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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 4, Issue of January 25, pp. 2835–2842, 2002

Printed in U.S.A.

Interaction between Not1p, a Component of the Ccr4-Not Complex, a Global Regulator of Transcription, and Dhh1p, a Putative RNA Helicase*

Received for publication, August 20, 2001, and in revised form, November 1, 2001
Published, JBC Papers in Press, November 5, 2001, DOI 10.1074/jbc.M107979200

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The Ccr4-Not complex is a global regulator of transcription that affects genes positively and negatively and is thought to regulate transcription factor IID function. Two components of this complex, Caf1p and Ccr4p, are directly involved in mRNA deadenylation, and Caf1p is associated with Dhh1p, a putative RNA helicase thought to be a component of the decapping complex. In this work, we tried to determine whether Dhh1p might interact with the Ccr4-Not complex. We found that, first, *not* mutations displayed severe synthetic phenotypes when combined with a *dhh1*-null mutation. Second, overexpression of Not1p was toxic in *dhh1*-null cells. Third, a *not* mutant phenotype was suppressed by deletion of *DHH1* and mimicked by overexpression of *DHH1*. Fourth, *dhh1*-null mutants displayed resistance to heat shock, a phenotype observed for all mutants that affect the Ccr4-Not complex. Finally, like Caf1p and Ccr4p, Dhh1p co-immunoprecipitated with the nonessential N-terminal domain of Not1p, and the levels of Caf1p and Dhh1p were dependent upon this Not1p domain. Taken together, our results suggest that the Ccr4-Not complex, via the N-terminal region of Not1p, is necessary for the maintenance of stable cellular levels of Dhh1p and Caf1p, thus contributing to regulation of mRNA decay in addition to transcription.

The *Saccharomyces cerevisiae* Ccr4-Not complex is a global regulator of gene expression that affects genes positively and negatively. The components of this complex were identified in separate genetic screens. The *NOT* genes were isolated in a selection for mutants that were 3-aminotriazole (AT)[1]-resistant when carrying a mutant, activation-defective derivative of Gnc4p instead of the endogenous wild-type protein (1–3). The selection was directed toward mutants that displayed increased *HIS3* transcription, and these mutants were additionally selected as displaying increased expression of a *HIS3-lacZ* reporter gene. The *not* mutants were shown to increase transcription preferentially from the TATA-less promoters of the *HIS3* and *HIS4* genes. Finally, both genetic and biochemical experiments demonstrated that the five Not proteins functioned together in a high molecular mass complex (2–4). In separate experiments, the Ccr4 protein and its associated factor Caf1p, also known as Pop2p (8), were identified as proteins required for glucose derepression of gene expression (5–8), in particular for alcohol dehydrogenase II expression. A large 1.2-MDa complex containing Caf1p, Ccr4p, and the five Not proteins was isolated (9). These seven proteins also co-fractionate in larger 2-MDa complexes (4). Whereas only Not1p is essential for yeast viability, the Ccr4-Not complex is probably essential for yeast vegetative growth, and only Not1p is absolutely essential for its integrity or function (4).

The role of the Ccr4-Not complex is still not clearly understood. It is generally thought of as a transcriptional regulator that affects TFIID function and belongs to the category of intermediary factors. In support of a role in transcription, interactions of given components of the complex with the TATA box-binding protein and particular yeast TBP-associated factors ($yTaf_{II}$ proteins) have been described (10–12).[2,3] There are also functional links to components of another $yTaf_{II}$-containing complex, namely the Spt-Ada-GCN5 acetyltransferase complex (13, 14), and to other proteins involved in transcription. Accordingly, Ccr4p itself is a component of the Paf1p holoenzyme (15), and some Ccr4-Not proteins are associated with the Srb9–11 proteins (16).

Certain observations are, however, not in agreement with a unique role for the Ccr4-Not complex in regulating transcription. Indeed, mutation or deletion of each of the different components of the complex displays a very unique set of phenotypes. For instance, all of the *not* mutants are AT-resistant, but neither *ccr4* nor *caf1* mutants are. This distinction can be correlated with the observation that, whereas Ccr4p and Caf1p are associated with the N-terminal domain of Not1p, the Not proteins are associated with the essential C-terminal domain of Not1p (4, 17). The Not1 protein itself is the only known component of the Ccr4-Not complex that is essential for yeast viability. The C-terminal domain of Not1p is sufficient for yeast vegetative growth, but wild-type yeast vegetative growth requires the interaction of the N- and C-terminal domains within Ccr4-Not complexes (4). Another point is that interactions between components of the Ccr4-Not complex and factors unrelated to transcription have been described. Both Caf1p and Ccr4p were shown to interact with the cell cycle-regulated protein kinase Dbf2p (16, 18). Furthermore, Caf1p and Ccr4p are associated with the major yeast cytoplasmic deadenylase

* This work was supported by Swiss National Science Foundation Grant 31-49808.96 (to M. A. C.) and by Federal Office for Education and Science Grant 96.0072 and a grant from Novartis (to M. A. C.) for the support of L. M. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: AT, 3-aminotriazole; TFIID, transcription factor IID; HA, hemagglutinin.

[2] C. Deluen, L. Maillet, M. Molinete, G. Theiler, M. Lenaire, N. Paquet, and M. A. Collart, submitted for publication.

[3] L. Maillet and M. A. Collart, manuscript in preparation.

TABLE I
S. cerevisiae strains

Strain	Genotype	Ref. or source
MY1	*MAT***a** *trp1Δ1 leu2*::*PET56 ura3–52 gcn4Δgal2*	2
MY2	Isogenic to MY1, except *MATα*	2
MY3	Isogenic to MY1, except *his3*::*TRP1*	2
MY4	Isogenic to MY3, except *MATα*	2
MY49	Isogenic to MY1, except *not1–2*	2
MY508	Isogenic to MY1, except *not3*::*URA3*	2
MY509	Isogenic to MY1, except *not1–1*	2
YOU123	Isogenic to MY1, except *not5–1*	3
YOU584	Isogenic to MY2, except *not4*::*LEU2*	3
YOU555	Isogenic to MY1, except *not5*::*LEU2*	3
MY1728	Isogenic to MY1, except *ccr4*::*URA3*	9
MY1729	Isogenic to MY1, except *caf1*::*LEU2*	9
MY2700	Isogenic to W303 *MAT***a**, except *dhh1*::*kanMX6*	F. Stutz
MY2811	Isogenic to MY509, except *dhh1*::*kanMX6 MATα*	This work
MY2834	Isogenic to YOU555, except *dhh1*::*kanMX6 MATα*	This work
MY2836	Isogenic to YOU123, except *dhh1*::*kanMX6 his3*::*TRP1*	This work
MY2859	Isogenic to MY1, except *dhh1*::*kanMX6*	This work
LY17	Isogenic to MY2, except *not1–1 caf1*::*LEU2*	4
LY26	Isogenic to MY2, except *not3*::*URA3 caf1*::*LEU*	4
LY29	Isogenic to MY2, except *not1–1 ccr4*::*URA3*	4
LY54	Isogenic to MY2, except *ccr4*::*URA3 caf1*::*LEU*	4
LY70	Isogenic to MY4, except *not3*::*URA3 ccr4*::*URA3*	4
LY176	Isogenic to MY3, except *not1*::*LEU2* + pLexANot1ΔN3	4
LY186	Isogenic to LY176, except +pNot1ΔC2	4
LY279	Isogenic to MY1, except *DHH1-HA*	This work
LY287	Isogenic to MY509, except *DHH1-HA*	This work
LY296	Isogenic to MY1729, except *DHH1-HA his3*::*TRP1 MATα*	This work
LY298	Isogenic to LY186, except *DHH1-HA*	This work
LY299	Isogenic to LY176, except *DHH1-HA*	This work
LY302	Isogenic to MY508, except *dhh1*::*kanMX6 MATα*	This work
LY375	Isogenic to LY176, except *DHH1-HA* + pNot1ΔN1	This work

