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The Ccr4-Not Complex Independently Controls both Msn2-Dependent Transcriptional Activation—via a Newly Identified Glc7/Bud14 Type I Protein Phosphatase Module—and TFIID Promoter Distribution

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The Ccr4-Not complex is a conserved global regulator of gene expression, which serves as a regulatory platform that senses and/or transmits nutrient and stress signals to various downstream effectors. Presumed effectors of this complex in yeast are TFIID, a general transcription factor that associates with the core promoter, and Msn2, a key transcription factor that regulates expression of stress-responsive element (STRE)-controlled genes. Here we show that the constitutively high level of STRE-driven expression in *ccr4-not* mutants results from two independent effects. Accordingly, loss of Ccr4-Not function causes a dramatic Msn2-independent redistribution of TFIID on promoters with a particular bias for STRE-controlled over ribosomal protein gene promoters. In parallel, loss of Ccr4-Not complex function results in an alteration of the posttranslational modification status of Msn2, which depends on the type 1 protein phosphatase Glc7 and its newly identified subunit Bud14. Tests of epistasis as well as transcriptional analyses of Bud14-dependent transcription support a model in which the Ccr4-Not complex prevents activation of Msn2 via inhibition of the Bud14/Glc7 module in exponentially growing cells. Thus, increased activity of STRE genes in *ccr4-not* mutants may result from both altered general distribution of TFIID and unscheduled activation of Msn2.

All living organisms are proficient at adapting within a defined physiological range to changing environmental conditions. Such adaptation processes are inherently coupled to changes in the expression of functional proteins which—particularly in eukaryotic cells—are based on regulation of different processes, such as transcription initiation, mRNA stability, translation, or posttranslational protein modification. Interestingly, the Ccr4-Not complex, which is conserved from yeast to human, acts on most of these processes to control the appropriate expression of functional proteins and may therefore play a critical role in adaptive responses to environmental challenges. The complex exists in at least two distinguishable forms of 1.2 and 2 MDa and harbors nine core subunits, which include Ccr4, Caf1, Caf40, Caf130, and Not1-5 (reviewed in references 11, 14, and 17). While the smaller complex may contain solely the core subunits, the larger form is likely to be associated with various additional proteins that are involved in transcription initiation, such as the SAGA-complex subunit Ada2 (4), the RNA polymerase II (Pol II) holoenzyme subunits Srb9, Srb10, and Srb11 (32), and the TFIID-complex subunits TBP, Taf13, and Taf1 (16, 28, 29, 44). The larger complex may further include proteins that are involved in mRNA degradation, such as the putative RNA helicase Dhh1, which resides in the decapping complex (35). Interestingly, both core subunits Ccr4 and Caf1 are also known to control initial steps in mRNA degradation as key components of the yeast deadenylase complex (8, 15, 52, 53). Finally, the Ccr4-Not complex has also been found to interact directly with the cell cycle-regulated Dbf2 protein kinase (33) as well as with the E2 ubiquitin-conjugating enzymes Ubc4 and Ubc5, which—by analogy to the situation in mammalian cells—are thought to interact with the putative E3 ubiquitin ligase Not4 (1).

The *NOT* genes were originally isolated in a selection for mutants that cause an increase in transcription of the *HIS3* gene (12, 13, 41). The *not* mutants displayed core promoter-specific defects which, together with the reported interaction between specific Not proteins and TFIID subunits, indicated that the *NOT* gene products may be involved in control of TFIID function. In line with this suggestion, it was recently found that Not5 not only associates with promoter DNA in a Taf1-dependent manner but also controls appropriate Taf1-DNA association, particularly during adaptation to nutrient-limiting conditions (16). In parallel, the Ccr4-Not complex may exert an additional control over transcription initiation by directly or indirectly inhibiting the function of the zinc finger transcription factor Msn2 (30), which is known to control expression from the stress response element (STRE) in response to environmental signals (20, 23, 38, 45). While in principle it is possible that the Ccr4-Not complex may simply regulate the

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TABLE 1. *S. cerevisiae* strains used in this study

Strain	Relevant genotype	Source or reference
MY1	*MAT***a** *ura3-52 trp1 leu2*Δ::*PET56 gcn4 gal2*	13
MY2	*MAT*α *ura3-52 trp1 leu2*Δ::*PET56 gcn4 gal2*	36
MY4	*MAT*α *ura3-52 trp1 leu2*Δ::*PET56 gcn4 gal2 his3*::*TRP1*	36
MY508	MY1 *not3*Δ::*URA3*	13
MY1719	MY2 *not5*Δ::*LEU2*	41
MY1728	MY1 *ccr4*Δ::*URA3*	36
MY1729	MY1 *caf1*Δ::*LEU2*	36
MY2050	MY1 *not4*Δ::*LEU2 his3*Δ::*TRP1*	This study
MY2051	MY2 *not4*Δ::*LEU2 his3*Δ::*TRP1*	This study
MY2052	MY1 *not4*Δ::*LEU2*	30
MY2053	MY2 *not4*Δ::*LEU2*	This study
MY2182	MY2 *not2*Δ::*kanMX6*	This study
MY2489	MY2 *his3*Δ::*TRP1 ura3*Δ::*STRE-lacZ-URA3*	30
MY2596	MY2050 *ura3*Δ::*STRE-lacZ-URA3*	This study
MY2904	MY1 *bud14*Δ::*kanMX6*	This study
MY2995	MY2 *his3*Δ::*TRP1 not4*Δ::*LEU2 bud14*Δ::*kanMX6*	This study
MY2998	MY2 *bud14*Δ::*kanMX6*	This study
MY3317	*MAT***a** *ura3-52 his3 glc7-133 not4*Δ::*LEU2*	This study
MY3234	MY2998 *ura3*Δ::*STRE-lacZ-URA3*	This study
MY3235	MY2995 *ura3*Δ::*STRE-lacZ-URA3*	This study
MY3362	KT1705 *ura3*Δ::*STRE-lacZ-URA3*	This study
MY3363	MY3317 *ura3*Δ::*STRE-lacZ-URA3*	This study
MY3496	MY2 *msn2*Δ::*TRP1*	This study
MY3498	MY1 *his3*Δ::*TRP1 msn2*Δ::*TRP1 not4*Δ::*LEU2*	This study
MY3590	MY1 *MSN2-myc13*::*kanMX6*	This study
MY3591	MY2050 *MSN2-myc13*::*kanMX6*	This study
MY3633	MY1 *not4*Δ::*LEU2 his3*Δ::*TRP1 bud14*Δ::*kanMX6 MSN2-myc13*::*kanMX6*	This study
MY3634	MY1 *his3*Δ::*TRP1 bud14*Δ::*kanMX6 MSN2-myc13*::*kanMX6*	This study
PD6517	*MAT*α *ade8 leu2 trp1 cdc35-10*	18
PE6	PD6517 *bud14*Δ::*kanMX2*	This study
SGY446	*MAT*α *tpk1*Δ::*ADE8 tpk2-63*(Ts) *tpk3*::*TRP1 ura3-52 his3 leu2-3,11 trp1 ade8*	48
PE15	SGY446 *bud14*Δ::*kanMX2*	This study
W303-1A	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	51
PEY78	W303-1A *msn2*-Δ3::*HIS3 msn4-1*::*TRP1*	38
PE27	W303-1A *bud14*Δ::*kanMX2*	This study
EGY48	*MAT***a** *his3 trp1 ura3 LEU2*::*pLexAop6-LEU2*	55
KT1112	*MAT***a** *ura3 leu2 his3*	50
KT1960	*MAT*α *ura3-52 leu2 his3 trp1*	K. Tatchell
KT1961	*MAT***a** *ura3-52 leu2 his3 trp1*	K. Tatchell
KT1703	KT1960 *glc7-132*	K. Tatchell
KT1705	KT1961 *glc7-133*	K. Tatchell
KT1706	KT1960 *glc7-133*	K. Tatchell
KT1708	KT1960 *glc7-127*	K. Tatchell
IP19	KT1961 *BUD14-myc13*::*kanMX6*	This study

presence of TFIID at Msn2-regulated promoters, an alternative model suggests that the Ccr4-Not complex, possibly in response to high protein kinase A (PKA) levels under conditions of nutrient abundance, inhibits Msn2 function via direct or indirect posttranslational modification (30). Notably, in this context, both subcellular localization and STRE-binding activity of Msn2 are regulated by phosphorylation and dephosphorylation processes that are likely to involve different protein kinases and yet-unknown protein phosphatases (9, 21, 23, 24, 26).

