6 and *EBS*1. These three factors, like CCR4, CAF1 and NOT4 are known or presumed to be involved in controlling mRNA export or degradation. These results suggest that defects in factors involved in post-transcriptional regulation of mRNA metabolism may result in corresponding decreases in transcriptional initiation. The CCR4 requirement at *ADH*2 may, therefore,

result from indirect effects involving the integration of mRNA synthesis with the subsequent nuclear export and degradation of mRNA.

2. MATERIALS AND METHODS

2.1. Yeast Strains and Growth Medium

Yeast strains are listed in **Table 1**. Strains were grown on YEP medium (1% yeast extract/2% Bacto-peptone) supplemented with 2% glucose and 2% agar (YD plates). Allyl alcohol was added to YD plates in the concentration indicated in the test. ADH assays were conducted as previously described [6].

2.2. Analysis of *spt*10 Suppressors

Standard genetic techniques were used to identify complementation groups. Yeast transformations were conducted with either an YCp50-LEU2, YEpl3, or YEp24 based genomic library. Western analysis was conducted as described [6] using antibody specific to CCR4-NOT protein components [23].

2.3. RNA Analysis

S1 nuclease protection assays were conducted to quantitate *ADH*2 mRNA levels as described [17,24]. The rates of *ADH*2 mRNA half-lives were determined following growth of yeast on YEP medium containing 2% glycerol/ 2% ethanol and shifting to YEP medium containing 8% glucose as previously indicated [17].

Table 1. Yeast strains.

Strain	Genotype
553-4b	*MATa adh*1-11 *adh*3 *cre*1-1 *ural leu*2 *trp*1
553-4b-10	*MATa adh*1-11 *adh*3 *cre*1-1 *ccr*4-10 *ural leu*2 *trp*1
43-2B	*MATα adh*1-11 *adh*3 *ural his*4
43-2B-M1	*MATα adh*1-11 *adh*2-1 *adh*3 *ural his*4
1076-2c	*MATα ura*3 *his*3 *leu*2 *trp*1 *ccr*4::*URA*3
1005-2-3c	*MATa adh*1-11 *his*3 *ura*3 *trp*1 *leu*2 *caf*1::*LEU*2 *spt*10::*trp*1
1005-2-3c-s	*MATα adh*1-11 *his*3 *ura*3 *trp*1 *leu*2 *caf*1::*LEU*2 *spt*10::*trp*1
BA1-1	*MATα adh*1-11 *leu*2 *asg*1-4-6
BA2-1	*MATα adh*1-11 *leu*2 *his*4 *ebs*1-1-42
BA3-1	*MATα adh*1-11 *leu*2 *lsm*6-5-17
BA4-1	*MATα adh*1-11 *leu*2 *trp*1 *asg*4-4-33
BA5-1	*MATα adh*1-11 *leu*2 *trp*1 *asg*5-5-37
BA6-1	*MATα adh*1-11 *trp*1 *nup*159-6-72
994-2	*MATα adh*1-11 *his*3 *ura*3 *leu*2 *spt*10::*TRP*1
994-2-ΔEBS1	*MATα adh*1-11 *his*3 *ura*3 *leu*2 *spt*10::*TRP*1 *ebs*1::*URA*3
LGY101	*MATα rat*7-1 *his*3Δ200 *ura*3-52 *leu*2Δ1
AEMY19	*MATα trp*1Δ1 *his*3Δ200 *ura*3-1 *leu*2-3,-112 *lsm*6::*URA*3

3. RESULTS

3.1. A Screen for Suppressors of *spt*10 at *ADH*2

In order to identify other factors that are functionally related to the CCR4-NOT proteins, we conducted a search for additional suppressors of the *spt*10 effects at the *ADH*2 locus using a strain carrying the defective *adh*1-1 allele. The increased expression of *ADH*2 caused by *spt*10 results in an inability of strains to grow in the presence of ally1 alcohol. Ally1 alcohol can be converted by ADH enzymes to acrolein that kills the cells. While *ccr*4 alleles were previously identified by such a screen [1], in order to identify new suppressors we used a modified selection process. In the previous screen, mutations that allowed growth on ally1 alcohol-containing plates were then tested for sensitivity to the respiratory inhibitor Antimycin A; complete loss of both ADH I and ADH II enzyme activity in the cell would require the utilization of oxidative phosphorylation rendering such cells Antimycin A sensitive. This secondary requirement resulted in the elimination of nearly 90% of the ally1 alcohol revertants [1]. Since there is a narrow range of ADH enzyme activities that allows growth on both ally1 alcohol and resistance to antimycin A we were intent on identifying new suppressors of *spt*10 that resulted in ADH II activity that fell into this range.