TABLE II
Phenotypes of double mutants

The phenotypes of the double mutants on yeast extract/peptone/dextrose at 30 °C are listed. Lethal means inviable; (−) indicates that the double mutant grows like the slower growing single mutants; and SYN(−) indicates a slightly slow growth phenotype compared with that of the slower growing single mutant. The strains used in the crosses are listed in Table I.

	not1–1	*not1–2*	*not3*Δ	*not4*Δ	*not5*Δ	*not5–1*	*ccr4*Δ	*caf1*Δ
*dhh1*Δ	(−)	Lethal	SYN(−)	Lethal	SYN(−)	SYN(−)	(−)	Lethal
*caf1*Δ	(−)	Lethal	SYN(−)	Lethal	Lethal	Lethal	(−)	
*ccr4*Δ	(−)	Lethal	(−)	Lethal	Lethal	Lethal		

(19), and a recombinant fragment of Caf1p was shown to degrade poly(A) *in vitro* (20), demonstrating that Caf1p is a nuclease. An additional link to mRNA degradation is the DEAD box putative RNA helicase Dhh1p (21), which is associated *in vivo* with Caf1p (22). This helicase is thought to be a component of the decapping complex (19) and indeed interacts in the two-hybrid assay with the decapping enzyme Dcp1p (23). It is hard to reconcile the large number of described partners for the Ccr4-Not complex with a unique function or even with a unique complex. In fact, we have evidence that, although the 1.2-MDa complex is a defined entity that can be purified (9), the previously described 2-MDa complex (4) is probably not a unique entity.[4]

In this work, we wanted to determine whether Dhh1p, probably involved in the degradation of mRNAs like Caf1p and Ccr4p, interacts with the Ccr4-Not complex. First, we demonstrated striking genetic interactions between *DHH1* and the *NOT* genes. Second, we were able to demonstrate that the N-terminal region of Not1p is indeed essential for the maintenance of stable levels of both Caf1p and Dhh1p *in vivo*. This correlates with an interaction between the N-terminal domain of Not1p and the two factors, an interaction that is likely to occur within the context of Ccr4-Not complexes. Taken together with the established links between the Ccr4-Not complex and transcription and our more recent finding that the Ccr4-Not complex contributes to regulation of stress responses and responses through the protein kinase A pathway,[5] this work provides a new view of the Ccr4-Not complex as a general coordinator of the regulation of gene expression via several pathways.

MATERIALS AND METHODS

Media and Strains—All media were standard. To obtain a *dhh1*-null strain, we backcrossed the strain received (MY2700) (Table I) three times with MY1 and selected for *dhh1*-null spores lacking *GCN4* after the first backcross. During these backcrosses, we noticed that *dhh1* could be ts^+ or ts^- at 37 °C for a reason that we could not assign to any known locus. We repeatedly chose the ts^+ spores. After the third backcross, the *dhh1*-null strain mostly isogenic to our strains was crossed once more with the various *ccr4-not* mutants, and the double mutants were obtained by tetrad analysis. To test for AT resistance, all of the strains that have a deletion of *GCN4* on the chromosome were transformed with a *LEU2* centromeric plasmid expressing an N-terminally deleted derivative of Gcn4p (C-163). Wild-type cells expressing this derivative cannot grow even on 5 mM AT, whereas *not* mutants grow on at least 40 mM AT. All AT tests were done on 20 mM AT. Wild-type cells or cells expressing in *trans* the C- and N-terminal domains of Not1p and expressing HA-tagged Dhh1p were created by transformation as described below. To obtain cells expressing only the C-terminal domain of Not1p and HA-tagged Dhh1p, the transformants obtained from the

[4] L. Maillet and M. A. Collart, unpublished data.

[5] E. Lenssen, U. Oberholzer, J. Labarre, C. de Virgilio, and M. A. Collart, *Mol. Microbiol.*, in press.

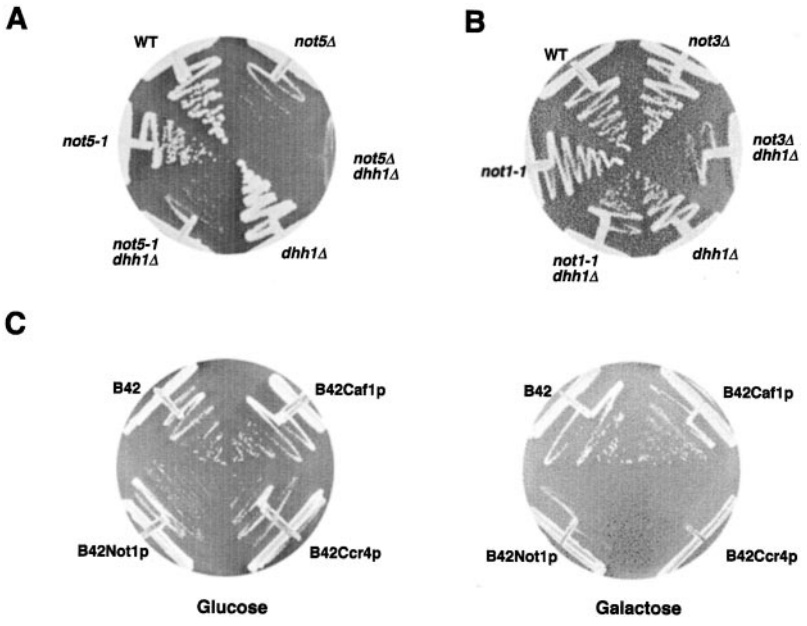

FIG. 1. **Synthetic growth in *dhh1*Δ cells carrying *not* mutations or a plasmid overexpressing Ccr4p or Not1p.** *A* and *B*, the indicated mutants were streaked onto glucose-rich plates for 3–5 days at 30 °C. *C*, *dhh1*Δ cells (MY2700; see Table I) carrying the plasmids expressing the indicated fusion proteins from the *GAL1* promoter were streaked onto glucose- or galactose-rich plates as indicated for 3–5 days at 30 °C. The B42Caf1p fusion carries only a partial Caf1p sequence (amino acids 147–433) capable of interacting (upon the two-hybrid analysis) with Ccr4p, Dhh1p, and Not1p and containing the RNase D domain. *WT*, wild type.

complemented cells were streaked onto 5-fluoroorotic acid, a drug that kills cells carrying a functional *URA3* gene.