It has been proposed that the Ccr4-Not complex may regulate mRNA levels of Msn2-controlled genes, such as *HSP12*, via more than one mechanism. Accordingly, Ccr4 negatively regulates *HSP12* mRNA stability (8), while the Not5 subunit of the Ccr4-Not complex controls the recruitment of Taf1 to the *HSP12* core promoter (16). Moreover, since posttranslational modification of Msn2 appeared different in those mutants of the Ccr4-Not complex in which Msn2-dependent transcription was increased, the Ccr4-Not complex may also directly or indirectly regulate the activity status of Msn2 (30). Here we study in more detail how the Ccr4-Not complex controls transcription of Msn2-dependent genes. We show that the complex acts independently on TFIID to control its promoter-specific distribution and on Msn2 to control its posttranslational modification, possibly via a newly identified Bud14/Glc7 protein phosphatase module. Thus, the Ccr4-Not complex regulates STRE-dependent transcription via at least two different mechanisms, namely, modification of TFIID distribution and modification of Msn2 activity.

MATERIALS AND METHODS

Yeast strains, media, and general methods. The *Saccharomyces cerevisiae* strains used in this work are listed in Table 1. Strains KT1960 and KT1961 are congenic to KT1112. Strains KT1703 and KT1708 carrying integrated *glc7-132* and *glc7-127* alleles, respectively (for details, see reference 54), and KT1705 and KT1706 carrying the integrated *glc7-133* allele were kindly provided by K. Tatch-

TABLE 2. Plasmids used in this study

Plasmid	Description[a]	Source or reference
pEG202	2μm *HIS3 ADH1-LexA-DBD*	55
pEG202-MSB2	pEG202 *ADH1-LexA-DBD-MSB2*	47
pSH18-34	2μm *URA3 LexAop(8)-lacZ*	25
pJG4-5	2μm *TRP1 GAL1-AD*	25
pJG4-5-MSB2	pJG4-5 *GAL1-AD-MSB2*	47
YCplac22	*CEN TRP1*	22
pAS24	*CEN LEU2 GAL1-HA2*	5
YCpADH1	*CEN LEU2 ADH1-promoter*	43
pCTT1-18/7x	*URA3 STRE(7X)-lacZ*	37
pFD668	*CEN LEU2 ADH1-GLC7*	This study
pCDV471	pEG202 *ADH1-LexA-DBD-GLC7*	This study
pIP760	pEG202 *ADH1-LexA-DBD-glc7-133*	This study
pCDV472	pJG4-5 *GAL1-AD-BUD14*	This study
pCDV690	pJG4-5 *GAL1-AD-REG1*	This study
pCDV697	pJG4-5 *GAL1-AD-REG2*	This study
pCDV688	pJG4-5 *GAL1-AD-REF2*	This study
pCDV692	pJG4-5 *GAL1-AD-GIP2*	This study
pAR498	YCplac22 *GAL1-HA2*	This study
pIP607	YCplac22 *GAL1-HA2-GLC7*	This study
pIP765	YCplac22 *GAL1-HA2-glc7-127*	This study
pIP766	YCplac22 *GAL1-HA2-glc7-132*	This study
pIP767	YCplac22 *GAL1-HA2-glc7-133*	This study

[a] 2μm indicates high-copy-number plasmids, and *CEN* indicates low-copy-number plasmids. *ADH1* and *GAL1* designate only the promoter regions of the corresponding genes.

ell. Mating of MY2052 with MY4 and subsequent sporulation of the resulting diploid yielded MY2050, MY2051, and MY2053. PCR-based gene deletions (*bud14*Δ::*kanMX2* transformed into W303-1A, SGY446, and PD6517 to create PE27, PE15, and PE6; *bud14*Δ::*kanMX6* transformed into MY1 to create MY2904; *not2*Δ::*kanMX6* transformed into MY2 to create MY2182; and *msn2*Δ::*TRP1* transformed into MY2 to create MY3496) and tagging of chromosomal genes (*BUD14-myc13-kanMX6* transformed into KT1961 to create IP19 and *MSN2-myc13-kanMX6* transformed into MY1 and MY2050 to obtain MY3590 and MY3591, respectively) were done as described previously (34). Mating of MY2904 with MY2051, KT1705 with MY2053, and MY3496 with MY2050 and subsequent sporulation of the resulting diploid strains yielded the segregants MY2995 and MY2998, MY3317, and MY3498, respectively. Mating of MY2998 with MY3591 and subsequent sporulation of the resulting diploids yielded the segregants MY3633 and MY3634. The linearized, NcoI-cut integrative vector pCTT1-18/7x (see below) was transformed into strains MY2050, MY2998, MY2995, KT1705, and MY3317 to construct MY2596, MY3234, MY3235, MY3362, and MY3363, respectively. Strains were grown at 30°C (except where noted) in standard rich yeast extract-peptone-dextrose (YPD) medium with 2% glucose (unless otherwise stated) or in synthetic defined media lacking specific amino acids as described previously (46). Yeast transformations, manipulation of *Escherichia coli*, and the preparation of bacterial growth media were performed as described previously (2).

Plasmid construction. Plasmids are listed in Table 2. To fuse the various genes to the LexA DNA-binding domain coding sequences in plasmid pEG202 and/or to the activation domain coding sequences in plasmid pJG4-5, yeast *glc7-133*, *GLC7*, *BUD14*, *REG1*, *REG2*, *REF2*, and *GIP2* full-length coding sequences were amplified by PCR using *Pfx* Platinum polymerase (Invitrogen) and genomic wild-type DNA as template DNA (except for *glc7-133*, where genomic DNA from strain KT1706 was used). Appropriate restriction sites were introduced with the primers. The PCR products were cloned at the EcoRI-XhoI sites of pJG4-5 (using *BUD14* and *REG1* to yield pCDV472 and pCDV690, respectively), the NcoI-XhoI sites of pJG4-5 (using *REG2*, *REF2*, and *GIP2* to yield pCDV697, pCDV688, and pCDV692, respectively), or the NcoI-XhoI sites of pEG202 (using *GLC7* and *glc7-133* to yield pCDV471 and pIP760, respectively). All constructs have the original start and stop codons of the fused genes. Plasmids pEG202-MSB2 and pJG4-5-MSB2 were described previously (47). To express *GLC7* under the control of the *ADH1* promoter, restriction sites were introduced immediately upstream (HindIII) and 616 nucleotides downstream (SacI) of the Glc7 coding sequence by PCR using wild-type genomic DNA (of strain KT1960) as template. The resulting amplicon was ligated into HindIII-SacI-digested YCpADH1 (43), thus creating pFD668. To obtain hemagglutinin (HA) epitope-tagged versions of Glc7, Glc7-127, Glc7-132, and Glc7-133, the corresponding full-length coding regions were amplified by PCR using genomic DNA of strains KT1960, KT1708, KT1703, and KT1706, respectively, as template. Restriction sites were introduced immediately upstream of the ATG codon (PstI) and 151 nucleotides downstream of the stop codons of the *GLC7*, *glc7-127*, *glc7-132*, and *glc7-133* coding regions (HindIII). The PCR products were cloned at the PstI-HindIII sites of pAR498, which is YCplac22 containing an EcoRI-HindIII *GAL1-HA2* fragment isolated from pAS24, to yield pIP607 (*GAL1-HA2-GLC7*), pIP765 (*GAL1-HA2-glc7-127*), pIP766 (*GAL1-HA2-glc7-132*), and pIP767 (*GAL1-HA2-glc7-133*). The *STRE-lacZ* reporter plasmid (pCTT1-18/7x) has been described previously (37).

