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Genetic Evidence Supports a Role for the Yeast CCR4-NOT Complex in Transcriptional Elongation

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ABSTRACT

The CCR4-NOT complex is involved in the regulation of gene expression both positively and negatively. The repressive effects of the complex appear to result in part from restricting TBP access to noncanonical TATAA binding sites presumably through interaction with multiple TAF proteins. We provide here genetic evidence that the CCR4-NOT complex also plays a role in transcriptional elongation. First, defects in CCR4-NOT components as well as overexpression of the *NOT4* gene elicited 6-azauracil (6AU) and mycophenolic acid sensitivities, hallmarks of transcriptional elongation defects. A number of other transcription initiation factors known to interact with the CCR4-NOT complex did not elicit these phenotypes nor did defects in factors that reduced mRNA degradation and hence the recycling of NTPs. Second, deletion of *ccr4* resulted in severe synthetic effects with mutations or deletions in the known elongation factors RPB2, TFIIS, and SPT16. Third, the *ccr4* deletion displayed allele-specific interactions with *rpb1* alleles that are thought to be important in the control of elongation. Finally, we found that a *ccr4* deletion as well as overexpression of the *NOT1* gene specifically suppressed the cold-sensitive phenotype associated with the *spt5-242* allele. The only other known suppressors of this *spt5-242* allele are factors involved in slowing transcriptional elongation. These genetic results are consistent with the model that the CCR4-NOT complex, in addition to its known effects on initiation, plays a role in aiding the elongation process.

EUKARYOTIC gene expression is characterized by the interaction of a number of factors and large protein complexes, each of which may have multiple roles in the formation of mRNA. For example, TAF_{II} proteins are components of both TFIID that is involved in transcription initiation and the SAGA complex that is proposed to affect chromatin accessibility (Grant *et al.* 1997; Sterner *et al.* 1999). TFIID has also been shown to recruit a factor important for polyadenylation of mRNA (Dantonel *et al.* 1997), suggesting a role for TFIID in 3′ end formation of mRNA. Individual subunits of TFIID have multiple functions. For instance, $TAF_{II}250$ can acetylate and ubiquitinate histones as well as regulate TBP access to DNA (Mizzen *et al.* 1996; Liu *et al.* 1998a; Pham and Sauer 2000). The TFIIH complex, in addition to its known roles in initiation and in promoter clearance (Kim *et al.* 2000), aids in DNA excision repair (Drapkin *et al.* 1994). The carboxy-terminal domain (CTD) of RNA polymerase II enjoys multiple functions both in initiation and in postinitiation processes such as mRNA capping, splicing, and polyadenylation (McCracken *et al.* 1997; Hirose and Manley 1998; Hirose *et al.* 1999; Komarnitsky *et al.* 2000; Schroeder *et al.* 2000). In addition, TFIIF is important for both initiation and elongation of mRNA (Tan *et al.* 1995; Francois *et al.* 1998). It is clear, therefore, that the multiple protein interactions possible for these proteins and protein complexes can engender many and varied roles for these factors. We report genetic evidence that the CCR4-NOT complex in addition to its known roles in transcriptional initiation has a function in transcriptional elongation.

The CCR4-NOT complex affects gene expression both positively and negatively (Denis 1984; Denis and Malvar 1990; Sakai *et al.* 1992; Collart and Struhl 1993, 1994; Liu *et al.* 1998b). The repressive effects of the complex have been linked to restricting TBP access to noncanonical TATAA sequences (Collart and Struhl 1994; Collart 1996), apparently through the interaction with multiple TAF proteins (Badarinarayana *et al.* 2000; Lemaire and Collart 2000), and more recently to effects on the degradation of mRNA (Tucker *et al.* 2001). However, the partial nonoverlap of phenotypes observed when different components of the CCR4-NOT complex are deleted (Bai *et al.* 1999) and the positive role for the complex in gene expression (Denis 1984; Liu *et al.* 1998b) suggest additional functions for the CCR4-NOT complex.