DNA—The multicopy plasmid expressing Dhh1p was constructed by cloning an *Eco*RV-*Sac*I fragment including the *DHH1* gene into pRS425. The plasmid overexpressing Caf1p used to assay for AT resistance was obtained by amplification of *CAF1* sequences from genomic DNA and cloning as a *Hin*dIII-*Bam*HI fragment into pRS426. The plasmids overexpressing partial Caf1p, Ccr4p, or Not1p in galactose medium or overexpressing Ccr4p in glucose medium have been described previously (2, 9). To create an HA-tagged version of Dhh1p, oligonucleotides F2 (5′-TTT CAT GGC GAT GCC ACC TGG TCA GTC ACA ACC CCA GTA TCG GAT CCC CGG GTT AAT TAA-3′) and R1 (5′-GCG TAT CTC ACC ACA GTA GTT ATT TTT TCT TAG ATA TTC TGA ATT CGA GCT CGT TTA AAC-3′) were used for PCR amplification from vector pFA6a-3HA-*kanMX6* (25), and the PCR product was transformed into the desired strain. The G418-resistant transformants were verified for the correct integration events using oligonucleotides within the *DHH1* sequence (5′-CCC ATT CCC GAT AGA GCA AC-3′) and within the *kanMX6* gene (5′-CCT CAG TGG CAA ATC CTA ACC-3′).

Protein Extracts and Western Blot Analyses—Protein extracts were prepared as described below, and equivalent amounts of extract were separated by 10% SDS-PAGE. After transfer of the SDS-polyacrylamide gels onto nitrocellulose, the desired proteins were revealed by probing with specific antibodies against LexAp (kind gift from Roger Brent) at 1:3000 dilution, Ccr4p at 1:3000, Caf1p at 1:3000, HA (Babco) at 1:3000, and Dob1p (kind gift from Patrick Linder) at 1:3000; and secondary antibodies conjugated with horseradish peroxidase (Bio-Rad) were used at 1:10,000 dilution.

Gel Filtration Assays—Total protein extracts were prepared as previously described (4) by bead-beating in 350 mM NaCl, 40 mM Hepes (pH 7.2), 0.1% Tween 20, 10% glycerol, and protease inhibitors, followed by clarification by ultracentrifugation. The Superose 6 gel filtration assays were carried out as previously described (4). Briefly, 300 μl of total cell extracts were loaded onto a Superose 6 gel filtration column equilibrated with 350 mM NaCl, 10% glycerol, 0.1% Tween 20, and 40 mM Hepes (pH 7.3). The column was run at 0.4 ml/min; and 400-μl fractions were collected starting at 16 min, trichloroacetic acid-precipitated, and analyzed by Western blotting. The position of the void volume was determined by the elution of salmon sperm DNA and corresponds to the fraction just prior to the fraction that we have labeled 1. The column was calibrated using Bio-Rad markers for gel filtration.

Immunoprecipitation—Total protein extracts were prepared as described above for the gel filtration experiments. 2 mg of extract were immunoprecipitated with 1 μl of goat anti-LexAp antibody (Babco) or 1 μl of rabbit anti-Not1p antibody (raised against amino acids 1–241), followed by protein G-Sepharose.

Assay for Heat Shock Resistance—To measure resistance to heat shock, cells were grown overnight in glucose-rich medium, diluted in the same medium to $A_{600\,nm}$ = 0.1 in the morning, allowed to grow to $A_{600\,nm}$ = 1.0, and incubated at 50 °C for various lengths of time. Equivalent amounts of cells after different time points were plated on glucose-rich medium and left to grow at 30 °C. The amount of cells forming colonies was then calculated. The amount of colonies formed from cells not subjected to the heat shock was calculated as 100%.

RNA Analyses—Total cellular RNA was extracted as previously described by the hot acid/phenol method (1), and 50 μg were hybridized to the *DHH1* probe (5′-CCC AAT GTA CGG ACG ACT TGG GAA GTT TGC AGA GCT AAC TCT CTT GTG GGA ACC CCG C-3′) and to the *DED1* probe (1) as an internal control. After digestion by S1 nuclease, the hybridized oligonucleotides were analyzed on a sequencing gel.

TABLE III
Growth on AT with C-163-Gen4p

Growth was monitored on minimal medium lacking histidine and supplemented with 20 mM AT. All strains are isogenic to MY1, except for the indicated allele, and carry the plasmid encoding the C-163-Gen4p derivative. p*DHH1*, p*CCR4*, and p*CAF1* are multicopy plasmids carrying the indicated genes. WT, wild type.

Genotype	AT resistance
WT	–
not1–1	+
*not3*Δ	+
*ccr4*Δ	–
*caf1*Δ	–
*dhh1*Δ	–
*not1–1 ccr4*Δ	–
*not1–1 caf1*Δ	–
*not1–1 dhh1*Δ	–
*not3*Δ *ccr4*Δ	–
*not3*Δ *caf1*Δ	–
*not3*Δ *dhh1*Δ	–
WT p*DHH1*	+
WT p*CCR4*	–
WT p*CAF1*	–

RESULTS

Synthetic Phenotypes Are Observed between dhh1-null and not Mutants—DHH1 was isolated as a multicopy suppressor of a *caf1*-null mutant (*caf1*Δ), and Dhh1p was shown to interact physically with Caf1p (22). Because Caf1p is a component of the Ccr4-Not complex, we wanted to determine first whether there were any genetic interactions between *DHH1* and the *NOT* genes. We introduced a *dhh1*-null mutation (*dhh1*Δ) into our genetic background (see "Materials and Methods") and created double mutants by crosses. Table II shows that like *caf1*- and *ccr4*-null mutations, a *dhh1*-null mutation displayed very dramatic synthetic phenotypes when combined with *not* mutations (Fig. 1, *A* and *B*). In particular, *not1-2*, which is