IP and immunoblotting. To perform coprecipitation experiments with Bud14 and wild-type or mutant Glc7 proteins, strain IP19 expressing genomically tagged Bud14-myc13 was transformed with a control plasmid (pAR498) and with pIP607, pIP765, pIP766, and pIP767, which express *HA2-GLC7*, *HA2-glc7-127*, *HA2-glc7-132*, and *HA2-glc7-133*, respectively, under the *GAL1* promoter. Overnight cultures grown at 30°C in synthetic defined medium with 2% (wt/vol) raffinose were diluted to an optical density at 600 nm (OD_{600}) of 0.4 in the same medium and grown for an additional 2 h. Subsequently, 4% (wt/vol) galactose was added to induce *GAL1*-driven expression of the various Glc7 and Bud14 proteins during an additional 4 h at 30°C. Cells were then harvested by centrifugation and resuspended in ice-cold lysis buffer (50 mM Tris-HCl [pH 7.5], 0.1 M NaCl, 1 mM EDTA, 1% NP-40, one tablet of Complete Protease Inhibitor Cocktail [Roche Diagnostics GmbH]). Following addition of an equal volume of acid-washed glass beads, cells were broken by subjecting them to four 30-s cycles in a cell disruptor (FastPrep FP120). The extracts were clarified three times by centrifugation for 10 min at 4°C in a microfuge (17,000 rpm). HA2-tagged Glc7 and Glc7 variants were purified from clarified extracts with a protein G-agarose immunoprecipitation (IP) kit (Roche Diagnostics GmbH) following the manufacturer's instructions using monoclonal mouse anti-HA antibodies (HA.11 and immunoglobulin G1; Covance). Protein G-agarose beads carrying immunoprecipitates were resuspended in 50 μl of 2× sample buffer (62.5 mM Tris [pH 6.8], 25% glycerol, 2% sodium dodecyl sulfate [SDS], 0.01% bromphenol blue, 5% β-mercaptoethanol), boiled for 5 min, and electrophoresed on 10% polyacrylamide–SDS gels. After electrophoresis, proteins were transferred to nitrocellulose for immunoblotting and subsequent detection (ECL system; Amersham) of coprecipitated myc-tagged Bud14 using monoclonal mouse anti-myc antibodies (Santa Cruz Biotechnology, Inc.). Immunoblot analyses of total lysates were carried out as described previously (42). For analyses of Msn2 electrophoretic mobility, proteins (of the equivalent of an OD_{600} of 1) of exponentially growing cells were extracted by the post-alkaline extraction method, separated on 7% polyacrylamide–SDS gels, transferred to nitrocellulose, and probed with anti-Msn2 antibodies (kind gift from E. Estruch).

Two-hybrid analyses. Quantitative β-galactosidase assays were performed as described previously (2) with reporter plasmid pSH18-34. For the assays (see Table 5), we used strain EGY48 that had been cotransformed with a pEG202-based plasmid and a pJG4-5-based plasmid.

mRNA preparation and synthesis of cDNA. Yeast strains were grown at 30°C in YPD medium. Overnight cultures of 5 ml were diluted to an OD_{600} of 0.2 and maintained in exponential growth phase ($OD_{600} < 1.0$) for a period of 12 h by repeated dilution in fresh YPD medium to ensure complete depletion of stationary phase-specific transcripts. At this point exponential-phase samples were harvested. Subsequently, the cultures were grown until glucose was exhausted in the medium, and diauxic shift samples were harvested 30 min after glucose exhaustion. Total RNA was then extracted using the RNApure kit (GeneHunter Corporation) according to the manufacturer's instructions. Radiolabeled cDNA probes were generated from 1 μg of total RNA by reverse transcription of mRNA using Superscript II (Invitrogen), an oligo(dT) primer (10- to 20-mer mixture; Research Genetics), and [^{33}P]dCTP. Labeled probes were purified by passage through Bio-Spin 6 Chromatography Columns (Bio-Rad) and denatured for 5 min at 95°C.

GeneFilter hybridization and data analysis. Yeast Index GeneFilters (Research Genetics-Invitrogen) were hybridized with the labeled probes according to the manufacturer's protocol. The filters were scanned by use of a PhosphorImager (Fuji BAS-1000) to obtain digital images. Images produced by MacBas (Fuji) were converted to TIFFs and imported into the Pathways version 4.0 software (Research Genetics) for subsequent normalization against all data points and quantification of spot intensities. The average ratio was calculated from $\log_2$ expression ratios during the exponential phase of growth relative to the diauxic-shift transition from two independent experiments using either wild-type or mutant strains. Noninterpretable spots were manually flagged and excluded. A selection from the remaining spots was made to include only those open reading frames (ORFs) for which the discrepancy between the two independent exper-

iments was less than 2.5-fold. Of the selected 3,466 ORFs, those with an average ratio in wild-type cells of at least 2.0 were analyzed for Bud14 and Msn2/Msn4 dependency. To this end, we calculated fold decrease values by dividing the average ratio in wild-type strains by the average ratio in *bud14*Δ and *msn2 msn4* mutant strains for any given ORF. Descriptions of gene products were derived from the *Saccharomyces* Genome Database and/or the Comprehensive Yeast Genome Database (MIPS). Original data are available upon request.

Miscellaneous. Glucose concentrations were determined by the glucose oxidase method (Roche Diagnostics, GmbH). DNA sequences were obtained using the BigDye primer cycle sequencing kit and an ABI 301 automated sequencer (Applied Biosystems) according to the manufacturer's instructions. Protein concentrations were measured by use of the Bio-Rad protein assays according to the manufacturer's instructions using bovine serum albumin as a standard. For β-galactosidase assays and analyses of mRNA levels, cells were grown exponentially in rich medium at 30°C to an OD_{600} between 0.8 and 1.2. Protein extracts (50 μg) were then tested for β-galactosidase activity as previously described (30). For analysis of mRNA levels, total cellular RNA was extracted by the hot acid phenol method, and *lacZ* transcript levels were measured by S1 analysis using a specific oligonucleotide as previously described (12). Chromatin IP (ChIP) and quantitative real-time PCR were also performed as previously described (16). Polyclonal antibodies against Taf3, Taf8, Taf9, Taf11, and Taf12 were raised in rabbits following purification of the corresponding recombinant proteins expressed in *E. coli* from pET15b-derived plasmids (Elevage Scientifique des Dombes). Oligonucleotide sequences for the specific promoter DNAs measured in this study are available upon request.

TABLE 3. Cross-linking of the TFIID subunits TBP, Taf1, and Taf8 to various promoters in wild-type and *not5*Δ cells[a]

Protein	Incubation temp (°C)	Strain	Relative amount of DNA of:						
			HSP12	*HSP26*	*HSP104*	*ADH1*	*RPS8A*	*RPS9B*	*BAT1*
TBP	30	WT	0.36	0.43	0.48	1.97	0.83	2.45	0.76
		*not5*Δ	1.18	0.83	1.12	2.27	0.97	1.82	0.94
	39	WT	6.10	11.80	16.40	6.37	1.35	1.17	1.10
		*not5*Δ	18.50	25.30	21.90	8.52	1.09	1.72	0.68
Taf1	30	WT	0.36	0.38	0.43	0.74	1.20	3.23	0.52
		*not5*Δ	0.62	0.46	0.61	0.89	0.93	2.31	0.41
	39	WT	0.95	1.33	1.42	1.04	0.53	0.49	0.45
		*not5*Δ	2.02	3.09	2.40	1.77	0.73	0.80	0.45
Taf8	30	WT	0.34	0.33	1.17	2.82	5.37	17.10	1.75
		*not5*Δ	0.62	0.37	0.48	0.69	0.99	2.27	0.42
	39	WT	3.47	4.29	5.01	2.75	1.14	1.11	1.18
		*not5*Δ	4.11	5.41	4.47	2.88	1.12	1.15	0.63

[a] Wild-type (WT) (MY1) and *not5*Δ (MY1719) cells were grown to exponential phase and cross-linked following incubation for 10 min at 30 or 39°C. The indicated proteins (i.e., TBP, Taf1, and Taf8) were immunoprecipitated from total cell extracts, and the amount of DNA of each of the indicated promoters in the immunoprecipitates was quantified by real-time PCR and expressed relative to the amount of DNA in the corresponding total extracts (in arbitrary units). One representative data set (of a total of three) (unpublished data) is shown.