The CCR4-NOT complex is present in at least two forms in yeast, 1.9×10^6 daltons (1.9 MD) and 1.0 MD (Liu *et al.* 1997, 1998b; Bai *et al.* 1999). The smaller, core 1.0-MD complex has been well characterized and consists of CCR4, CAF1, the five NOT proteins (NOT1–5), and two new proteins, CAF40 and CAF130 (Draper *et al.* 1994, 1995; Liu *et al.* 1998b; Bai *et al.* 1999; J. Chen,

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Y.-C. Chiang, J. Rappsilber, P. Russell, M. Mann and C. L. Denis, unpublished data). Although the 1.9-MD complex remains uncharacterized, several proteins such as DBF2, MOB1, CAF4, and CAF16 have been linked to interactions with CCR4-NOT proteins and are likely components of this larger complex (Liu *et al.* 1997; Komarnitsky *et al.* 1998; Liu *et al.* 2001). The arrangement of the proteins in the 1.0-MD CCR4-NOT complex have been analyzed (Liu *et al.* 1998b; Bai *et al.* 1999; Maillet *et al.* 2000). CCR4 and CAF1 bind to a central region of NOT1 whereas NOT2, NOT5, and NOT4 associate through the C terminus of NOT1. CAF40 and CAF130, although their locations are less clear, associate with a different part of the complex than that with which either CCR4 and CAF1 or NOT2, NOT5, and NOT4 are associated (J. Chen, Y.-C. Chiang, J. Rappsilber, P. Russell, M. Mann and C. L. Denis, unpublished data). Because NOT2 and NOT5 appear primarily responsible for TFIID interactions (Badarinarayana *et al.* 2000; Lemaire and Collart 2000), CCR4 may be making contacts and playing roles in the cell other than that evinced by NOT2 and NOT5.

A number of factors have been shown to play possible roles in eukaryotic transcriptional elongation (Chavez and Aguilera 1997; Uptain *et al.* 1997; Hartzog *et al.* 1998; Wada *et al.* 1998; Chavez *et al.* 2000; Costa and Arndt 2000). In yeast defects in many of these factors affecting elongation such as SPT5, SPT4, SPT6, RTF1, RPB2, RPB1, TFIIS, and ELP1 elicit 6-azauracil (6AU) and mycophenolic sensitive phenotypes (Archambault *et al.* 1992; Exinger and Lacroute 1992; Powell and Reines 1996; Hartzog *et al.* 1998; Lennon *et al.* 1998; Otero *et al.* 1999; Costa and Arndt 2000). 6AU sensitivities result from lowering of GTP and/or UTP levels in the cell that is presumed to impair elongation (Exinger and Lacroute 1992). We have observed that a deletion of *CCR4* or other components of the CCR4-NOT complex gives rise to a 6AU sensitive phenotype. In confirmation of a role for CCR4 in elongation, the *ccr4* deletion causes synthetic and allele-specific phenotypes with several known defects in elongation factors. Moreover, a *ccr4* deletion or overexpression of *NOT1* suppresses an *spt5-242* defect that has been shown previously to be suppressed by the slowing of elongation. These and other results suggest a role for components of the CCR4-NOT complex in controlling transcriptional elongation.

MATERIALS AND METHODS

Yeast strains and growth conditions: Yeast strains are listed in Table 1. Strains were grown on YEP plates (1% yeast extract/2% Bacto-peptone/2% agar) supplemented with either 2% glucose (YD plates) or 3% glycerol (YG plates). Minimal plates were prepared lacking uracil as described (Badarinarayana *et al.* 2000) and were supplemented with 100 μg/ml 6AU, 40 μg/ml mycophenolic acid, or 600 μg/ml guanine. IMPDH assays were conducted as previously described for ADH assays (Liu *et al.* 1998b), except the reaction conditions contained 20 mm IMP, 50 mm glutathione, and 50 mm Tris, pH 7.5 instead of 0.3 m ethanol and 50 mm pyrophosphate buffer. Standard errors of the mean were <20% in all cases.

RESULTS

Defects in CCR4-NOT complex components elicit 6AU sensitive phenotypes: It was previously observed that a *ccr4* deletion could give rise to 6AU sensitivity (Chang *et al.* 1999). We examined this further by analyzing a number of different strains and their corresponding *ccr4* derivatives for growth on medium containing 6AU. In each case, a *ccr4* defect resulted in 6AU sensitivity (data not shown), indicating that 6AU sensitivity was another phenotype, like caffeine sensitivity, cold sensitivity, and glycerol 37° sensitivity, that was associated with a *ccr4* disruption (Denis and Malvar 1990; Liu *et al.* 1997). We also examined whether defects in other components of the CCR4-NOT complex elicited 6AU sensitivity (Figure 1). In addition to *ccr4*, we found that *caf1*, *not1-2*, *not2-1*, *not4*, and *not5* mutations each gave rise to a 6AU phenotype (Figure 1). While a *not3* deletion did not result in a 6AU sensitive phenotype (Figure 1), the *not3-2* allele did (data not shown). This is in agreement with previous results showing that the *not3-2* allele generally results in more severe phenotypes than the *not3* deletion (Liu *et al.* 1998b; our unpublished data). As 6AU sensitivity in yeast has been generally correlated with defects in transcriptional elongation, although not exclusively so, these results suggest that the CCR4-NOT complex plays a role in elongation in addition to its known role in controlling initiation (Denis and Malvar 1990; Sakai *et al.* 1992; Collart and Struhl 1994).