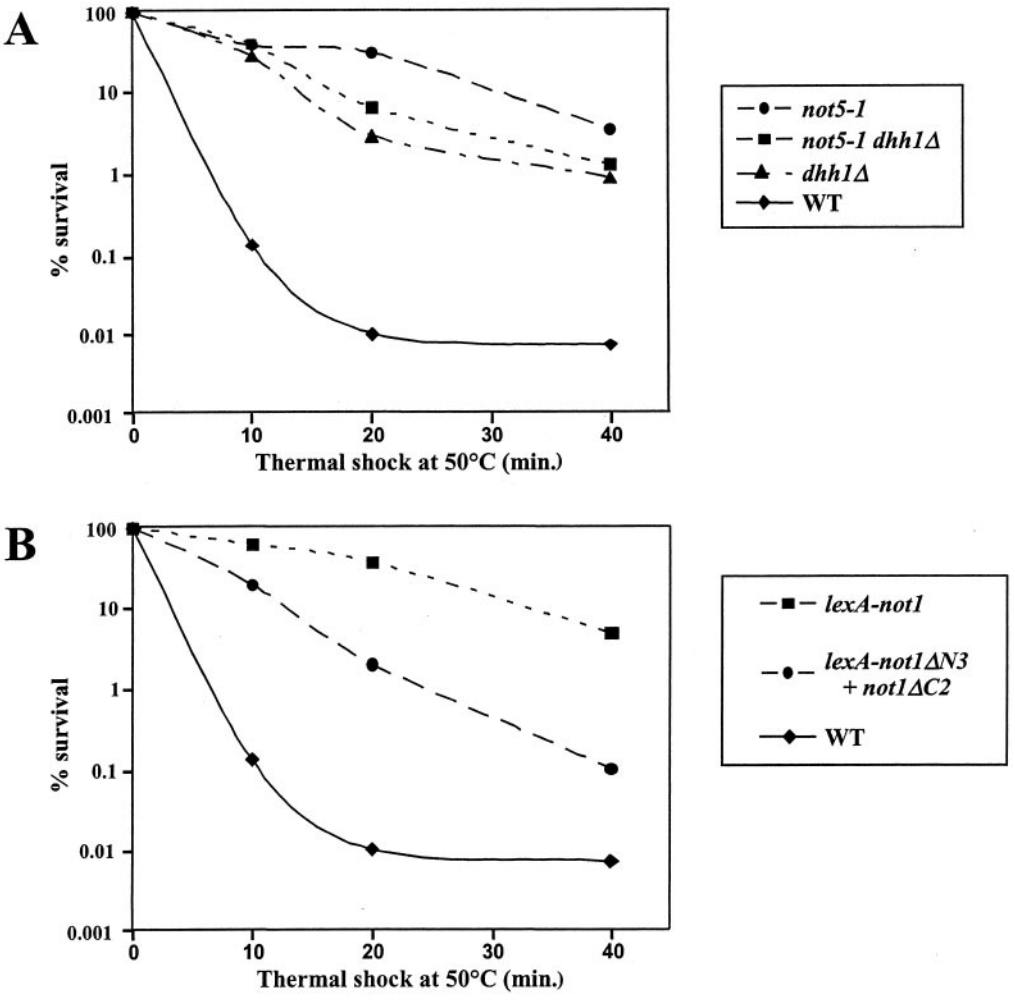

FIG. 2. ***dhh1Δ* and *not* mutants are resistant to heat shock.** Cells from the indicated strains were grown overnight in glucose-rich medium, diluted in the morning to $A_{600\ nm} = 0.1$ in glucose-rich medium, allowed to grow to $A_{600\ nm} = 1$, and transferred to 50 °C for 40 min. At the indicated times, a constant number of cells was plated in 10-fold serial dilutions on glucose-rich medium and allowed to grow at 30 °C. The amount of colony-forming units was calculated as 100% at $t = 0$ and evaluated at subsequent time points. The results are plotted for each strain. *WT*, wild type.

synthetically lethal when combined with any mutation in another *CCR4-NOT* gene, except *NOT3*, was lethal when combined with a *dhh1*-null mutant. This synthetic lethality could be confirmed by introduction of a wild-type *NOT1* gene on a *URA3* plasmid into the diploid prior to dissection. Indeed, double-deleted spores carrying the *URA3* plasmid could be obtained, but could not grow on plates with 5-fluoroorotic acid, a drug that kills cells carrying a functional *URA3* gene. Interestingly, we found that *dhh1*Δ was also synthetically lethal when combined with a *caf1*-null mutation (Table II), in contrast to what has been previously published (22). This could be verified by introduction of either the wild-type *CAF1* or *DHH1* gene on a *URA3* plasmid prior to dissection and recovery of double mutant spores carrying the *URA3* plasmid. These spores could not grow on 5-fluoroorotic acid.

These first experiments revealed surprisingly that the *dhh1*Δ *not1*Δ double mutant spores carrying an episomal copy of *NOT1* grew slower than the *dhh1*Δ single mutant. To investigate this finding further, we transformed *dhh1*Δ cells with a multicopy plasmid overexpressing *NOT1* specifically in galactose-containing medium. The transformed cells grew much slower than *dhh1*Δ cells transformed with the parental vector on galactose-containing medium, but not on glucose-containing medium (Fig. 1*C*). Surprisingly, the expression of *CCR4* from a multicopy plasmid had the same phenotype, but not that of *CAF1* (Fig. 1*C*). The multicopy clone expressing *CAF1* did not, however, encode full-length Caf1p, although it did encode the region necessary for interaction with Not1p, Dhh1p, and Ccr4p (9, 22) and the RNase D domain (20). Taken together, these results show that cells lacking Dhh1p are particularly sensitive to alterations of the Ccr4-Not complex or to expression of specific components of the Ccr4-Not complex from multicopy plasmids.

A not Mutant Phenotype Is Suppressed by Deletion of DHH1 and Mimicked by Overexpression of DHH1—The results above could be explained if the function of Dhh1p is sensitive to modifications of Caf1p function because Caf1p is a known component of the Ccr4-Not complex and is also associated with Dhh1p. To investigate a possible more direct functional relationship between Dhh1p and the Ccr4-Not complex, we wanted to determine whether any of the mutant phenotypes associated with mutations in the *NOT* genes might require Dhh1p. In particular, *not* mutants expressing an activation-defective derivative of Gcn4p are resistant to AT. As mentioned above, this phenotype is unique to the *not* mutants and furthermore requires the Ccr4 and Caf1 proteins (4, 17). We thus analyzed AT resistance of single and double mutants that grew well enough to determine growth on AT plates, namely *not3*Δ *dhh1*Δ and *not1-1 dhh1*Δ (see Fig. 1*B*). These double mutants and the single mutants were transformed with a plasmid expressing

the mutant Gcn4p derivative and analyzed for growth on AT. Table III shows that, first, a deletion of *DHH1*, like the deletion of *CCR4* or *CAF1*, did not lead to AT resistance; and second, it completely suppressed AT resistance of *not* mutants.

To determine whether AT resistance in *not* mutants might be due to increased functional levels of Dhh1p, Ccr4p, or Caf1p, we analyzed AT resistance in wild-type cells expressing each of the three proteins from multicopy plasmids. Interestingly, expression of *DHH1*, but not that of either *CCR4* or *CAF1* (full-length in this case), from a multicopy plasmid rendered wild-type cells AT-resistant. Taken together, these results suggest that AT resistance in *not* mutants could be due to increased activity of Dhh1p in the context of wild-type Ccr4p and Caf1p.

Dhh1p Interacts with the N-terminal Domain of Not1p—To address further whether Dhh1p might be associated with the Ccr4-Not complex, we determined whether a deletion of *DHH1* might render cells resistant to heat shock. Indeed, in a recent study, we found that this is a phenotype shared uniformly by any mutation that affects the Ccr4-Not complex.[5] Fig. 2*A* shows that a deletion of *DHH1* rendered cells resistant to heat shock at 50 °C, as did a mutation of *NOT5*, and that the effects were not additive. Thus, in this case, a deletion of *DHH1* has the same phenotype as a deletion of a *NOT* gene. By this criterion, we can expect that Dhh1p might be associated with the Ccr4-Not complex.

We thus examined whether Dhh1p is physically associated with Not1p, the only known essential protein of the Ccr4-Not complex. We performed immunoprecipitation experiments using strains expressing full-length Not1p or a derivative of Not1p lacking the N-terminal sequences of Not1p (Not1ΔN1p) against which antibodies were raised. We also used a strain that expressed full-length Not1p but that lacked the *CAF1* gene. In all three strains, Dhh1p was HA epitope-tagged at its genomic locus (see "Materials and Methods"). Immunoprecipitation with anti-Not1p antibodies demonstrated specific co-immunoprecipitation of tagged Dhh1p with Not1p independent of Caf1p (Fig. 3*A*).