Strain 553-4b, containing a *spt*10 mutant allele called *cre*1-1, was chosen for this screen since the *cre*1-1 allele, unlike an *spt*10 deletion, does not result in phenotypes typically associated with *ccr*4 or *not* defects: these phenotypes include temperature sensitivity, slow growth, and sensitivity to caffeine. Approximately 1×10^8 cells of strain 553-4b were spread onto YD plates containing 10 mM ally1 alcohol. 198 ally1 alcohol resistant colonies were selected for analysis. Complementation analysis, following mating each of these revertants to strain 43-2B-M1 carrying a defective *adh*2 allele, identified 99 of these revertants as containing mutations in the *ADH*2 gene (data not shown).

Since *ccr*4 and defects in other CCR4-NOT complex components are sensitive to caffeine and a number of other factors such as high temperature and elevated salt concentration, the remaining 99 revertants were tested for sensitivity to these conditions. Sixteen of the 99 showed sensitivity to one or more of the following conditions: 8 mM caffeine, 37°C, 16°C, 0.75 M MgCl$_2$, or 0.2 M LiC1 (**Table 2**; data not shown) and were chosen for further analysis.

### 3.2. Identification of Known Suppressors of *spt*10

To test if any of these 16 revertants generated in this screen were *ccr*4 mutations, Western analysis was con-

ducted on each strain to determine if the CCR4 protein was absent. Extracts from only one revertant, 553-4b-1-65, lacked detectable CCR4 protein (data not shown; see also **Figure 1**). In addition, genetic complementation analysis was conducted by crossing a *ccr*4-containing strain to all the revertants. In this case, only revertant 553-4b-1-65 failed to complement a *ccr*4 allele. Similarly, a *caf*1 strain, 1005-2-3c-s, was crossed to all revertants to identify those that contained *caf*1 mutations. Re-

Table 2. Phenotypic characterization of *spt*10 revertants.

Strain	Allele	8 mM Caff	YD 37°	0.2 M LiCl
553-4b-10	*ccr*4-10	–	w/+	–
1-65	*ccr*4-1-65	–	w/–	–
4-26	*caf*1-4-26	–	–	–
1-42	*ebs*1-1-42/*asg*2	+	–	–
5-17	*lsm*6-5-17/*asg*3	–	–	+
6-72	*nup*159-6-72/*asg*6	–	+	+
4-6	*asg*1-4-6	–	+	+
1-36	*asg*1-1-36	–	+	+
1-52	*asg*1-1-52	–	+	+
1-53	*asg*1-1-53	–	+	+
1-58	*asg*1-1-58	–	+	+
4-11	*asg*1-4-11	–	+	+
5-15	*asg*1-5-15	–	+	+
5-21	*asg*1-5-21	–	+	+
5-84	*asg*1-5-84	–	+	+
6-16	*asg*1-6-16	–	+	+
4-33	*asg*1-4-33	–	+	+

Yeast strains were grown on YEP medium containing 2% glucose supplemented with 8 mM caffeine (8 mM caff) or 0.2 M LiC1. Growth was at 30°C except as indicated. "+": very good growth; "w/+": good growth; "w/–": poor growth; "–": no growth.
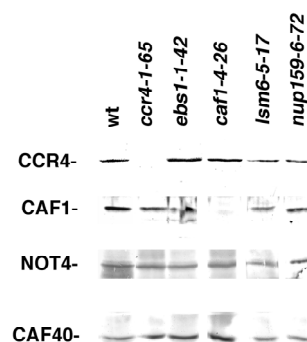
Figure 1. *nup*159, *lsm*6 and *ebs*1 alleles do not affect the expression of CCR4-NOT components. Western analysis was conducted on yeast extracts with antibodies directed against CCR4, CAF1, NOT4, and CAF40. Left lane-(wt) strain 553-4b; other lanes-strain 553-4b except containing the allele as indicated.