6AU sensitivity appears to arise from 6AU lowering the levels of GTP and UTP in the cell (Exinger and Lacroute 1992). Provision of excess guanine has been found to overcome 6AU sensitivity (Exinger and Lacroute 1992). Similarly, we found that excess guanine could rescue the 6AU sensitive phenotype observed by defects in *CCR4-NOT* genes (data not shown). Mycophenolic acid similarly acts to decrease GTP levels in the cell and *ccr4*, *caf1*, and *not4* deletions also resulted in sensitivity to mycophenolic acid (data not shown), indicating that it was not some specific interaction between 6AU and defects in the CCR4-NOT complex components that elicited the above described phenotypes. Defects in other factors known to associate with the 1.9-MD CCR4-NOT complex but which are not components of the 1-MD CCR4-NOT complex, such as CAF4, CAF16, and DBF2, did not elicit a 6AU sensitive phenotype (Figure 1; data not shown). These data indicate that it is the functionality of the core CCR4-NOT components that are important to the 6AU sensitivity phenotype.

The 6AU sensitivity observed by defects in CCR4-NOT proteins was not, however, found to occur when other

TABLE 1

Yeast strains

Strain	Genotype
EGY188	*MAT***a** *ura3 his3 trp1 LexA*$_{op}$*-LEU2* [YEp13 *URA3*]
EGY188-1a	Isogenic to EGY188 except *ccr4::URA3*
EGY188-c1	Isogenic to EGY188 except *caf1::URA3*
EGY188-c4	Isogenic to EGY188 except *caf4::URA3*
EGY188-S9-U9	Isogenic to EGY188 except *srb9::URA3*
KY803	*MAT***a** *ura3-52 leu2:PET56 trpl*Δ*1 gcn4* [YEp13 *URA3*]
MY8	Isogenic to KY803 except *not1-2* and *MAT*α [YEp13 *URA3*]
MY16	Isogenic to KY803 except *not2-1* [YEp13 *URA3*]
MY25	Isogenic to KY803 except *not3-2* [YEp13 *URA3*]
MY508	Isogenic to KY803 except *not3::URA3*
MY537	Isogenic to KY803 except *not4::URA3*
MY1735	Isogenic to KY803 except *not5::URA3*
MY1738	Isogenic to KY803 except *not1::LEU2* and [pRS426-NOT1-1319-2108-*URA3*]
KY803-c40	Isogenic to KY803 except *caf40::URA3*
KY803-c130	Isogenic to KY803 except *caf130::URA3*
DY103	*MAT***a** *ura3-52 leu2-3,112 his3*Δ*200 rpb*Δ*297::HIS3* [pRP214 (*LEU2 RPB2*)] [pRS316 (*URA3*)]
Z96-1a	Isogenic to DY103 except *ccr4::URA3* and lacks [pRS316 (*URA3*)]
DY104	Isogenic to DY103 except [pRP2-4L (*LEU2 rpb2-4*)]
Z100-1a	Isogenic to DY104 except *ccr4::URA3* and lacks [pRS316 (*URA3*)]
DY105	Isogenic to DY103 except [pRP2-10L (*LEU2 rpb2-10*)]
DY106	Isogenic to DY103 except *dstl::hisG*
Z106-1a	Isogenic to DY105 except *ccr4::URA3* and lacks [pRS316 (URA3)]
DY106-1-1a	Isogenic to DY106 except *ccr4::URA3* and lacks [pRS316 (URA3)]
Z103/316	Isogenic to DY103 except [pRP2-7L (*LEU2 rpb2-7*)]
Z103-1a	Isogenic to Z103/316 except *ccr4::URA3* and lacks [pRS316]
FY1635	*MAT*α *his4-912*δ *lys2-128*δ *leu2*Δ*1 ura3-52 spt5-242*
GHY149	*MAT***a** *his4-912*δ *lys2-128*δ *leu2*Δ*1 ura3-52 rpb1-244*
FY1638	*MAT***a** *his4-912*δ *lys2-128*δ *leu2*Δ*1 ura3-52 rpb1-221*
FY1642	*MAT***a** *his4-912*δ *lys2-128*δ *leu2*Δ*1 ura3-52* SPT5-FLAG
FY1635-1a	Isogenic to FY1635 except *ccr4::URA3*
GHY149-1a	Isogenic to GHY149 except *ccr4::URA3*
FY1638-1a	Isogenic to FY1638 except *ccr4::URA3*
FY1642-1a	Isogenic to FY1642 except *ccr4::URA3*
H154	*MAT*α *ura3-52 his 4-912*δ *spt16-197*
H154-1a	Isogenic to H154 except *ccr4::URA3*
GHY180	*MAT*α *ura3-52 leu2*Δ*1 his 4-912*δ *lys2-128*δ *spt4*Δ*2::HIS3*
FY276-UT	*MAT***a** *leu2*Δ*1 ura3::TRP1 his4-9128 lys2-128*δ *spt5-8*
GHY180-1a	Isogenic to GHY180 except *ccr4::URA3*
FY276-uT-1a	Isogenic to FY276-UT except *ccr4::URA3*
W303-1a-elp1::L2	*MAT***a** *ura3 his3 leu2 trp1 ade2 elp1:::LEU2*
W303-la-elp1::L21a	Isogenic to W303-la-elp1::L2 except *ccr4::URA3*