Genetically, Dhh1p has been associated with Caf1p, and the two proteins are known to physically interact. Within the Ccr4-Not complex, the Caf1 protein is associated with the N-terminal nonessential domain of Not1p (17). To determine whether Dhh1p might be associated with the same domain of Not1p, the genomic copy of *NOT1* was replaced by two plasmids expressing separately the N-terminal (amino acids 1–1318) and tagged C-terminal (amino acids 1319–2108) domains of Not1p (4) in a strain in which Dhh1p was HA epitope-tagged at its genomic locus. Such a strain is wild type for growth on all the media and at all temperatures we have tested. However, it is probably not entirely wild type as far as the integrity of the Ccr4-Not complexes is concerned (4), and this was confirmed by our observation that this strain still displayed a certain degree of heat shock resistance compared with a wild-type strain (Fig. 2*B*). We performed an immunoprecipitation experiment in which we immunoprecipitated either the N- or C-terminal domain of Not1p. We found that Dhh1p co-immunoprecipitated with the N-terminal (but not C-terminal) domain of Not1p (Fig. 3*B*). Thus, Dhh1p is indeed associated with the N-terminal domain of Not1p *in vivo* and probably within the context of Ccr4-Not complexes.

The N-terminal Domain of Not1p Is Essential to Independently Maintain Wild-type Levels of Dhh1p and Caf1p in Vivo—A possible interpretation for the observations presented so far could be that the Ccr4-Not complex controls the levels of functional Dhh1p *in vivo*. To investigate how this may occur, we first analyzed Dhh1, Ccr4, and Caf1 protein levels in wild-type cells and in several mutant strains, all of which expressed

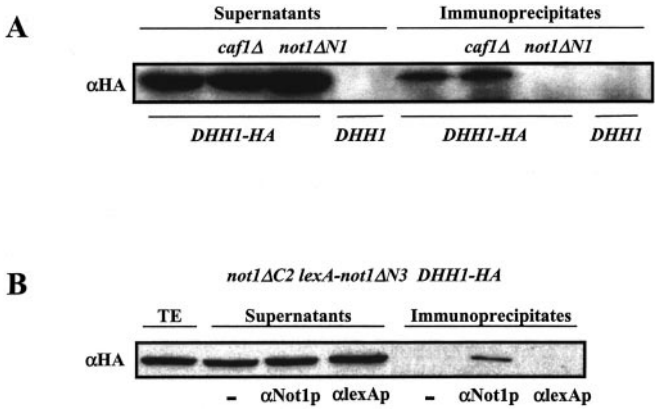

FIG. 3. **Dhh1p co-immunoprecipitates with the N-terminal domain of Not1p.** *A*, wild-type cells, cells in which the *CAF1* gene was deleted (MY1729), or cells in which the *NOT1* gene was deleted and replaced by a plasmid carrying *not1ΔN1* expressing a derivative of Not1p lacking the 394 N-terminal amino acids (LY375) carried additionally a genomically HA-tagged copy of Dhh1p. 2 mg of total protein extracts from these cells and wild-type cells expressing untagged Dhh1p were incubated with 1 μl of anti-Not1p antibody (recognizing only the N-terminal 241 amino acids of Not1p), followed by protein G-Sepharose. *B*, cells carrying a disruption of the genomic copy of *NOT1* and carrying two different plasmids, one expressing the N-terminal domain of Not1p (Not1ΔC2p) and one expressing the C-terminal domain of Not1p fused to LexAp (LexANot1ΔN3p), carried additionally a genomically HA-tagged copy of Dhh1p (LY186). 2 mg of total protein extracts were incubated without antibody (−), with 1 μl of anti-Not1p antibody, or with 1 μl of anti-LexAp antibody as indicated, followed by protein G-Sepharose. 50 μg of total protein extract (*TE*), 50 μg of the supernatants, or the total immunoprecipitates were loaded as indicated onto 10% SDS-polyacrylamide gels and transferred to nitrocellulose for the detection of HA-tagged Dhh1p by Western blot analysis with antibodies against HA.

an HA epitope-tagged Dhh1p. We analyzed cells lacking Caf1p, Ccr4p, Dhh1p, or the N-terminal domain of Not1p; cells expressing the N- and C-terminal domains of Not1p in *trans*; or cells carrying a mutation in the C-terminal domain of Not1p (*not1-1*) (4). Fig. 4*A* shows that the stable level of Caf1p was drastically reduced in cells lacking the N-terminal sequences of Not1p, as has been previously observed (17). The stable level of Dhh1p was also severely reduced in these cells and still somewhat reduced in cells expressing both domains of Not1p in *trans*. There was a slight decrease of Dhh1p in cells lacking Caf1p and possibly a very slight decrease of Caf1p in cells lacking Dhh1p. Ccr4p was not dramatically decreased in any of the strains, nor was Dob1p, a protein involved in ribosome biogenesis (24) measured to control for protein loading (Fig. 4*A*). Thus, the N-terminal domain of Not1p is necessary for maintaining the stable cellular levels of Dhh1p and Caf1p. To make sure that this effect was not due to transcriptional regulation, we analyzed *DHH1* mRNA levels in the different mutants. There was no modulation of *DHH1* mRNA levels in any of the mutants (data not shown).

We looked next at Dhh1p in cells expressing the N- and C-terminal domains of Not1p in *trans* or in cells lacking the N-terminal region of Not1p by gel filtration of total cell extracts. In the complemented cells, Dhh1p fractionated with a very broad profile throughout the gradient, from the void volume (>2 MDa) to fraction 25 (118 kDa), with a peak in fractions 23–25 (Fig. 4*B*, *upper left panel*). This profile was similar to that in wild-type cells expressing integral Not1p (Fig. 4*C*). As previously published (4), the C-terminal domain of Not1p also eluted with a broad profile (Fig. 4*B*, *lower left panel*). In contrast, Dob1p eluted mostly with a discrete peak in fractions 9–11 and a second weaker peak in fractions 21–23 (Fig. 4*B*, *middle left panel*). In cells expressing only the C-terminal domain of Not1p, there was, as mentioned above, much less