RESULTS

The Ccr4-Not complex controls differential TFIID promoter association. We have previously shown that cross-linking of Taf1 (expressed from a centromeric plasmid) to the *HSP12* promoter was increased in *not5*Δ cells (16). To investigate this effect in more detail, we decided to perform a series of ChIP experiments again using wild-type and *not5*Δ cells yet with different TFIID subunits and a larger set of promoters. The cross-linking of TBP and of the TFIID-specific Taf1 and Taf8 proteins (expressed from their own promoters) to the promoters of *HSP12*, *HSP26*, *HSP104*, *ADH1*, *RPS8A*, *RPS9B*, and *BAT1* in unstressed and heat-shocked (10 min at 39°C) wild-type and *not5*Δ cells is shown in Table 3. In unstressed wild-type cells, we found that TBP, Taf1, and Taf8 cross-linking to the promoters of the highly expressed *ADH1* or the *RPS8A* and *RPS9B* ribosomal protein genes was high compared to the observed cross-linking to the promoter of the weakly expressed *HSP12* gene. TBP and Taf proteins (Tafs) are therefore differentially distributed across different promoters (e.g., the amount of Taf8 cross-linked to the *RPS9B* promoter is about 50-fold higher than the amount cross-linked to the *HSP12* promoter) (Table 3), and their distribution pattern correlates well with the expression pattern from the corresponding promoters. When wild-type cells were subjected to a brief heat shock, cross-linking of TBP and Tafs strongly increased on highly expressed heat shock gene promoters (*HSP12*, *HSP26*, and *HSP104*) and generally decreased on repressed ribosomal protein gene promoters (*RPS8A* and *RPS9B*) (Table 3). Accordingly, in wild-type cells subjected to heat stress, the distribution pattern of TBP and Tafs also correlated well with the expression pattern from the corresponding promoters.

Loss of Not5 caused a dramatic change in the distribution of TBP and Tafs on various promoters in unstressed cells (Table 3). In general, the differential distribution of TBP, Taf1, and Taf8 was significantly reduced in *not5*Δ mutant cells (e.g., while the largest difference in cross-linking of Taf8 is 50-fold in wild-type cells [17.1 and 0.34 for the *RPS9B* and *HSP12* promoters, respectively], this value is reduced to 6-fold in *not5*Δ cells [2.27 and 0.37 for the *RPS9B* and *HSP26* promoters, respectively]). In particular, Taf1 and Taf8 cross-linking appeared to increase on the *HSP12* promoter and to decrease on the *RPS8A/9B* promoters in *not5*Δ cells. Since we obtained similar results for additional TFIID-specific Tafs (Taf3 and Taf11) and Tafs that are shared between TFIID and SAGA (Taf9 and Taf12) (data not shown), our data indicate that Not5 is involved in recruitment and/or stabilization of TFIID on ribosomal protein gene promoters and in detraction and/or destabilization of TFIID on promoters of stress genes in unstressed cells. This assumption is further supported by additional experiments (data available on request), where the amount of TBP and Tafs cross-linked to various promoters was normalized to the corresponding amount cross-linked to the *HSP12* promoter. Accordingly, loss of Not5 consistently caused a significant increase of the amount of TBP, Taf1, and Taf8 cross-linked to the *HSP12* promoter relative to that cross-linked to the *RPS9B* promoter. It is worth mentioning that the heat shock-induced changes in distribution of Taf8 (and other TFIID subunits) (data not shown) across promoters remained largely unaffected by the loss of Not5 (Table 3) (unpublished data), indicating that Not5 exerts its control over TFIID distribution mainly under nonstress conditions.

Finally, as with the loss of Not5, loss of various additional Ccr4-Not complex subunits (i.e., Not2, Not3, Not4, Ccr4, or Caf1) significantly increased the amount of both TBP (Fig. 1A) and Taf1 (Fig. 1B) that was cross-linked to the *HSP12* promoter relative to that cross-linked to the *RPS9B* promoter (similar results were obtained when using an alternative pair of an STRE-controlled gene [*HSP26*] and a ribosomal protein

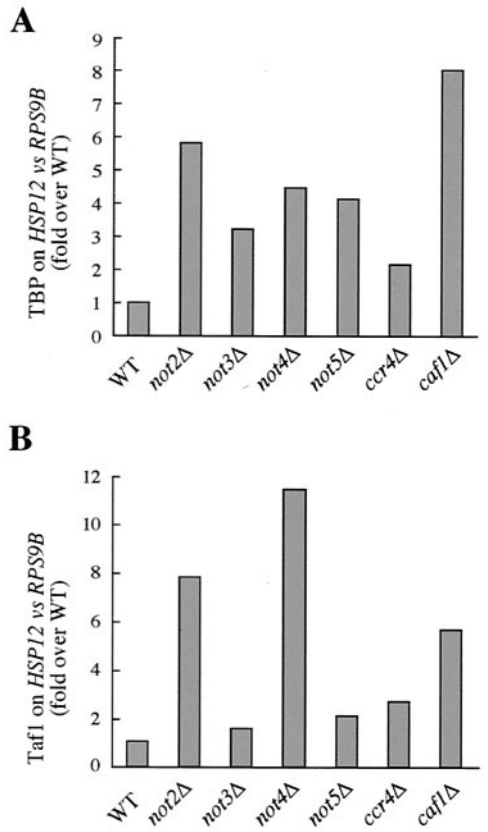

FIG. 1. Distribution of TBP (A) and Taf1 (B) on *HSP12* and *RPS9B* promoters in wild-type (WT) and various *ccr4-not* mutant cells. Wild-type (MY1), *not2*Δ (MY2182), *not3*Δ (MY508), *not4*Δ (MY2052), *not5*Δ (MY1719), *ccr4*Δ (MY1728), and *caf1*Δ (MY1729) strains were grown to exponential phase at 30°C. Following chromatin IP from whole cell extracts using either anti-TBP or anti-Taf1 antibodies, the levels of *HSP12* and *RPS9B* promoter DNA in the immunoprecipitates relative to their levels in whole cell extracts were determined via reverse transcription-PCR. The values represent ratios of the levels of *HSP12* to *RPS9B* promoter DNA precipitated with either anti-TBP or anti-Taf1 antibodies. Each value has been brought arbitrarily to the wild-type value (WT = 1). Results from one representative experiment are shown (similar results were obtained three times).

gene [*RPS8A*]) (data not shown). The control over differential distribution of TFIID across promoters appears therefore to reflect a general function of the Ccr4-Not complex.

The Ccr4-Not complex controls TFIID distribution independently of Msn2. To address the question of whether the redistribution of TFIID in *ccr4-not* mutants may result from constitutive activation of Msn2, we analyzed the amount of Taf8 that was cross-linked to the *HSP12* promoter relative to that cross-linked to the *RPS9B* promoter in wild-type, *not4*Δ, *msn2*Δ, and *not4*Δ *msn2*Δ cells before and after heat shock (10 min at 39°C). Cross-linking of Taf8 to the *HSP12* promoter relative to the *RPS9B* promoter was similarly increased in both exponentially growing *not4*Δ and *not4*Δ *msn2*Δ cells when compared to wild-type cells (Table 4), indicating that the changes in Taf8-promoter association following loss of Not4 do not require the presence of Msn2 (in unstressed cells). Under heat shock conditions, loss of Msn2 significantly reduced cross-linking of Taf8 to the *HSP12* promoter relative to the *RPS9B* promoter in both wild-type and *not4*Δ cells (Table 4). This effect was mainly due to a decrease in cross-linking of Taf8 to *HSP12* following heat shock (data not shown). Thus, while Msn2 is involved in recruitment of Taf8 to the *HSP12* promoter under heat-shock conditions, it is dispensable for the redistribution of TFIID following inactivation of the Ccr4-Not complex in unstressed cells.