known factors involved in transcriptional initiation were deleted. Deletion of *PAF1* or *CDC73*, whose protein products have been shown to be part of an RNA polymerase II complex containing CCR4, did not elicit a 6AU phenotype (Chang *et al.* 1999; data not shown). Similarly, deletion of RNA polymerase II holoenzyme components, *SRB5*, *SRB9*, *SRB10*, *SRB11*, *GAL11*, or *SIN4*, or SAGA components *ADA2* or *GCN5* failed to result in 6AU sensitivity (data not shown).

We further examined whether defects in factors that cause a decreased rate of RNA degradation and hence a reduction in recycling of NTPs could result in a 6AU sensitivity phenotype. Defects in proteins known to be involved in mRNA degradation, such as XRN1 (Muhlrad *et al.* 1995), or the exosome components SKI8, SKI6, SKI2, or SKI3 (Van Hoof *et al.* 2000) did not result in 6AU sensitivity phenotypes (data not shown).

Disruption in CCR4-NOT function has been previously observed either by deleting specific components of the complex or by overexpressing an individual component of the complex (Badarinarayana *et al.* 2000). This latter phenomenon probably results from disturbing the balance within the complex and thereby interrupting its functional contacts (Badarinarayana *et al.* 2000). We consequently tested the effect of overproducing each of the components of the CCR4-NOT complex on sensitivity to 6AU. Only *NOT4* overexpression resulted in poor growth on medium containing

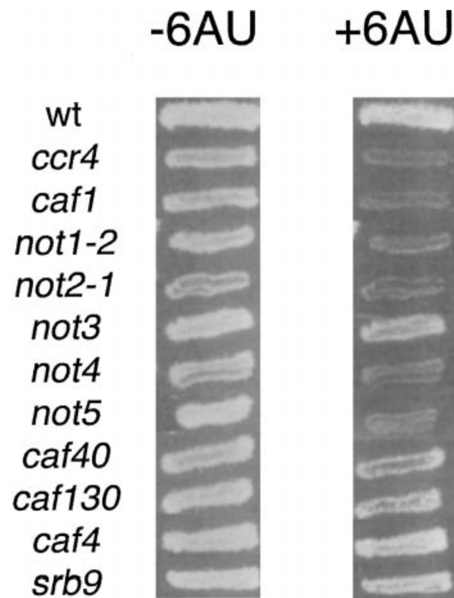

FIGURE 1.—Defects in CCR4-NOT components result in 6AU sensitivity. All yeast strains were grown on minimal medium lacking uracil (−6AU) that was supplemented with 100 μg/ml 6AU (+6AU). Growth was monitored after 5 days at 34° although similar results were obtained at 30° and 25°. All strains were isogenic to either EGY188 (wt) or KY803 (whose growth was identical to EGY188). It should be noted that all strains grew well on minimal medium containing both 6AU and uracil.

6AU, although it had no major effect on growth on medium not containing 6AU (data not shown). These results suggest that it is the functionality and potentially the balance of components within the CCR4-NOT complex that is critical to the 6AU sensitivity phenotype.

The *ccr4* mutation displays synthetic interactions with defects in the RPB2, TFIIS, and SPT16 elongation factors: If CCR4 were to be involved in transcriptional elongation processes, defects in CCR4 might be expected to result in synergistic defects or lethalities when combined with defects in other known elongation factors. Previously we had shown that combining the *ccr4* deletion with that of *hprl*, encoding a factor known to control elongation in yeast (Chavez and Aguilera 1997; Chavez *et al.* 2000; Y. Cui and C. L. Denis, unpublished data), results in a synthetic lethality (Chang *et al.* 1999). We extended these results by analyzing the effect of *ccr4* in combination with defects in elongation factors RPB2, TFIIS, and SPT16 (a component of the FACT elongation complex; Orphanides *et al.* 1999).