vertants 553-4b-4-26 and -6-72 partially failed to complement the *caf*1 mutation, although not completely, so that we were unable to conclusively identify these revertants as containing *caf*1 mutations. To clarify this result, the mutation in revertant 553-4b-4-26 was cloned by complementation using a genomic library and the complementing gene was shown to contain the complete *CAF*1 gene following DNA sequence analysis. Subsequent retransformation of the *CAF*1 containing genomic clone into revertant 553-4b-6-72 failed to complement its defects, demonstrating that 553-4b-4-26 is the only *caf1* mutation produced by this screen. Crosses with strains containing either a *dbf*2 or *not*4 mutation showed that none of the remaining revertants carried mutations in either of these genes.

Additional complementation analysis grouped the remaining 14 revertants that displayed pleiotropic effects into four complementation groups (**Table 2**). Most of the complementation groups were represented by a single revertant, whereas complementation group designated *asg*1 contained 10 members.

ADH II enzyme activity assays were conducted on the 16 revertants from each of these complementation groups, the parent strain 553-4b, and 553-4b-10, which contains the previously characterized *ccr*4-10 allele (**Table 3**). The ADH II assay values that were obtained confirm that growth on ally1 alcohol was due to reduced expression of *ADH*2. As expected, values for many of the revertants were not as low as the *ccr*4-containing strain 553-4b-10 since many of the revertants identified were not Antimycin A sensitive (data not shown). On glucose, most rever-

Table 3. *ADH*2 expression in *spt*10 revertants.

Allele	ADH II enzyme activity		*ADH*2 mRNA	
	Glc	EtOH	Glc	EtOH
wt	52	2400	1.0	1.0
*ccr*4-10	16	620	0.32	ND
*ccr*4-1-65	22	730	ND	ND
*caf*1-4-26	19	250	0.12	0.39
*ebs*1-1-42	27	1700	0.59	0.64
*lsm*6-5-17	15	1400	0.50	0.38
*nup*159-6-72	26	240	0.41	0.49
*asg*1-4-6	24	570	0.41	ND
*asg*1-4-33	43	940	ND	ND

ADH II enzyme activities were conducted as described previously following growth in YEP medium supplemented with 8% glucose (Glc) or 3% ethanol (EtOH) [46]. The standard error of the mean (SEM) for all ADH assays was less than 20% and the values represent the average of at least four determinations. *ADH*2 mRNA levels were quantitated using an S1 nuclease protection assay as described in **Figure 2** using *ACT*1 mRNA levels as a standard. The *ADH*2 mRNA levels were normalized against levels found in the parent strain 553-4b which is given a value of 1.0. The SEMs for the *ADH*2 mRNA levels were less than 20% except for *lsm*6 and *caf*1 under ethanol growth conditions in which the values were 37% and 28%, respectively.

tants displayed at least a two-fold reduction of ADH II activity compared to the parental strain (**Table 3**). Assay values for these revertants grown in ethanol medium showed a wide range of values. Most of the activity levels in these revertants fell into an intermediate range around 1000 mU/mg of ADH II. For the ten alleles of *asg*1 the range of ADH II activities under ethanol conditions was from 440 to 1200 mU/mg. These results indicate that the *spt*10 revertants, like previously identified *ccr*4 and *caf*1 alleles, suppressed both *spt*10-induced *ADH*2 expression and the ability of *ADH*2 to derepress under non-fermentative growth conditions.

3.3. Cloning of *ASG*2, 3, and 6

In order to clone the respective genes for the remaining unidentified complementation groups, a representative from each group was transformed with either or both low and high copy yeast genome libraries. Depending on the phenotype of the representative mutant, selection for complementing plasmids was conducted on different media. Repeated attempts to complement *asg*1 failed, however, and complementing clones were only obtained for *asg*2, *asg*3 and *asg*6.