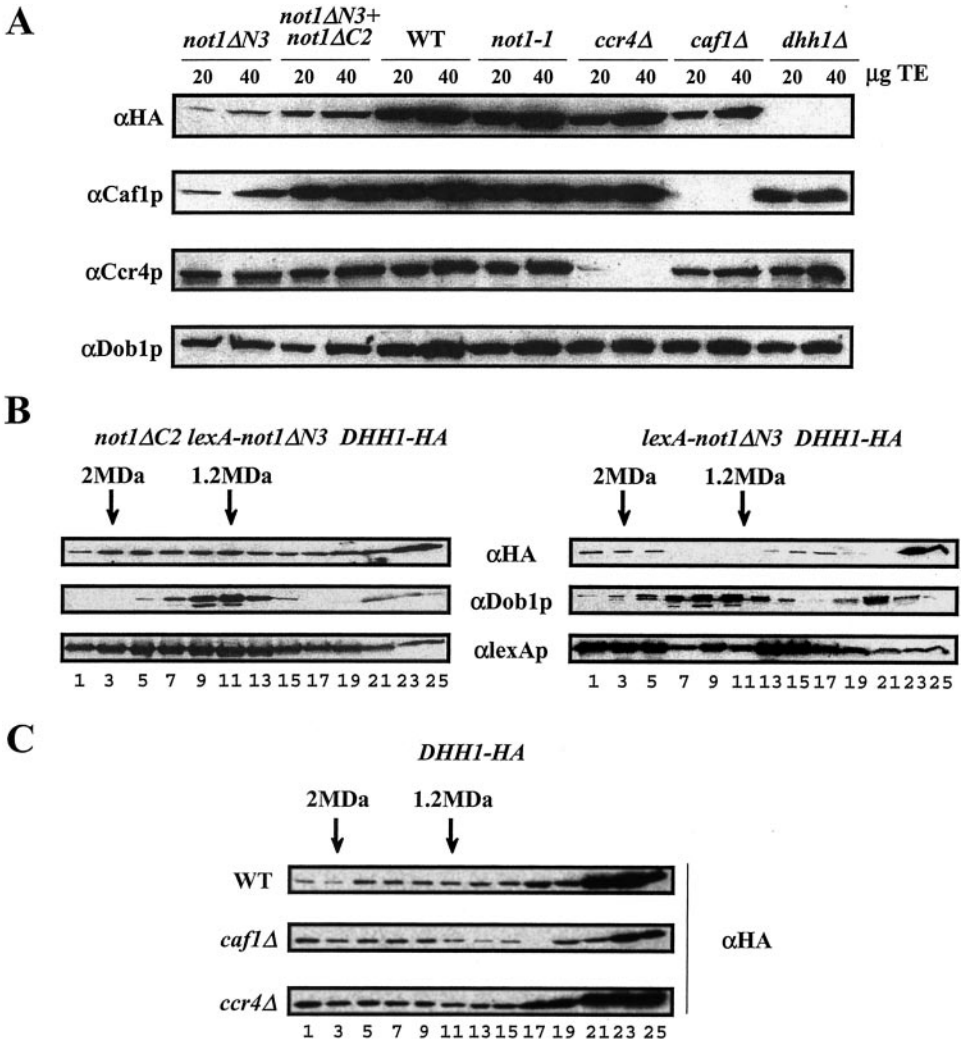

FIG. 4. **Dhh1p and Caf1p levels are decreased in cells lacking the N-terminal domain of Not1p, and this corresponds to the decrease of Dhh1p in specific large complexes.** *A*, total protein extracts (*TE*; 20 or 40 μg as indicated) from the indicated strains were loaded onto 10% SDS-polyacrylamide gels and analyzed by Western blotting for the levels of Dhh1p (with anti-HA antibodies), Caf1p, Ccr4p, and Dob1p as indicated. *B* and *C*, total protein extracts from cells expressing HA epitope-tagged Dhh1p, cells expressing the N- and C-terminal domains of Not1p in *trans* or expressing only the C-terminal domain of Not1p (as indicated) (*B* only), or wild-type cells (*WT*) or cells with the *CAF1* or *CCR4* gene deleted (*C* only) were analyzed by Superose 6 gel filtration. After trichloroacetic acid precipitation, the odd-numbered fractions were analyzed by Western blotting for the presence of Dhh1p (anti-HA antibodies), Dob1p, or the C-terminal domain of Not1p (anti-LexAp antibodies) as indicated.

Dhh1p (Fig. 4*B*, *upper right panel*). A peak of Dhh1p was still very apparent in fractions 23–25. However, instead of the broad elution pattern seen in the complemented and wild-type cells, Dhh1p was missing in fractions 7–11 and also decreased in fractions 19–21. Interestingly, the C-terminal domain of Not1p was also decreased in fractions 7–11 while still displaying a broad elution pattern (Fig. 4*B*, *lower right panel*). Fractions 7–11 were not underloaded, as could be seen by the reproducible elution of Dob1p in these fractions (Fig. 4*B*, *middle right panel*). Taken together, these results show that, in cells lacking the N-terminal domain of Not1p, the total cellular levels of Dhh1p are decreased, and this corresponds to a decrease in complexes containing Dhh1p and the C-terminal domain of Not1p of similar sizes.

In cells lacking Ccr4p, the level and elution pattern of Dhh1p were no different from those in wild-type cells (Fig. 4*C*). In contrast, in cells lacking Caf1p, the small decrease in the total levels of Dhh1p correlated with a decrease of Dhh1p in complexes eluting mostly in fraction 17 (Fig. 4*C*). These complexes were different from those that were decreased in cells lacking the N-terminal domain of Not1p (Fig. 4*B*).

DISCUSSION

Dhh1p Functionally and Physically Interacts with the Ccr4-Not Complex—In this work, we have presented experiments that demonstrate a functional and physical interaction between the Ccr4-Not complex and Dhh1p. Prior experiments had associated Dhh1p only with Ccr4p and Caf1p (22). Thus, it was unclear whether the Not proteins, associated with Caf1p and Ccr4p in Ccr4-Not complexes, were functionally related to Dhh1p. We describe here synthetic phenotypes when *not* mutations are combined with a deletion of the *DHH1* gene. The particularly striking phenotype is the synthetic lethality observed between the *not1-2* and *dhh1*-null mutants. Indeed, *not1-2* is a nonsense mutation localized in the middle of the *NOT1* gene (4). *not1-2* mutant cells express very little full-length Not1p due to readthrough of the stop codon and dramatically overexpress the N-terminal domain of Not1p (14). In this mutant, the amount of integral Not1p is limited, as are probably the Ccr4-Not complexes for which Not1p is essential (4). In contrast, the overexpressed N-terminal domain probably tends to sequester factors with which it interacts away from the