The *rpb2-10* and, to a lesser degree, the *rpb2-4* allele have been shown to slow RNA polymerase II elongation *in vitro* (Powell and Reines 1996). They and the *rpb2-7* allele (which had no effect on *in vitro* elongation) all result in 6AU sensitive phenotypes (Powell and Reines 1996). When *ccr4* was combined with each of these three alleles, *rpb2-10 ccr4*- and *rpb2-4 ccr4*-containing strains displayed reduced growth at 30° and an inability to grow at 39°, normally a permissive temperature for *ccr4* or *rpb2* mutants (Figure 2). The *rpb2-7* allele when combined with *ccr4* did not cause reduced growth at 30°, but did exhibit a temperature-sensitive phenotype at 37°, slightly lower than that observed for *ccr4 rpb2-4* or *ccr4 rpb2-10* combinations (Figure 2). The temperature-sensitivity phenotypes observed when the *rpb2* alleles were combined with *ccr4* were found, however, to be suppressed by growth on minimal medium, suggesting that slowing growth allowed the cell to overcome the block caused by the combination of *ccr4* and *rpb2* defects (Figure 2). Moreover, each of the three *rpb2* alleles when combined with *ccr4* resulted in a failure to grow on nonfermentative carbon sources (Figure 2). This latter phenotype is not a result of permanent damage to the mitochondria since revertants capable of growing on nonfermentable carbon sources were obtained in *ccr4 rpb2-10* and *ccr4 rpb2-4* backgrounds (data not shown). We also observed that the *rpb2-10 ccr4*-containing strain was capable of growth on medium containing 6AU after 7 days whereas even after 7 days of growth the strains harboring the *rpb2-4 ccr4* or *rpb2-7 ccr4* alleles remained 6AU sensitive (data not shown). This latter phenomenon suggests an allele-specific interaction between loss of CCR4 function and the *rpb2-10* allele.

We extended these analyses by analyzing the effect of combining *ccr4* with a deletion in *DST1*, the gene encoding TFIIS. As observed for the *rpb2* alleles, *dstl* when combined with *ccr4* resulted in 39° temperature sensitivity (Figure 2), a phenotype that was suppressed by growth on minimal medium (data not shown), and a nonfermentative growth defect (Figure 2). Similarly, we observed that a *ccr4* deletion when combined with the *spt16-197* allele gave rise to a 34° temperature-sensitive phenotype that was not observed with either *ccr4* or *spt16-197* alone (Figure 2). However, combining *ccr4* with defects in elongation factors did not always result in severe phenotypes. No augmentation of phenotype was observed when *ccr4* was combined with *elp1*, *spt4*, or the *spt5* alleles, *spt5-8* or *spt5-242* (data not shown). These above results indicate two important points. First, combining *ccr4* with mutations in *RPB2*, *DST1*, or *SPT16* that are known or presumed to be defective in elongation results in more severe phenotypes. Combining *ccr4* with the *rpb2-3* allele that affects transcriptional initiation events (Lee *et al.* 1998) did not result in synergistic effects (data not shown). Second, *ccr4* displays an allele-specific effect with the *rpb2-10* allele, suggesting a functional link between the corresponding proteins. These results support a role for CCR4 in transcript elongation.

***rpb1* alleles involved in elongation display allele-specific interactions with *ccr4*:** Two *rpb1* alleles (*rpb1-221* and *rpb1-244*) that have been previously described cause synthetic defects with a *dst1* deletion (Hartzog *et al.* 1998). These *rpb1* alleles also suppress the cold-sensitive defect associated with the *spt5-242* allele, confirming a role for these alleles in elongation processes controlled by the SPT4-SPT5 complex (Hartzog *et al.* 1998). We subsequently examined the effect of combining a *ccr4*