For strain 553-4b-6-72 (*asg*6), complementing plasmids were selected on 8 mM caffeine plates. A single plasmid (6-72-1), derived from a YCp50-LEU2 genomic library, was identified and was shown to contain an 8.4 Kbp fragment of Chromosome IX. Three genes were identified on this plasmid that could potentially be responsible for complementing 553-4b-6-72: YIL113w, *POR*2 and *NUP*159. Given the function of *NUP*159 as involved in mRNA transport out of the nucleus and that a defect in such a gene could result in lowered gene expression, a *nup*159 strain (LGY101) was obtained carrying the *rat*7-1 mutation (*RAT*7 is the same as *NUP*159). Crossing strain LGY101 to strain 553-4b-6-72, resulted in diploids that failed to grow on 8 mM caffeine plates, indicating that the *asg*6-6-72 mutation could not complement the *rat*7-1 mutation. *asg*6-6-72 was therefore designated as an allele of *NUP*159. The NUP159 protein has recently been shown to play an important role in mRNA transport by aiding DBP5 remodeling of the mRNP complex [25], but otherwise has no direct role in mRNA expression.

Revertant 553-4b-5-17 (*asg*3) when transformed with the yeast genomic library YEp13 yielded one transformant that was caffeine resistant and that was also unable to grow on 10 mM ally1 alcohol media. The insert contained in plasmid 5-17-1 was determined to be a 6.5 Kbp fragment of chromosome IV containing three genes, *ATP*17, *LSM*6 and *RGA*2. RGA2 has been described as a GTPase activating protein, LSM6 is involved in RNA splicing processing and mRNA decapping [26,27] and

ATP17 is a subunit of ATP synthase. A *BamHI-SalI* fragment of plasmid 5-17-1 that contained *ATP*17 and *LSM*6 was subcloned into pRS315, a single copy plasmid containing a *LEU*2 gene. This plasmid was able to complement the caffeine sensitive phenotype of 5-17, showing that one of these two genes, but not *RGA*2, was responsible for this complementation. Also, following transformation of a YCp50-LEU2, single copy yeast genomic library, into 553-4b-5-17, a single caffeine resistant colony was obtained that contained a plasmid with both *ATP*17 and *LSM*6 genes (data not shown). An *lsm*6-containing strain, AEMY19, was crossed to 5-17, and the resulting diploid strain failed to grow in the presence of 8 mM caffeine. This failure to complement indicates that the *asg*3-5-17 mutation is an allele of *lsm*6.

Strain 553-4b-1-42 (*asg*2) after transformation with a YEp13 high copy yeast genomic library yielded a single plasmid that could complement the ability to grow at 37°C and the allyl alcohol phenotype. The insert was found to be a 4.3 Kbp region of chromosome IV containing a single gene, *EBS*1. EBS1 was initially reported to be involved in telomere maintenance based on its sequence similarity to EST1 [28] but more recently it has been shown to be the ortholog of the higher eukaryotic nonsense-mediated decay protein SMG7 [29]. Its involvement in nonsense mediated decay may be only one means that its is involved in mRNA expression, as it has also been demonstrated that EBS1 binds eIF4E, the mRNA cap binding protein required for translational initiation. In fact, EBS1 is a negative regulator of translation [30]. EBS1 also binds DCP1 [31], a component of the mRNA decapping complex [20]. Its exact role in decapping is not understood, as it does not clearly affect mRNA degradation rates (R. Parker, pers. comm), and more recent studies indicate that it does not affect *MFA*2 decapping [29]. Removal of amino acids 456-783 from EBS1, that overlap a putative RNA recognition motif, resulted in an EBS1-expressing plasmid that was unable to complement the 37°C growth defect of the 553-4b-1-42, demonstrating that the *EBS*1 gene complemented 553-4b-1-42 and that this deleted region is required for EBS1 function. We also showed that deleting *ebs*1 gave the same phenotype as the *ebs*1-1-42 allele in that it was capable of suppressing an *spt*10 deletion in its effects on either *ADH*2 or *HIS*4 (data not shown).

3.4. *spt*10 Suppressors Affect *ADH*2 Expression at the Transcriptional Level

The identification of three additional suppressors of *spt*10 as factors involved in mRNA degradation (LSM6 and EBS1), translation (EBS1), and transport (NUP159) coincide with the role of the other *spt*10 suppressors, CCR4, CAF1 and NOT4, that have known roles in post-transcriptional control of mRNA metabolism, including