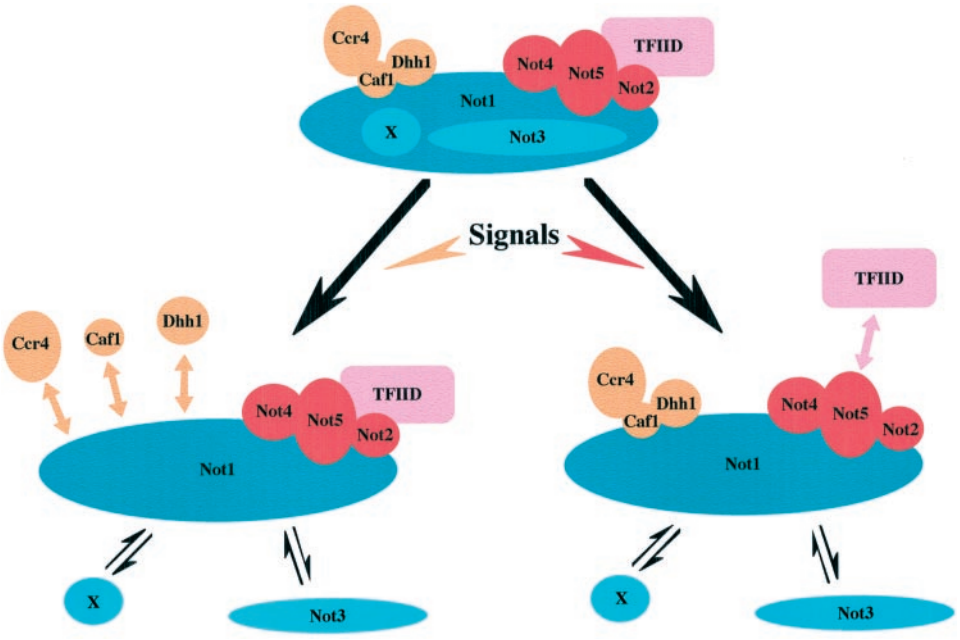

FIG. 5. **Model for the interactions of the regulatory proteins in the Ccr4-Not complex.** The components of the Ccr4-Not complexes depicted at the top were drawn according to the data concerning their respective interactions with the Not1 protein. TFIID is thought to interact with the C-terminal domain of Not1p probably via Not2p and Not5p. Distinct signals (*red* and *orange arrows*) could induce a variety of changes (indicated by the *double arrows* in the complexes depicted at the bottom) in some components (*e.g.* Not3p (see last paragraph under "Discussion") or unknown protein X) of the Ccr4-complex and consequently affect their interactions with the Ccr4-Not complex. These distinct signals could affect specifically the function of the C-terminal domain of Not1p in the regulation of transcription (proteins indicated by *red* and *pink shapes*) or the role of the N-terminal domain of Not1p in the regulation of mRNA stability (proteins indicated by *orange circles*), or both functions. A variation in TFIID-, Dhh1p-, Caf1p-, or Ccr4-dependent activities is symbolized by the separation of these proteins from Not1p and the *double-headed arrows*. The regulation of activity could take place by post-translational modification; dissociation of the proteins from the complex; moving the proteins from one cellular compartment to another; or yet by other mechanisms, any one of which would ultimately lead to a variation of the level of active proteins in the cell.

integral Ccr4-Not complexes. This may explain why *not1-2* is lethal when combined with mutations in genes encoding components interacting with the N-terminal domain (Ccr4p and Caf1p) or with the C-terminal domain (Not2p, Not4p, and Not5p) of Not1p. Thus, the finding that a null mutation in *DHH1* is also lethal when combined with *not1-2* supports the idea that Dhh1p is also a component of the Ccr4-Not complexes. The finding that overexpression of Not1p or Ccr4p is toxic in cells lacking Dhh1p can also be understood if Dhh1p is a component of Ccr4-Not complexes. Indeed, the disruption of these essential complexes is expected to be greater if when one component is removed, another is overexpressed and can titer away yet other components.

It is possible that all of the synthetic phenotypes observed and mentioned above result from Dhh1p and the Not proteins contributing separately to a common function; and indeed, Dhh1p contributing to mRNA decay while the Not proteins contribute to transcription would be in line with such an idea. However, we consider this unlikely because the *not5* mutants display much more dramatic transcriptional phenotypes (and any other phenotype that we have ever investigated) compared with *not1-2*, yet the former are viable when combined with a *dhh1*-null mutant, but the latter are not.

We further demonstrated by co-immunoprecipitation experiments that Dhh1p is associated with the N-terminal domain of Not1p *in vivo*. This is probably in large complexes of >1.2 MDa, as one can infer from the gel filtration experiments of extracts from cells lacking the N-terminal domain of Not1p in which specific Dhh1p- and Not1p-containing complexes are depleted. Again, one can argue that it could be that Not1p interacts with Dhh1p independently of the Ccr4-Not complexes. The experimental evidence argues against this possibility. First, a deletion of *DHH1* leads to heat shock resistance, as does any mutation that affects the Ccr4-Not complex.[5] Second, a phenotype associated with mutations in any of the *NOT* genes, and indeed the phenotype that led to their isolation, namely AT resistance, is Dhh1p-dependent. One could suggest that AT resistance requires Dhh1p function, but is not mediated by Dhh1p. However, this is contradicted by the observation that overexpression of Dhh1p leads to AT resistance. AT resistance of *not* mutants also requires the Caf1 and Ccr4 proteins. It has recently been described that Caf1p and Ccr4p are directly involved in mRNA deadenylation that occurs before mRNA decapping, in which Dhh1p is thought to be involved (19, 20). Thus, if AT resistance results from alterations in mRNA decay provoked by increased Dhh1p function, this will naturally require prior functional deadenylation and thus both Caf1p and Ccr4p. Hence, the most likely model, considering all of the above observations, is that Dhh1p function is derepressed in *not* mutants and usually repressed by the Ccr4-Not complex via its interaction with the N-terminal domain of Not1p.

The N-terminal Domain of Not1p Is Necessary to Maintain Stable Cellular Levels of Both Dhh1p and Caf1p—In trying to understand how the Ccr4-Not complex may control Dhh1p function, we made the surprising observation that, in cells that lack the N-terminal domain of Not1p, Dhh1p levels are dramatically decreased, as are those of Caf1p (17). Neither of these decreases can be accounted for indirectly by the decrease in the other protein. Indeed, even a total absence of Caf1p does not lead to as dramatic a decrease in Dhh1p as a deletion of the N-terminal domain of Not1p, and the complete absence of Dhh1p has very little effect, if any, on Caf1p levels. Furthermore, the relative level of Dhh1p in complexes of a specific size (>1.2 MDa) is decreased when the N-terminal sequences of Not1p are deleted, and such an effect is not observed in a *caf1*-null mutant. We also found that, whereas the fraction-

ation of Ccr4p in large complexes is dependent upon Caf1p (17), the association of Dhh1p in large complexes is clearly not dependent upon Caf1p. Finally, Dhh1p is associated with Not1p independently of Caf1p. These experiments suggest that the N-terminal domain of Not1p is necessary to maintain stable cellular levels of Dhh1p and Caf1p and for the association of Dhh1p in complexes >1.2 MDa.

These effects of the N-terminal domain of Not1p on Dhh1p could be indirect, especially because the Not1 protein is a global transcriptional regulator. Nevertheless, because both Dhh1p and Caf1p physically interact with this N-terminal domain of Not1p, it seems reasonable to suggest that this physical interaction is likely to be directly responsible for maintaining the wild-type levels of Dhh1p and Caf1p.

One can infer from these results that the loss of N-terminal sequences of Not1p is likely to result in decreased Dhh1p- and Caf1p-dependent activity if Dhh1p and Caf1p are limiting *in vivo*. Alternatively, if both Dhh1p and Caf1p contribute to mRNA degradation, even if neither protein is limiting *in vivo*, they could become so when the other one is decreased. In support of such a possibility is our observation that a *dhh1*-null mutation is lethal in cells lacking Caf1p. It has also been mentioned previously that a deletion of *CAF1* displays a synthetic lethal phenotype with a mutant of the *DCP1* gene encoding the decapping enzyme, which interacts with Dhh1p (19, 23). It will be interesting to determine whether indeed, as expected, mRNA degradation is decreased in cells lacking the N-terminal domain of Not1p.