TABLE 4. Relative cross-linking of Taf8 to the *HSP12* and *RPS9B* promoters in unstressed and heat-shocked wild-type and various mutant cells[a]

Strain	Relative *HSP12*/*RPS9B* association ratio at:	
	30°C	39°C for 10 min
Wild type	1.0	1.0
*not4*Δ	2.5	0.8
*msn2*Δ	0.8	0.2
*not4*Δ *msn2*Δ	2.5	0.1
*bud14*Δ	0.7	2.2
*not4*Δ *bud14*Δ	2.9	1.0

[a] Wild-type (MY3590), *not4*Δ (MY3591), *msn2*Δ (MY3496), *not4*Δ *msn2*Δ (MY3498), *bud14*Δ (MY3634), and *not4*Δ *bud14*Δ (MY3633) cells were grown to exponential phase at 30°C and treated for 10 min at 39°C. The relative association of Taf8 to the promoters of *HSP12* and *RPS9B* was evaluated from the average of three independent ChIP experiments. The ratio *HSP12*/*RPS9B* before (30°C) and after (10 min at 39°C) heat shock was brought arbitrarily to 1.0 in wild-type cells, and the other ratios are expressed relative to the wild-type ratio.

Activation of Msn2-dependent transcription in *ccr4-not* mutants requires normal protein phosphatase type I function. While the altered distribution of TFIID in *not* mutants appears to be independent of the presence of Msn2, the constitutively high expression of Msn2-controlled genes in these mutants strongly depends on the presence of Msn2 (30). Since we observed earlier that the loss of Ccr4-Not function resulted in aberrant posttranslational modification of Msn2, we considered the possibility that the Ccr4-Not complex may—via control of the activity of a protein phosphatase or kinase—regulate the capacity of Msn2 to activate target genes. In this context, we discovered that loss of Not4, similar to defined mutations in the essential type I protein phosphatase Glc7 (54), resulted in synthetic lethality when combined with loss of the two partially redundant Ppz1 and Ppz2 protein phosphatases (data not shown). One interpretation of these genetic data is that Not4 and Glc7 act in a common pathway that (e.g., due to a role in repression of Msn2-controlled, growth-inhibitory genes) becomes essential for cellular fitness in the absence of Ppz1 and Ppz2. To study both the epistatic relationship between *not4*Δ and *glc7* and the potential contribution of Glc7 in Msn2-dependent transcription, we determined the effect of *glc7* alleles on STRE-dependent gene expression in wild-type and *not4*Δ cells. As shown in Fig. 2, we found that the glucose-derepressed, recessive *glc7-133* allele (3) dramatically reduced the constitutive STRE-dependent transcription in *not4*Δ mutant cells while having little impact on STRE-dependent transcription in unstressed wild-type cells. These data show that normal Glc7 function is required for the increased STRE-controlled gene expression in *ccr4-not* mutants and suggest that Glc7 may act directly or indirectly downstream of the Ccr4-Not complex to regulate Msn2 function. Intriguingly, in this context, we isolated the catalytic protein phosphatase do-

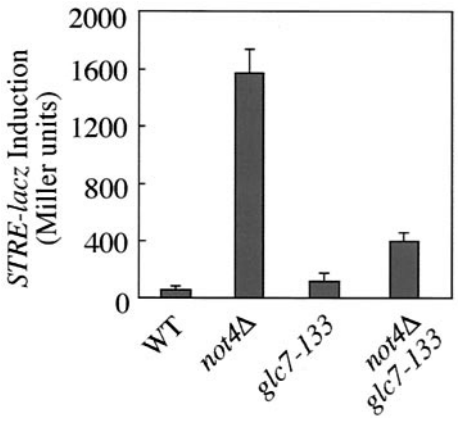

FIG. 2. Constitutive activation of STRE-dependent transcription in *not4*Δ mutant cells depends partially on the function of the Glc7 phosphatase. Proteins were extracted from wild-type (MY2489), *not4*Δ (MY2596), *glc7-133* (MY3362), and *not4*Δ *glc7-133* (MY3363) cells growing exponentially (OD_{600} of 0.8 to 1.2) at 30°C on rich medium. Protein extracts (50 μg of proteins in total) were tested for β-galactosidase activity (indicated in Miller units).

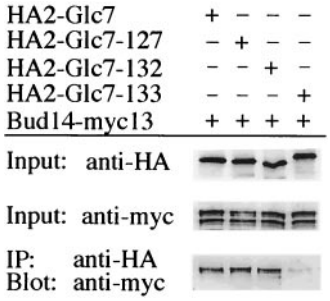

FIG. 3. Interaction between Bud14 and Glc7. Strain IP19 expressing genomically tagged Bud14-myc13 was transformed with a control plasmid (pAR498) and with pIP607, pIP765, pIP766, and pIP767, which express *HA2-GLC7*, *HA2-glc7-127*, *HA2-glc7-132*, and *HA2-glc7-133*, respectively, under the *GAL1* promoter. Cell lysates (Input) and immunoprecipitates (IP) were subjected to PAGE, and immunoblots were probed using anti-HA or anti-myc antibodies. Note that the faster-migrating second band revealed in the anti-myc input blot may represent a degradation product of Bud14-myc13, which appears to poorly bind Glc7.

main of Ppz1, which is highly conserved between Ppz1, Ppz2, and Glc7 (54), in a two-hybrid screen for Not1 interactors (data not shown). Thus, even though Glc7 and the Ccr4-Not complex may independently converge on Msn2 function, an attractive model posits that Glc7 may act as an effector of the Ccr4-Not complex.

Bud14, a new Glc7-binding protein, is involved in control of Msn2 function by the Ccr4-Not complex. Glc7 itself has little substrate specificity, and it has been proposed that its specificity is dictated by different regulatory subunits that target the catalytic subunit to its site of action and/or regulate its substrate specificity (49). Consequently, we sought to isolate the corresponding Glc7 subunit(s) that specifies Glc7 activity in its presumed effector function for the Ccr4-Not complex. To this end, we performed a screen that was based on the assumption that specific activation of Glc7 due to overproduction of the appropriate regulatory subunit may mimic downregulation of the Ccr4-Not complex. Since *SSA3* is one of the genes that is most strongly induced in exponentially growing *ccr4-not* complex mutants (30), we screened for genes that, when overexpressed from a 2μm plasmid, resulted in enhanced transcription of an *SSA3-lacZ* reporter gene (6). One of the genes isolated in this screen (i.e., *BUD14*) encodes a 78.3-kDa protein containing a potential SH3 domain and a V/IXF motif (at positions 377 to 379) that generally serves as a recognition site for the type 1 protein phosphatase Glc7 (10, 19). Both two-hybrid and co-IP assays confirmed that Bud14 specifically associates with Glc7 (Fig. 3; Table 5). Interestingly, the protein encoded by the *glc7-133* allele, unlike two other Glc7 mutant proteins (i.e., Glc7-127 and Glc7-132) (3), was seriously defective for Bud14 binding (Fig. 3). Furthermore, while Glc7-133 was almost entirely defective for Bud14 binding (the Glc7-133-Bud14 interaction was reduced by more than 99% compared to the corresponding Glc7-Bud14 value), it was only partially defective for binding of several other known subunits, including Reg1, Reg2, Ref2, and Gip2 (Table 5) (for a review, see reference 49). The Glc7-Bud14 interaction is therefore particularly sensitive to the mutations encoded by the *glc7-133* allele, which was able to suppress a *ccr4-not* mutant phenotype (see above).