that of mRNA degradation, translation, and elongation [17-20,32]. We subsequently investigated whether the effects of these new *spt*10 suppressors were the result of reducing *ADH*2 mRNA expression. *ADH*2 mRNA levels were quantitated by S1 analysis and are summarized in **Table 3** (see also **Figure 2**, where the zero time points are representative assays conducted under ethanol growth conditions). Each of the *lsm*6-17, *ebs*1-1-42, *nup*159-6-72 and *asg*1-4-6 alleles reduced *spt*10-enhanced *ADH*2 mRNA expression by around two-fold or more under glucose growth conditions and by a similar level following ethanol growth conditions. It should be mentioned that the *spt*10 allele does not affect ADH II enzyme levels following ethanol growth conditions [1,3]. Therefore, the effects of these suppressor mutations on ADH II activity following non-fermentative growth represent direct effects on *ADH*2 expression. These results indicate that these alleles reduce ADH II enzymatic levels by primarily reducing the steady state levels of *ADH*2 mRNA levels. Therefore, as previously found for CCR4 and CAF1 [3,11] (**Table 3**), these new *spt*10 suppressors are required for activating *ADH*2 expression. It should be noted, however, that under ethanol growth conditions the actual steady state reduction in *ADH*2 mRNA levels by *lsm*6 and *nup*159 did not appear to directly correlate with the degree of reduction in the levels of ADH II enzyme activity (**Table 3**). The cause for this is unclear but could result from the unknown nature of the mutation or possibly even from pleiotropic effects on the translatability or expression of the mRNA.

3.5. *lsm*6, *ebs*1, and *nup*159 Alleles Do Not Affect Expression of CCR4-NOT Complex Components

One likely cause for the suppression of *spt*10 and of *ADH*2 gene expression by the *lsm*6, *ebs*1 and *nup*159 alleles is that they reduce the levels of key CCR4-NOT complex components. The levels of CCR4-NOT complex proteins were subsequently determined by Western analysis in strains carrying these mutant alleles. As shown in **Figure 1**, *lsm*6, *ebs*1 and *nup*159 had no effect on the relative abundance of CCR4, CAF1, and other complex components. We also found that the integrity of the CCR4-NOT complex, as evidenced by co-immunoprecipitation, to be unaffected by the *lsm*6, *ebs*1, and *nup*159 alleles (data not shown). These data indicate that *lsm*6, *ebs*1, and *nu*159 affect *ADH*2 expression by a means other than through indirect effects on the CCR4-NOT complex.

3.6. *lsm*6, *ebs*1, *nup*159 Decrease or Have No Effect on *ADH*2 mRNA Half-Lives

Both LSM6 and EBS1 have known or suggested roles in
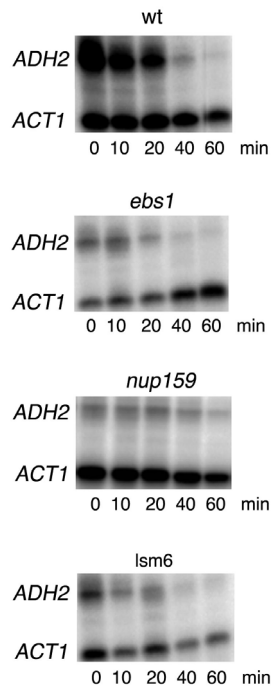
Figure 2. *nup*159, *lsm*6 and *ebs*1 alleles increase or have no effect on *ADH*2 mRNA stability. S1 nuclease protection assays were performed on 40 µg of total RNA using an oligonucleotide specific to the 5' region of *ADH*2 and to *ACT*1 [17]. *ADH*2 mRNA stability was determined as previously described [17] following shifting of yeast from ethanol-containing medium to glucose-containing medium at time zero. The *ebs*1, *nup*159 and *lsm*6 alleles are the same as described in **Table 2**.

controlling the rate of mRNA degradation, with *lsm*6 slowing mRNA decapping and EBS1 interacting with the decapping complex or controlling at least nonsense mediated decay [26,27,29]. It was theoretically possible, however, that the *lsm*6 and *ebs*1 alleles identified in this *spt*10 screen were mutations that enhanced the rate of *ADH*2 mRNA degradation, thereby suppressing *spt*10 effects on the initiation of *ADH*2 synthesis and reducing *ADH*2 expression under non-fermentative growth conditions. To test this hypothesis we examined the rate of *ADH*2 mRNA degradation in each of these mutated strains. *ADH*2 mRNA half-lives were determined following pre-growth of yeast under ethanol (non-fermentative conditions) and shifting them to glucose growth conditions upon which *ADH*2 syntheses is rapidly shut off. Although in an *spt*10 background there is a low level of *ADH*2 expression under glucose growth conditions, this level is much less than found under non-fermentative conditions (**Table 3** and **Figure 2**) allowing mRNA half-lives to be readily calculated.