What Is the Function of the Ccr4-Not Complex?—Our results show that, although the *not1ΔN3* mutant leads to decreased Dhh1p levels, in contrast, other *not* mutants display the same phenotype (AT resistance) as increased Dhh1p levels. One model that can reconcile these apparently contradictory results is that the interaction of Dhh1p (and Caf1p) with the N-terminal domain of Not1p is essential, on one hand, to render Dhh1p (and Caf1p) stable and, on the other hand, to limit the amount of functional Dhh1p at any given time in the cell (see model on Fig. 5). A similar role of the Ccr4-Not complex in sequestering TFIID has previously been suggested (2, 10, 12), and TFIID is included on the model of Fig. 5.

The position of Dhh1p *versus* TFIID relative to the Not1 protein on Fig. 5 stems from the knowledge obtained so far. Indeed, our present results assign a role to the nonessential N-terminal domain of Not1p in controlling the levels of two proteins important for mRNA degradation *in vivo*. It could be that control of mRNA degradation is the activity necessary for optimal growth mediated by this N-terminal domain of Not1p. This domain is not essential, and Dhh1p and Caf1p are not essential proteins, although the cell cannot live if both proteins are absent, but the deletion of the N-terminal domain does not lead to a total absence of both proteins, only a decrease. In contrast, the C-terminal essential region of Not1p is more likely to be important in regulation of transcription because Not5p and Not2p are associated with this domain and have been shown to interact with components of the general transcription factors TFIID (10, 12) and Spt-Ada-Gcn5 acetyltransferase complex (13). Furthermore, we have recently obtained evidence that the C-terminal essential function of Not1p is related to its interaction with TFIID.[3] This domain is essential, as are the components of the TFIID complex.

Why would a single core complex contribute to these two (and maybe more) important functions? One could imagine that its role is to coordinately modify these activities according to the physiological conditions of the cell. This model is supported by our recent finding that components of the Ccr4-Not complex, in particular Not3p, are modified in response to decreased nutrient levels and stress and subsequently degraded.[5] One expects then that, under these conditions, Dhh1p activity might be increased and affect mRNA decay. Indeed, in this work, we have shown that at least one of the phenotypes of a *not3Δ* strain (AT resistance) is mimicked by increased Dhh1p levels and requires Dhh1p. A similar type of alteration might occur with TFIID activity because we have already shown that there is a promoter-specific transcriptional alteration in *not3Δ* mutant cells (2). In such a model, the role of the Ccr4-Not complex would be much broader than initially suggested, as it would serve to coordinately regulate different cellular machineries in response to changes in the cellular environment.

Acknowledgments—We thank Mauricio Leal for technical assistance. We thank Françoise Stutz for the *dhh1*-null strain, Clyde Denis for plasmid Yep13-*CCR4* and both pJG45-*CAF1* and pJG45-*CCR4*, Claudio de Virgilio for a plasmid containing the *DHH1* locus, Roger Brent for antibodies against LexAp, and Patrick Linder for antibodies against Dob1p. We are very grateful to Bertrand Séraphin for critical reading of the manuscript and comments on the work.

REFERENCES

1. Collart, M. A., and Struhl, K. (1993) *EMBO J.* **12,** 177–186
2. Collart, M. A., and Struhl, K. (1994) *Genes Dev.* **8,** 525–537
3. Oberholzer, U., and Collart, M. A. (1998) *Gene (Amst.)* **207,** 61–69
4. Maillet, L., Tu, C., Hong, Y. K., Shuster, E. O., and Collart, M. A. (2000) *J. Mol. Biol.* **303,** 131–143
5. Draper, M. P., Salvadore, C., and Denis, C. L. (1995) *Mol. Cell. Biol.* **15,** 3487–3495
6. Denis, C. L. (1984) *Genetics* **108,** 833–834
7. Denis, C. L., and Malvar, T. (1990) *Genetics* **124,** 283–291
8. Sakai, A., Chibazakura, Y., Shimizu, Y., and Hishinuma, F. (1992) *Nucleic Acids Res.* **20,** 6227–6233
9. Liu, H.-Y., Badarinarayana, V., Audino, D. C., Rappsilber, J., Mann, M., and Denis, C. L. (1998) *EMBO J.* **17,** 1096–1106
10. Lemaire, M., and Collart, M. A. (2000) *J. Biol. Chem.* **275,** 26925–26934
11. Lee, T. I., Wyrick, J. J., Koh, S. S., Jennings, E. G., Gadbois, E. L., and Young, R. A. (1998) *Mol. Cell. Biol.* **18,** 4455–4462
12. Badarinarayana, V., Chiang, Y.-C., and Denis, C. L. (2000) *Genetics* **155,** 1045–1054
13. Benson, J. D., Benson, M., Howley, P. M., and Struhl, K. (1998) *EMBO J.* **17,** 6714–6722
14. Collart, M. A. (1996) *Mol. Cell. Biol.* **16,** 6668–6676
15. Chang, M., French-Cormay, D., Fan, H.-Y., Klein, H., Denis, C. L., and Jaehning, J. (1999) *Mol. Cell. Biol.* **19,** 1056–1067
16. Liu, H.-Y., Chiang, Y.-C., Pan, J., Chen, J., Salvadore, C., Audino, D. C., Badarinarayana, V., Palaniswamy, V., Anderson, B., and Denis, C. L. (2001) *J. Biol. Chem.* **276,** 7541–7548
17. Bai, Y., Salvadore, C., Chiang, Y.-C., Collart, M. A., Liu, H.-Y., and Denis, C. L. (1999) *Mol. Cell. Biol.* **19,** 6642–6651
18. Liu, H.-Y., Toyn, J. H., Chiang, Y.-C., Draper, M. P., Johnston, L. H., and Denis, C. L. (1997) *EMBO J.* **16,** 5289–5298
19. Tucker, M., Valencia-Sanchez, M. A., Staples, R. R., Chen, J., Denis, C. L., and Parker, R. (2001) *Cell* **104,** 377–386
20. Daugeron, M.-C., Mauxion, F., and Séraphin, B. (2001) *Nucleic Acids Res.* **29,** 2448–2455
21. Strahl-Bolsinger, S., and Tanner, W. (1993) *Yeast* **9,** 429–432
22. Hata, H., Mitsui, H., Liu, H., Bai, Y., Denis, C. L., Shimizu, Y., and Sakai, A. (1998) *Genetics* **148,** 571–579
23. Uetz, P., Giot, L., Cagney, G., Mansfield, T. A., Judson, R. S., Knight, J. R., Lockshon, D., Narayan, V., Srinivasan, M., Pochart, P., QureshiEmili, A., Li, Y., Godwin, B., Conover, D., Kalbfleisch, T., Jijayadamodar, G., Yang, M. J., Johnston, M., Fields, S., and Rothberg, J. M. (2000) *Nature* **403,** 623–627
24. de la Cruz, J., Kressler, D., Tollervey, D., and Linder, P. (1998) *EMBO J.* **17,** 1128–1140
25. Longtine, M. S., McKenzie, A., Demarini, D. J., Shah, M. G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J. R. (1998) *Yeast* **14,** 953–961

Interaction between Not1p, a Component of the Ccr4-Not Complex, a Global Regulator of Transcription, and Dhh1p, a Putative RNA Helicase

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J. Biol. Chem. 2002, 277:2835-2842.
doi: 10.1074/jbc.M107979200 originally published online November 5, 2001