To determine whether the newly identified Bud14/Glc7 module might positively regulate Msn2-dependent transcription downstream of the Ccr4-Not complex, we deleted the nonessential *BUD14* gene in both wild-type and *not4*Δ mutant cells and assayed STRE-dependent transcription in these strains. Interestingly, loss of Bud14—like introduction of the Bud14-binding-deficient *glc7-133* allele—significantly reduced the observed derepression of STRE-dependent transcription in the *not4*Δ mutant (Fig. 4A) while having little impact on STRE-dependent transcription in unstressed wild-type cells. Moreover, the intrinsically high level of thermotolerance observed in *not4*Δ cells, which depends on Msn2 (data not shown), was significantly reduced following loss of Bud14 or introduction of *glc7-133* (Fig. 4B). Together, these results formally place Bud14/Glc7 downstream of or in parallel to Not4 and support the assumption that Bud14/Glc7 and the Ccr4-Not

TABLE 5. Two-hybrid interactions between Glc7, Glc7-133, and various regulatory subunits[a]

AD fusion gene	β-Galactosidase activity (Miller units) with indicated DBD fusion gene[b]		
	GLC7	*glc7-133*	*MSB2*
BUD14	**2,098**	19	23
REG1	**365**	**77**	1
REG2	**445**	**98**	5
REF2	**120**	**55**	5
GIP2	**1,785**	**851**	1
MSB2	12	2	14

[a] Two-hybrid assays were performed as described in Materials and Methods. AD, activation domain; DBD, DNA-binding domain.

[b] β-Galactosidase activities were measured in three independent isolates of each strain after growth for 16 h at 30°C in minimal medium containing 1% raffinose and 2% galactose. The average activities (in Miller units) are shown. Values that are at least 10-fold higher than those for each of the corresponding controls (pJG4-5-*MSB2* and pEG202-*MSB2*) are shown in boldface.

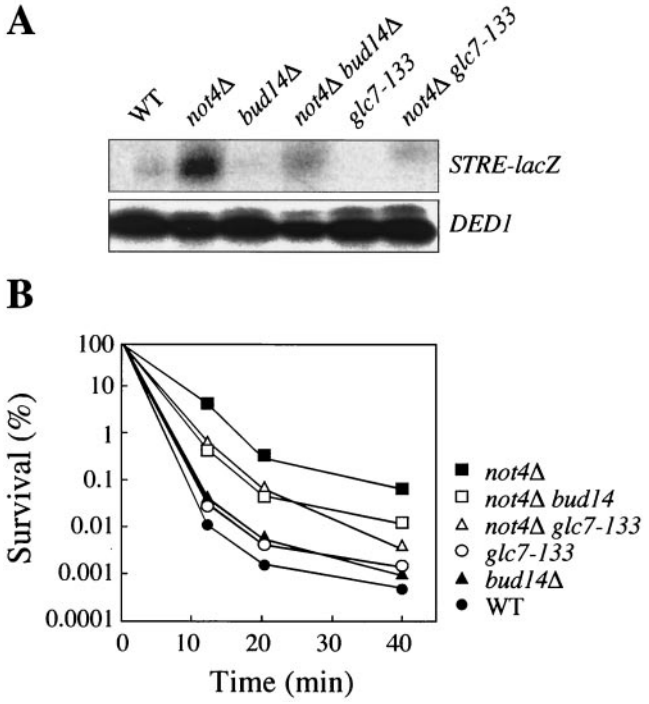

FIG. 4. Genetic interaction between *NOT4* and *GLC7*/*BUD14*. (A) Constitutive activation of STRE-dependent transcription in *not4*Δ cells depends on the function of the Glc7/Bud14 module. Total RNA was extracted from wild-type (WT) (MY2489), *not4*Δ (MY2596), *bud14*Δ (MY3234), *not4*Δ *bud14*Δ (MY3235), *glc7-133* (MY3362), and *not4*Δ *glc7-133* (MY3363) cells growing exponentially (OD_{600} of 0.8 to 1.2) at 30°C on rich medium. *LacZ* transcript levels were measured via S1 analysis using *DED1* as a control. (B) Mutations in *GLC7* and *BUD14* suppress the constitutive thermotolerance in *not4*Δ cells. The thermotolerance of exponentially growing cells (same strains as shown in panel A) was measured as survival following incubation at 50°C for the times indicated.

complex have a common role in the regulation of STRE-dependent transcription.

Bud14 and Glc7 control Msn2 posttranslational modification. In an attempt to define whether the Bud14/Glc7 module is implicated in posttranslational modification of Msn2, we next analyzed the Msn2 isoform pattern in wild-type and various mutant cells. As shown in Fig. 5, Msn2 exhibited a higher electrophoretic mobility in *not4*Δ than wild-type cell extracts. Msn2 mobility was also altered in *bud14*Δ and *glc7-133* cell extracts, supporting the notion that the Bud14/Glc7 phosphatase module is required for appropriate Msn2 modification in unstressed wild-type cells. Importantly, the high electrophoretic mobility of Msn2 in *not4*Δ cell extracts appears to be reversed by loss of Bud14 or introduction of the *glc7-133* allele. Thus, the Bud14/Glc7 module (directly or indirectly) affects Msn2 posttranslational modifications in both wild-type and *not4*Δ cells. Notably, the fact that Msn2 electrophoretic mobility is similarly high in cells exhibiting strong (*not4*Δ) or weak (*bud14*Δ and *glc7-133*) STRE-driven transcription indicates that Msn2 electrophoretic mobility per se is not indicative of Msn2 activity. Since similarly migrating isoforms of Msn2 may even represent differentially phosphorylated proteins, resolution of this issue will ultimately depend on identification of the residues that are responsive to the Ccr4-Not complex, to Bud14/Glc7, and to the various protein kinases that have been implicated in Msn2 phosphorylation (9, 21, 23, 24, 26). Nevertheless, our data allow us to conclude at present that both the Ccr4-Not complex and the Glc7/Bud14 module, which is required for the enhanced Msn2-driven transcription following loss of Ccr4-Not function, control the posttranslational modification of Msn2.

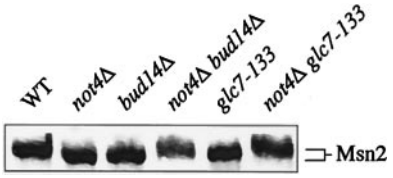

FIG. 5. Altered posttranslational modifications of Msn2 in *not4*Δ mutant cells depend partially on the function of the Glc7/Bud14 module. Proteins (of the equivalent of an OD_{600} of 1) of exponentially growing cells were extracted by the post-alkaline lysis method, separated on SDS–7% PAGE, transferred to nitrocellulose, and probed with anti-Msn2 antibodies. WT, wild type.

TABLE 6. Cross-linking of Msn2 to *HSP26* STRE in unstressed and heat-shocked wild-type, *not4*Δ, *bud14*Δ, and *not4*Δ *bud14*Δ mutant cells[a]

Strain	Relative amount of *HSP26* STRE promoter DNA at:	
	30°C	39°C for 10 min
Wild type	1.3	3.1
*not4*Δ	1.1	2.7
*bud14*Δ	1.0	6.4
*not4*Δ *bud14*Δ	1.6	5.0

[a] Wild-type (MY3590), *not4*Δ (MY3591), *bud14*Δ (MY3634), and *not4*Δ *bud14*Δ (MY3633) cells were grown to exponential phase at 30°C and treated for 10 min at 39°C. Msn2 was immunoprecipitated by use of a myc tag using monoclonal anti-myc antibodies. The levels of *HSP26 STRE* promoter DNA in the immunoprecipitates of unstressed (30°C) or heat-shocked (10 min at 39°C) cells are expressed relative to their corresponding presence in the total cell extract.

Neither the Ccr4-Not complex nor Bud14 contributes to Msn2 DNA association. To answer the question of whether the Ccr4-Not complex and the Bud14/Glc7 module may impinge on the same Msn2 control mechanism, we studied both nuclear localization and promoter affinity of Msn2 in wild-type cells and corresponding mutant cells. We found that Msn2 was normally localized (i.e., cytoplasmic in exponentially growing cells) in various *not*Δ, *bud14*Δ, and *glc7-133* mutants (data not shown). In addition, *not4*Δ, *bud14*Δ, and *not4*Δ *bud14*Δ cells exhibited a similar level of Msn2 cross-linked to the STRE region of the *HSP26* promoter in both exponentially growing and heat-shocked wild-type cells (Table 6). Thus, neither a change in subcellular localization of Msn2 nor altered STRE-promoter affinity is sufficient to explain the dramatic increase in STRE-dependent transcription observed in exponentially growing *not* mutants or the suppression of this phenotype following loss of Bud14.