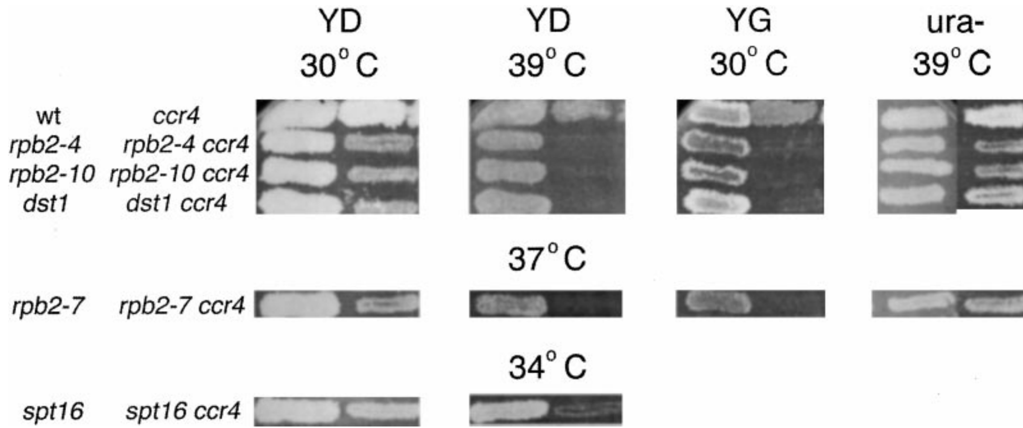

FIGURE 2.—Synthetic interaction between *ccr4* and *rpb2*, *dst1*, and *spt16* defects. Yeast strains were grown on YD, YG, or ura− plates at the temperatures indicated. wt, strain Z96 (isogenic to DY103 except it lacks the *URA3* plasmid); all other strains were isogenic to DY103 except *spt16* (H154) and *spt16 ccr4* (H154-1a).

deletion with the *rpb1-221* and *rpb1-244* alleles. We found that combining a *ccr4* deletion with the *rpb1-244* allele resulted in a severity of phenotypes greater than that for *ccr4* combined with *rpb1-221* (Figure 3). Strains containing *ccr4 rpb1-244* displayed weaker growth at 30° and no growth at 37° (Figure 3) and were unable to grow on nonfermentative carbon sources (data not shown). The poor viability of *rpb1-244* with *ccr4* is similar to that observed between *rpb1-244* and a *dst1* deletion (Hartzog *et al.* 1998). We also observed that the *ccr4 rpb1-244* synthetic lethalities were completely relieved by reintroduction of a wild-type *CCR4* gene into the strain. In addition, when *ccr4* was combined with the *rpb1-1* allele, known to affect initiation of transcription (Holstege *et al.* 1998), no synergistic effects were obtained (data not shown).

The *spt5-242* cold-sensitive allele is suppressed by *ccr4* or overexpression of *NOT1*: The above described *rpb1-244* and *rpb1-221* alleles were initially identified as suppressors of the cold-sensitive phenotype associated with *spt5-242*. Similarly, the *rpb2-10* allele, which causes an *in vitro* defect for transcriptional elongation (Powell and Reines 1996), is also capable of suppressing *spt5-242* (Hartzog *et al.* 1998). Slowing of elongation *in vivo* by the addition of 6AU also suppressed *spt5-242*. Because of the known role of human homologs of SPT5 in elongation (Wada *et al.* 1998), it was postulated that slowing of elongation allows rescue of the *spt5-242* elongation defect (Hartzog *et al.* 1998). We therefore tested if a *ccr4* deletion or overexpression of individual CCR4-NOT complex components could also rescue the *spt5-242* cold-sensitive phenotype. As summarized in Table 2, the *spt5-242* cold-sensitive phenotype was capable of being suppressed by a *ccr4* deletion. Also, overexpression of the *NOT1* gene specifically suppressed the *spt5-242* phenotype (Table 2). Overexpression of the *NOT1* gene resulted in about three- to fourfold more NOT1 protein in the cell than normally was present (data not shown). These results support the model that CCR4 and the NOT proteins affect transcriptional elongation.

One simple model for the effect of *ccr4* on elongation would be that components of the CCR4-NOT complex, as transcriptional regulators, affect the expression of the rate-limiting enzyme IMPDH in the synthesis of GTP (Glesne *et al.* 1991; Shaw and Reines 2000). Decreasing GTP levels would be expected to slow elongation. Because of the existence of four separate genes encoding IMPDH (Shaw and Reines 2000), we chose to quantitate the effect of CCR4-NOT defects directly on IMPDH enzyme levels in the cell. IMPDH enzyme levels (100 milliunits/mg in wild type) were found, however, to be relatively unchanged with the *ccr4* (120 milliunits/mg IMPDH), *not1-2* (100 milliunits/mg), *not2-1* (80 milliunits/mg), or *not4* (120 milliunits/mg) alleles, although a *caf1* deletion did result in a twofold drop in IMPDH enzyme levels to 56 milliunits/mg.