The *ADH*2 mRNA half-life was found to be about 12 min in agreement with previous results [17,33]. Both

*lsm*6-5-17 and *nup*159-6-72 slowed *ADH*2 mRNA degradation (**Figure 2** and summarized in **Table 4**) while the *ebs*1-1-42 allele had no effect on *ADH*2 mRNA stability. These results indicate that *lsm*6, *nup*159, *ccr*4 [17,33] and *caf*1 (data not shown) all increase the stability of *ADH*2 mRNA. Their effects on reducing the steady state levels of *ADH*2 mRNA must hence even be greater than the effects that are summarized in **Table 3**. Clearly, the reduction in *ADH*2 mRNA levels caused by these alleles is not occurring through direct effects on enhancing *ADH*2 mRNA degradation. These results, in contrast, suggest that interfering with post-transcriptional processes can reduce the level of mRNA synthesis.

4. DISCUSSION

CCR4 has been considered an activator of transcription since it first was identified as a suppressor of *spt*10 effects at *ADH*2 and as being required for *ADH2* derepression following non-fermentative growth [1,3]. The mechanism by which it and other factors related to it, such as CAF1 or NOT4, are required for maintaining *ADH*2 mRNA levels has remained, however, obscure [5, 6,11,21]. The identification of the CCR4-NOT complex as involved in mRNA deadenylation, with CCR4 and CAF1 as deadenylases [17-20,32,34], indicate that *ccr*4 and *caf*1 deletions actually stabilize *ADH*2 mRNA and hence raise its mRNA levels, not lower them [17,33]. In this report we identify three new factors, NUP159, LSM6 and EBS1, whose defects, like those of *caf*1 and *ccr*4, suppress *spt*10-enhanced *ADH*2 expression and decrease the ability of *ADH*2 to derepress. The known or putative roles of NUP159, LSM6, and EBS1 in the post-transcriptional regulation of mRNA suggests, therefore, a functional link to CCR4 and CAF1 and a common mechanism by which they all affect *ADH*2 expression.

The *lsm*6-5-17, *nup*159-6-72 and *ebs*1-1-42 alleles effect on *ADH*2 mRNA levels were not the result of increasing the rate of *ADH*2 mRNA degradation. In fact, *lsm*6 and *nup*159 stabilized *ADH*2 mRNA, indicating that their effects on the synthesis of the mRNA was at least two-fold greater than that observed from monitoring *ADH*2 steady state mRNA levels. Similar conclusions

Table 4. Effect of *spt*10 revertants on *ADH*2 mRNA half-life.

Allele	*ADH*2 mRNA half-life (min)
wt	12 ± 0.40
*ebs*1-1-42	13 ± 0.90
*lsm*6-5-17	29 ± 9.1
*nup*159-6-72	26 ± 0.16

*ADH*2 mRNA half-lives were calculated as described [17]. *ADH*2 mRNA levels were quantitated by S1 nuclease protection assays. The value ± SEMs represent the average of three to five determinations except for *lsm*6 whose value is the average of two determinations.

can be made for *ccr*4 and *caf*1 defects that caused increased stabilization of *ADH*2 mRNA and yet, concurrently, reduced overall *ADH*2 mRNA levels [3,17,33] (**Table 3**). We also showed that the *lsm*6, *nup*159 and *ebs*1 alleles did not alter the abundance of CCR4-NOT complex components, indicating that their effects were not indirectly through lowering CCR4 and CAF1 abundance in the cell. In addition, the role of EBS1 in negatively regulating translation [30] cannot directly explain how it would function as an activator of *ADH*2.