Since our results indicated that the Ccr4-Not complex controls Msn2 activity and TFIID distribution via two independent pathways, we also tested whether Bud14 may, in addition to playing a role in Msn2 activation, be involved in TFIID distri-

TABLE 7. Bud14- and Msn2/Msn4-dependent gene induction at the diauxic transition

ORF	Gene	Description	Fold decrease in[a]:	
			*bud14*Δ cells	*msn2 msn4* cells
YOR135C	*IDH2*	Oxidoreductase	4.7	3.5
YGR101W		Involved in energy generation	4.3	2.4
YEL070W		Mannitol-1-phosphate 5-dehydrogenase	4.1	4.2
YML087C		Molecular function unknown	4.1	3.9
YGR110W		Molecular function unknown	3.9	3.8
YLR116W		Branch point bridging protein	3.8	2.7
YLR070C		Putative sugar dehydrogenases	3.2	2.7
YER096W		Sporulation-specific protein	3.1	1.8
YHR211W		Flocculation-specific protein	3.1	1.8
YDR497C	*ITR1*	Major *myo*-inositol permease	3.0	1.6
YML120C	*NDI1*	NADH-ubiquinone-6 oxidoreductase	2.9	1.9
YKR052C	*MRS4*	Mitochondrial membrane transporter	2.9	4.1
YBR280C		Molecular function unknown	2.8	4.6
YBL075C	*SSA3*	Heat shock protein of HSP70 family	2.8	4.8
YNL125C		Molecular function unknown	2.8	4.3
YJL225C		ATP-dependent DNA helicase	2.8	2.4
YPR026W	*ATH1*	Acid trehalase	2.7	2.3
YDL078C	*MDH3*	Malate dehydrogenase	2.7	2.0
YCLX05C		Molecular function unknown	2.7	1.7
YKL146W		Neutral amino acid transporter	2.6	3.2
YIR043C		Molecular function unknown	2.6	1.8
YOR005C	*DNL4*	DNA ligase	2.6	2.2
YHR033W		Putative glutamate 5-kinase	2.6	1.1
YLR178C	*TFS1*	Cdc25-dependent nutrient response regulator	2.6	3.5
YER060W-A	*FCY22*	Purine/cytosine permease	2.6	3.3
YJL082W		Molecular function unknown	2.5	2.0
YHR006W	*STP2*	Transcription factor	2.5	2.9
YBR139W		Protease	2.5	3.7

[a] Genes with a fold decrease in *bud14*Δ (PE27) cells of >2.5 were selected from the pool of genes that were at least two-fold induced (average ratio, > 2) in wild-type cells at the diauxic transition. The corresponding fold decrease in *msn2 msn4* mutant (PEY78) cells is also shown.

bution. As shown in Table 4, cross-linking of Taf8 to the *HSP12* promoter relative to the *RPS9B* promoter was increased in *not4*Δ cells independently of the presence or absence of Bud14, indicating that Bud14, like Msn2, is not involved in the redistribution of TFIID in *not4*Δ mutant cells. Finally, Bud14 also was not required for the increased recruitment of TFIID to heat shock promoters following heat shock (Table 4), which is in line with our observation that loss of Bud14 or introduction of the *glc7-133* allele did not significantly alter the induction of STRE-controlled genes following a 10-min heat shock at 39° (data not shown).

The Ccr4-Not complex may prevent Msn2 activation by negative control of the Bud14/Glc7 module under high-PKA conditions. We have previously shown that the Ccr4-Not complex may function as an effector of the PKA pathway that contributes to downregulation of Msn2-dependent transcription of growth-inhibitory genes under conditions of high PKA (30, 42, 48). Based on the results shown above, the Ccr4-Not complex may perform this function at least in part via inhibition of the Glc7/Bud14 module, which—particularly following release from repression by the Ccr4-Not complex (for instance, under conditions of low PKA at the diauxic shift)—may positively regulate Msn2. In line with this model, we found that the dosage-dependent effect of Bud14 on *SSA3-lacZ* transcription was mainly apparent in cells that have entered the diauxic shift (data not shown), which temporally coincides with the time of Ccr4-Not complex downregulation (30). Furthermore, whole-genome array analysis confirmed a positive role of Bud14 in regulation of Msn2-controlled genes at the diauxic transition. Accordingly, we found that of 375 genes that were induced in wild-type cells at the diauxic transition, a large fraction (54.6% or 205 genes) required Msn2/Msn4 for induction, which is in accordance with previously published data (7). Importantly, 57 (27.8%) of these Msn2/Msn4-dependent genes also required Bud14 for induction, and application of a less stringent cutoff value for Bud14-dependent genes resulted in an almost complete overlap of the Bud14- and Msn2/Msn4-dependent gene sets. This is further illustrated in Table 7, which shows the entire set of genes that were most strongly dependent on Bud14 for induced expression at the diauxic shift (i.e., at least 2.5-fold reduced in *bud14*Δ cells compared to wild-type cells), including the corresponding values for fold decrease in *bud14*Δ and *msn2 msn4* mutant cells. The fact that the defect of *bud14*Δ mutant cells for induction of Msn2/Msn4-dependent genes at the diauxic transition was on average much lower (2.1-fold decrease) than the corresponding defect of *msn2 msn4* mutant cells (3.3-fold decrease) indicates the presence of additional (possibly redundant) regulatory mechanisms, which allow nutrient limitation-induced activation of Msn2/Msn4-dependent transcription in the absence of Bud14. Our observation that loss of Bud14 did not affect the expression levels of STRE-controlled genes in later postdiauxic growth phases (data not shown) supports the idea that the main role of Bud14 in regulation of transcription is confined to the diauxic transition phase.

To determine whether Bud14, as expected for an activator of

Msn2, also modulates PKA-dependent growth, we then studied whether loss of Bud14 could suppress growth defects that are associated with attenuated PKA activity. To this end, a temperature-sensitive PKA mutant strain with only one functional *tpk2*(Ts) gene was transformed with the *bud14*Δ::*kanMX2* cassette. Even though loss of Bud14 did not restore growth of the *tpk2*(Ts) cells at the nonpermissive temperature, it increased the growth rate of the *tpk2*(Ts) cells at a semipermissive temperature (31°C) from $0.107 \pm 0.010\ h^{-1}$ (SGY446) to $0.287 \pm 0.023\ h^{-1}$ (PE15). Loss of Bud14 therefore at least partially relieves dependence on PKA function. Overproduction of Bud14, in contrast, strongly inhibited the growth at 31°C of *tpk2*(Ts) cells (similar results were obtained using temperature-sensitive Ras GTP exchange factor and adenylate cyclase mutant strains harboring *cdc25*(Ts) and *cdc35*(Ts) mutations, respectively) (data not shown). Together, these results show that Bud14, possibly via Glc7, antagonizes PKA-mediated cell proliferation control. This is also in line with a previous report in which overexpression of *GLC7* was found to prevent growth of *ras1 ras2*(Ts) mutant cells (39) and our own observation that Glc7 overproduction (using an *ADH1-GLC7* construct, pFD688) strongly reduced the growth rate at 36°C of *cdc35*(Ts) (PD6517) cells (i.e., from $0.0730 \pm 0.003\ h^{-1}$ to $0.0014 \pm 0.003\ h^{-1}$), while it only slightly reduced the growth rate of *cdc35*(Ts) *bud14*Δ (PE6) cells (i.e., from $0.083 \pm 0.004\ h^{-1}$ to $0.068 \pm 0.003\ h^{-1}$). Thus, Glc7 antagonizes PKA-mediated growth at least in part through Bud14. Taken together, our combined genetic and molecular experiments support a model in which PKA-dependent repression of Msn2 is mediated at least in part by the Ccr4-Not complex, possibly through control of the Glc7/Bud14 module.