DISCUSSION

Genetic evidence for CCR4 involvement in transcriptional elongation: We have provided genetic evidence that supports a role for CCR4 and components of the CCR4-NOT complex in regulating transcriptional elongation. This novel role for these proteins is supported

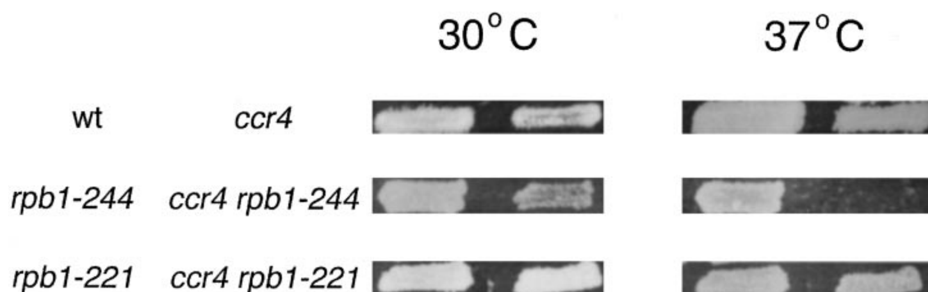

FIGURE 3.—Allele-specific interactions between the *ccr4* and *rpb1* allele. Yeast strains were grown on YD plates at the temperatures indicated. wt, strain FY1642; *rpb1-244*, strain GHY-149; *rpb1-221*, strain FY1638; *ccr4* derivatives were isogenic to the above three strains.

TABLE 2

The cold-sensitive phenotype of *spt5-242* is suppressed by *ccr4* and increased expression of *NOT1*

	30°	15°
wt	+	+
ccr4	+	+
spt5-242	+	w
ccr4 spt5-242	+	+
YEp13-U *spt5-242*	+	w
pCCR4 *spt5-242*	+	w
pNOT1 *spt5-242*	+	+
pNOT2 *spt5-242*	+	w
pNOT3 *spt5-242*	+	w
pNOT4 *spt5-242*	+	w

Growth was monitored on ura− plates at the temperature given. wt, strain 1642; *spt5-242*, strain 1635; all other strains were isogenic to 1635 except for indicated *ccr4* allele or high copy plasmid: YEp13-U (vector YEp13 plasmid converted to *URA3*); pCCR4 (YEp13-U-CCR4); pNOT1 (pRS426-NOT1); pNOT2 (pRS426-NOT2); pNOT3 (pRS426-NOT3); pNOT4 (pRS426-NOT4). +, good growth; w, weak growth.

by several observations. First, defects in nearly all of the individual components of the 1.0-MD core CCR4-NOT complex as well as overexpression of NOT4 elicited 6AU and mycophenolic sensitive phenotypes. These phenotypes were not generally associated with defects in other factors involved in transcriptional initiation and mRNA degradation, or with CCR4-NOT complex components (CAF4, CAF16, and DBF2) not part of the 1.0-MD CCR4-NOT complex. Second, deletion of *ccr4* resulted in severe synthetic effects with defects in several known elongation factors: *hprl*, *rpb2*, *rpb1*, *dst1*, and *spt16*. Because the biochemical mechanism of action by several of these elongation factors remains largely unknown, it is difficult to ascertain at what step or pathway CCR4 is involved. The spectrum of synthetic defects observed with *ccr4* suggests that the CCR4 protein may play a role in a novel aspect of this regulation.

Third, a *ccr4* deletion displayed an allele-specific interaction with the *rpb1-244* allele that has been suggested to play a role in elongation (Hartzog *et al.* 1998). This result suggests a functional interaction between CCR4 and RPB1 in terms of elongation and is supported by the previous observations that CCR4 is a component of the PAF1-containing RNA polymerase II transcription complex (Chang *et al.* 1999) and that components of the 1.9-MD CCR4-NOT complex display multiple physical interactions with the SRB9-11 proteins of the RNA polymerase II holoenzyme (Liu *et al.* 2001). Finally, we showed that a *ccr4* deletion or overexpression of *NOT1* suppressed the cold-sensitive phenotype associated with the *spt5-242* allele, suggesting that they slow the rate of elongation (Hartzog *et al.* 1998). The particular effect of *NOT1* overexpression may result from its role as the scaffold for the CCR4-NOT complex and thereby affect the integrity of the complex by its overexpression (Bai *et al.* 1999).