*spt*10 results in a low level of *ADH*2 expression under glucose growth conditions by an unknown mechanism that could involve affecting *ADH*2 chromatin structure [2, 35,36]. The observation that *lsm*6, *nup*159, *ebs*1, *ccr*4 and *caf*1 can each suppress *spt*10 effects at *ADH*2 could suggest that these factors function downstream of *spt*10 on chromatin structure. However, our previous analyses indicated that CCR4 acted independently of SPT10 to affect *ADH*2 expression [36]. Also, the fact that *lsm*6, *nup*159, *ebs*1, *caf*1 and *ccr*4 reduce *ADH*2 expression under ethanol growth conditions when *spt*10 has no effect on *ADH*2 transcription further supports an effect of these alleles on *ADH*2 independently of *spt*10.

One possible model for how CCR4 and CAF1 function in activation is that they influence action of the RNA polymerase II holoenzyme through their contacts to SRB-9, -10, and -11 mediator components [21]. Alternatively, CCR4 and CAF1 could serve as coactivators by somehow mediating the action of activators such as ADR1 at *ADH*2. However, this latter model seems unlikely in that ADR1 is not required for *spt*10-enhanced *ADH*2 expression [1,11].

Based on the results presented in this paper, we suggest a different model for how CCR4, CAF1, LSM6, NUP159 and EBS1 act as activators at the *ADH*2 locus. The primary roles for these factors appear to be the mRNA degradative process, in mRNA transport from the nucleus, or in mRNA elongation. Studies have increasingly linked transcription initiation processes with post-transcriptional processing and transport of mRNA [37]. It is, therefore, conceivable that interfering with mRNA degradation and transport could result in slower processing and movement of mRNA through the cell. These effects could then result indirectly in reducing transcriptional initiation of particularly sensitive genes. A variant of this model would be that proteins involved in post-transcriptional mRNA processing are loaded onto the mRNA during its synthesis. Defects in these processing components would interfere with this loading process that again indirectly influences the corresponding initiation of transcription from certain promoters. In this regard CCR4-NOT complex is known to play a critical role in mRNA elongation [15,16,38]. Its function as an activator of transcriptional elongation would therefore be a very

direct way for components of this complex to function as activators of *ADH*2 expression. For example, if transcription through the *ADH*2 gene were particularly prone to transcriptional arrests that the CCR4-NOT complex would be especially able to bypass by restimulating transcription [16], then a positive role for CCR4-NOT proteins would be substantiated. But no evidence for *ADH*2 susceptibility to transcriptional arrest is known and the EBS1, NUP159 and LSM6 factors do not have clear roles in elongation.

Finally, it has been recently shown that the nuclear pore factors may have diverse and direct effects on chromatin structure and hence gene expression [39]. Given these interactions between mRNA transport and transcription, the NUP159 protein and the CCR4-NOT complex, which has been shown to interact with the mRNA export pathway [40], may exert their respective effects on *ADH*2 chromatin structure and expression by virtue of their effects on mRNA export. Since the SPT10 protein is specifically involved in affecting chromatin structure [2,35,36], this could be one means by which defects in NUP159 and CCR4-NOT components suppress *spt*10 effects.

In contrast to the above models, given that CCR4, CAF1, LSM6, NUP159 and EBS1 defects might be expected to have broad affects on the mRNA degradation and/or transport of many genes [39-41], it remains possible that the mRNA for certain key transcriptional initiation factors might be specifically sensitive to corresponding defects in mRNA degradation or transport. For instance, the mRNA for holoenzyme or core initiation components might be specifically affected by such defects, resulting again, indirectly, in reduced *ADH*2 gene expression. However, whole genome microarray analysis with *ccr*4, *caf*1 and *not*4 deletions did not identify specific core transcriptional factors under the regulation of any of the CCR4-NOT factors [10].

In that *ccr*4, *caf*1, *lsm*6 and *nup*159 defects all reduce *ADH*2 steady state mRNA levels and at the same time reduce the rate of *ADH*2 mRNA degradation indicates an additional difficulty with interpreting the actual mechanisms by which factors control gene initiation. A number of factors thought to control initiation of transcription (HPR1, RTF1, PAF1, SPT5) have been shown to be involved in post-initiation processes [33,38,42-45]. Whether these original effects on transcription initiation were merely the consequences of indirect effects derived from their post-initiation sites of action has not been determined. At least for the case of CCR4-NOT factors understanding how their effects on *ADH*2 mRNA degradation rates can exert opposite effects on *ADH*2 synthesis may shed new light on the total integration of mRNA metabolism.

5. ACKNOWLEDGEMENTS

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