DISCUSSION

In this work, we have studied the association of TFIID subunits across different promoters in wild-type cells or cells with deletions of various subunits of the Ccr4-Not complex. In accordance with our previous study (16), which was focused on the analysis of Taf1, we show here that loss of Ccr4-Not complex subunits dramatically changes the overall promoter distribution pattern of several TFIID subunits. Accordingly, in unstressed exponentially growing wild-type cells, we found a high level of differential promoter cross-linking of Tafs (27, 31, 40), with a particular bias for promoters of ribosomal protein genes, whose expression is strong and Taf dependent, and against promoters of heat shock genes, whose expression is weak and Taf independent under these conditions. In Ccr4-Not complex mutant cells, in contrast, we found a generally low level of differential promoter cross-linking of Tafs, with a particular bias against promoters of ribosomal protein genes. Thus, the Ccr4-Not complex functions to ensure that the differential promoter association of Tafs remains high and appears to particularly stabilize ribosomal protein gene promoter association of TFIID under nonstress conditions (Fig. 6). Since we have previously demonstrated that the dramatically increased expression of STRE-controlled genes in *ccr4-not* mutants was dependent on the presence of Msn2, we reasoned that the TFIID promoter distribution defect in *ccr4-not* mutants may result indirectly from constitutive activation of Msn2. Interestingly, however, the observed changes in TFIID promoter distribution due to mutations in the Ccr4-Not complex appeared similar in the presence or absence of Msn2, indicating that the Ccr4-Not complex controls TFIID promoter association independently of Msn2.

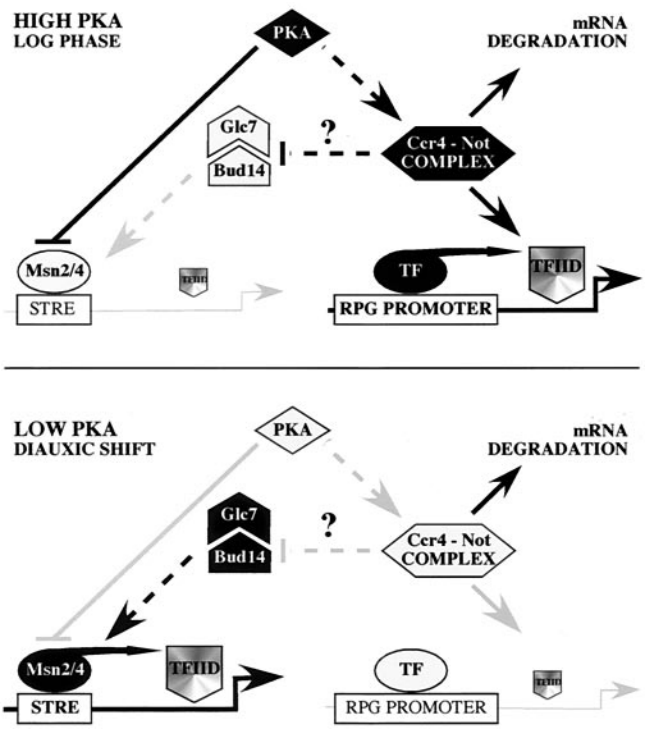

FIG. 6. Model for the function of the Ccr4-Not complex. (Top) Cells growing exponentially (high-PKA conditions). The level of TFIID on STRE-controlled promoters is low compared to the level on RPG promoters. In parallel, STRE gene expression is low, and ribosomal protein gene (*RPG*) expression is high. The Ccr4-Not complex positively regulates TFIID presence on RPG promoters and may negatively control the Bud14/Glc7 module. TF, presumed transcription factor(s). (Bottom) Cells entering the diauxic shift (low-PKA conditions). The level of TFIID on STRE-controlled promoters is increased compared to the level on RPG promoters. In parallel, STRE gene expression increases, and *RPG* expression decreases. The Glc7/Bud14 module is relieved from repression and may activate STRE genes following posttranslational modification of Msn2. Note that this activation may actually occur before both the exhaustion of glucose and the subsequent transfer of the major portion of Msn2 to the nucleus. Arrows and bars denote positive and negative interactions, respectively. Grey and black bars and symbols denote low and high activity, respectively.

While in principle it is possible that Msn2 activation results from the altered TFIID distribution in *ccr4-not* mutant cells, we also considered the possibility that the Ccr4-Not complex may activate Msn2 via a mechanism that is separate from its function in TFIID promoter distribution. Definition of such an independent mechanism requires the identification of signaling molecules that are necessary for proper STRE-dependent transcription without affecting the general distribution of TFIID across promoters. In this context, we isolated a new type I protein phosphatase module in yeast (i.e., Bud14/Glc7) that fulfilled both of these criteria (i.e., it is required for proper STRE-dependent transcription at the diauxic shift and does not appear to affect TFIID promoter association). Intriguingly,

we found that both the dramatic, constitutive Msn2/STRE-dependent transcription and the constitutive thermotolerance of various *ccr4-not* mutants are strongly dependent on the presence of Bud14 or a wild-type Glc7 protein. Moreover, mutations in the Ccr4-Not complex caused Msn2 posttranslational modifications that appeared to be reverted by loss of Bud14 or a Glc7-133 mutant protein. The simplest model that emerges from our genetic analyses is therefore that the Ccr4-Not complex may regulate the activity of Msn2 via a Glc7/Bud14-dependent, TFIID-independent mechanism (Fig. 6). It must be noted however, that while our results do not exclude additional, more complex models (particularly involving further intermediary steps or nonlinear interactions), our simple linear model provides an initial framework which predicts a variety of biochemical interactions that can be tested in the future.

How does the Ccr4-Not complex impinge on Msn2 activation? During heat shock, Msn2 activation appears to be accomplished through both nuclear accumulation and enhanced STRE binding (24, 26). We found that the Ccr4-Not complex and Bud14/Glc7 regulate neither nucleocytoplasmic localization of Msn2 nor its recruitment to STRE-controlled promoters. From these observations, we infer that the mechanism of Msn2 activation following both inactivation of the Ccr4-Not complex and activation of the Bud14/Glc7 complex differs significantly from the one observed following heat stress. (This is also in line with our findings that (i) enhanced Taf8 cross-linking to the *HSP12* promoter is independent of Msn2 following loss of Not4 yet strongly dependent on Msn2 following heat stress and (ii) STRE-driven gene expression is strongly dependent on Bud14 following loss of Not4 yet independent of Bud14 following heat stress). One possibility is that the Ccr4-Not complex primarily impinges on the ability of Msn2 to communicate with the general transcription machinery, particularly in response to the availability of nutrients. Strikingly, Msn2 has previously been shown to interact with Srb10, a component of the RNA Pol II holoenzyme (9), suggesting that Msn2 may communicate with the SRB-Mediator complex. Thus, a likely scenario that is based on our present and previously published data (16, 30) is that the Ccr4-Not complex and Bud14/Glc7, rather than controlling the subcellular localization or STRE binding of Msn2, may control the ability of Msn2 to activate Pol II-dependent transcription following nutrient limitation. Accordingly, in exponentially growing (high-PKA) cells, the Ccr4-Not complex may serve to prevent activation of Msn2 (possibly via inactivation of Bud14/Glc7), while inactivation of the Ccr4-Not complex during the diauxic shift (low PKA) may allow activation of Msn2 (possibly via Bud14/Glc7) (Fig. 6). This model is also in line with two additional observations, namely, that Bud14 increases in abundance (>10-fold) and—like Msn2—accumulates in the nuclei of cells entering the diauxic shift (data not shown). In summary, the Ccr4-Not complex may, as proposed previously, function as an effector of the PKA pathway that contributes, via inactivation of Bud14/Glc7, to downregulation of Msn2-dependent transcription of growth-inhibitory genes in cells growing on glucose (30, 42, 48). Elucidation of the precise nature of the biochemical interactions between the proteins in this proposed separate effector pathway is warranted to provide further insight into how the Ccr4-Not complex contributes to the control of yeast cell growth.

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