While the above evidence supports a direct role for CCR4-NOT proteins in affecting some aspect of elongation, it remains possible that the described interactions result from indirect effects of CCR4-NOT factors on transcription initiation processes. Although it is difficult to formally eliminate this alternative explanation, the above multiple correlations between *ccr4* and elongation defects and the observation that the CCR4-NOT proteins do not significantly affect the overall enzyme levels of IMPDH in the cell makes this suggestion seem unlikely. In addition, whereas it has been suggested that the *dst1* deletion causes sensitivity to 6AU due to its effect on the transcription of the *SSM1* gene (Shimoaraiso *et al.* 2000), we have found that *ccr4* has no effect on *SSM1* mRNA synthesis (H. Baker and C. L. Denis, unpublished data).

Relationship of CCR4-NOT function in initiation to that of elongation: The CCR4-NOT proteins have been implicated in the control of transcriptional initiation by a number of studies (Denis and Malvar 1990; Sakai *et al.* 1992; Collart and Struhl 1994). The most critical of this evidence is the enhanced transcription from the TATAA-less promoter at *HIS3* (Collart and Struhl 1993, 1994), the suppression by *ccr4* and *caf1* of *spt10*-enhanced *ADH2* expression but not of *ADR1*^{c}-enhanced *ADH2* expression (Denis 1984; Draper *et al.* 1995), and the effect of CCR4-NOT proteins on promoters placed in front of different reporter genes (Liu *et al.* 1998b). Moreover, *ccr4* does not affect the degradation rate of *ADH2* mRNA or elongation through the *ADH2* gene (Y. Cui and C. L. Denis, unpublished data), indicating that the effects of *ccr4* on *ADH2* expression must be at the level of initiation of transcription. In addition, the CCR4-NOT proteins exhibit multiple contacts to proteins playing important roles in controlling initiation (TFIID, ADA2, and SRB9-11; Benson *et al.* 1998; Lee *et al.* 1998; Badarinarayana *et al.* 2000; Lemaire and Collart 2000; Liu *et al.* 2001).

Yet, the multiple roles played by other initiation factors in such processes as DNA repair, promoter clearance, transcriptional elongation, polyadenylation, and 3′ end formation suggests that factors controlling initiation can be utilized in other facets of DNA/RNA metabolism. In addition to the genetic evidence described herein, the CCR4-NOT proteins display several characteristics suggestive of roles in aspects of RNA formation other than that of initiation. First, CCR4 is part of the PAF1-RNA polymerase II complex (Chang *et al.* 1999), which contains HPR1, a protein involved in elongation rather than in initiation (Chavez and Aguilera 1997; Chavez *et al.* 2000; our unpublished data). Second, CCR4-NOT components interact with the subset of proteins SRB9, -10, and -11 of the RNA polymerase II holoenzyme (Liu *et al.* 2001). While this complex does function in initiation, the importance of SRB10 in phos-

phorylation of RNA polymerase II (Hengartner *et al.* 1998) and the dependency of elongation on this phosphorylation (Payne *et al.* 1989), suggests a critical role for SRB10 in creating a competent elongating form of RNA polymerase II. Although the SRB9, -10, and -11 proteins generally act as repressors, which has been linked to preinitiation control of RNA polymerase II, they can also function as activators (Holstege *et al.* 1998; Liu *et al.* 2001) and may be involved in another aspect of RNA formation. As suggested previously (Akhtar *et al.* 1996), activators or coactivators like the CCR4-NOT proteins could aid in setting up processive polymerases at the promoter and thereby generate more active or increased numbers of elongating polymerases.

Third, components of the CCR4-NOT complex appear to display functions linked to direct RNA/DNA contacts. CCR4 and CAF1 display sequence homology and enzymatic activities related to exo- and endonucleases (Moser *et al.* 1997; Dlakic 2000; Tucker *et al.* 2001; J. Chen, Y.-C. Chiang and C. L. Denis, unpublished data) and NOT4 contains a putative RNA binding domain (Albert *et al.* 2000; our unpublished data). It is, therefore, likely that these proteins are involved directly in contributing to some facet of RNA synthesis, degradation, or monitoring. Such interactions are consistent with possible roles of these proteins in several aspects of elongation.

Recently, another presumed initiation factor, RTF1, has been found to display multiple genetic interactions with elongation factors and display 6AU sensitive phenotypes (Costa and Arndt 2000). The intrinsic overlap between initiation and elongation suggests that identifying factors like RTF1 and CCR4-NOT proteins that could act in both processes will not be unique to these factors. These proteins could act to affect promoter clearance, elongation procession, rescue of stalled complexes, or interaction with chromatin rearrangement factors. Identification of specific target genes controlled at the level of elongation by these factors would be one step toward elucidating their precise mechanisms of action.

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