A SAGA-Independent Function of *SPT3* Mediates Transcriptional Deregulation in a Mutant of the Ccr4-Not Complex in *Saccharomyces cerevisiae*

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ABSTRACT

The conserved multi-subunit Ccr4-Not complex regulates gene expression in diverse ways. In this work, we characterize the suppression of temperature sensitivity associated with a mutation in the gene encoding the scaffold subunit of the Ccr4-Not complex, *NOT1*, by the deletion of *SPT3*. We determine that the deletion of *SPT3*, but not the deletion of genes encoding other subunits of the SAGA complex, globally suppresses transcriptional defects of *not1-2*. We find that transcriptional activation in *not1-2* is associated with increased binding of TFIID and SAGA at promoters of upregulated genes, and this is suppressed by the deletion of *SPT3*. Interestingly, Spt3p-dependent activation of transcription occurs in *not1-2* even if the SAGA complex is disrupted by the deletion of *SPT7* that encodes a subunit of SAGA required for its integrity. Consistent with a SAGA-independent function of Spt3p, the deletion of *SPT3* displays synthetic phenotypes when combined with a deletion of *SPT7*. Taken together, our results provide a new view of the Spt3 protein by identifying a SAGA-independent function of this protein that is functionally linked to the Ccr4-Not complex.

THE Ccr4-Not complex is a multi-protein complex consisting of at least nine subunits (Not1p–Not5p, Caf1p, Caf40p, Caf130p, and Ccr4p) conserved from yeast to human and required for the appropriate control of gene expression (for reviews see COLLART 2003; DENIS and CHEN 2003; COLLART and TIMMERS 2004). It exists in several forms >1 MDa and carries at least two enzymes: the Ccr4p-Caf1p deadenylase (TUCKER *et al.* 2001) and the Not4p E3 ligase (ALBERT *et al.* 2002). The specificity of these enzymes has not yet been clearly established. Ccr4p contributes to the degradation of transcripts encoding ribosomal proteins and ribosome assembly factors (GRIGULL *et al.* 2004). Caf1p, endowed with nuclease activity *in vitro* (DAUGERON *et al.* 2001), does not seem to contribute to deadenylation *in vivo* (TUCKER *et al.* 2002; VISWANATHAN *et al.* 2004), but its recruitment to an mRNA is sufficient to decrease the half-life of this transcript (FINOUX and SÉRAPHIN 2006). For Not4p, the only substrate described to date is the nascent polypeptide-associated (EGD/NAC) complex (PANASENKO *et al.* 2006).

The function of the Ccr4-Not complex, however, extends beyond its known enzymatic activities. Indeed, many studies in the yeast *Saccharomyces cerevisiae* have demonstrated that the complex is involved in the pos-

itive and negative regulation of transcription in diverse ways (for reviews see (COLLART 2003; DENIS and CHEN 2003; COLLART and TIMMERS 2004). For instance, integrity of the Ccr4-Not complex is important for the appropriate distribution of TFIID across promoters (LENSSEN *et al.* 2005) and Not5p associates with promoters in a Taf1p-dependent manner (DELUEN *et al.* 2002). Other studies showed that transcriptional activation by Gcn4p leads to the recruitment of several subunits of the Ccr4-Not complex to the *ARG1* promoter (SWANSON *et al.* 2003) or that appropriate recruitment of the transcription machinery to *RNR* genes in response to DNA damage requires the Ccr4-Not complex (MULDER *et al.* 2005). The Ccr4-Not complex also controls the post-translational status of the Msn2p stress transcription factor via the Glc7 type I protein phosphatase, thereby inhibiting its transcriptional activity (LENSSEN *et al.* 2005). Other genetic studies connect the Ccr4-Not complex to transcription, since, for instance, mutations in *CCR4-NOT* genes lead to the functional expression of *his4-912δ*, similar to mutations in *SPT15* encoding the TATA-binding protein TBP (BADARINARAYANA *et al.* 2000).

One interesting genetic interaction linking the Ccr4-Not complex to transcription is the suppression of a temperature-sensitive mutation in the gene encoding the scaffold subunit of the Ccr4-Not complex, *NOT1*, by the deletion of *SPT3*, encoding a subunit of the SAGA complex (COLLART 1996). SAGA is a conserved multi-protein complex that is required for the normal transcription of many genes (for review see TIMMERS and TORA 2005). It is modular in structure and a functional

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organization (Sterner *et al.* 1999; Wu *et al.* 2004). Genetic analyses of the nonessential subunits show that they fall into several classes by mutant phenotypes. One class consists of the Spt7p, Spt20p, and Ada1p subunits that are essential for the integrity of the complex, and deletion of these subunits can have severe growth defects (Sterner *et al.* 1999). A second class consists of Spt3p and Spt8p, which have very similar profiles of gene expression and synthetic genetic interactions (Ingvarsdotir *et al.* 2005). Their functions have generally been linked to interactions of SAGA with TBP. A third class consists of the Gcn5p, Ada2p, and Ada3p, which contribute to the histone acetyl-transferase (HAT) activity of the complex. Indeed, Gcn5p is the HAT enzyme and acetylates histones H2B and H3, whereas Ada2p and Ada3p are required for Gcn5p to acetylate nucleosomal histones (Grant *et al.* 1997; Sterner *et al.* 1999; Balasubramanian *et al.* 2002). Sgf11p and Ubp8p constitute a discrete, more recently identified functional module within SAGA that provides deubiquitination activity. Ubp8p is the catalytic subunit and Sgf11p is required for its association in the SAGA complex (Powell *et al.* 2004; Ingvarsdotir *et al.* 2005). Recently, Sus1p, thought to bridge transcription and mRNA export (Rodriguez-Navarro *et al.* 2004), and Chd1p, important for HAT activity (Pray-Grant *et al.* 2005), were identified as other SAGA subunits. The SAGA complex also has essential subunits such as Tra1p, thought to be involved in interactions with acidic activators (Grant *et al.* 1998) and a subset of TBP-associated factors and Tafp's (which are also subunits of the TFIID complex), required for nucleosome acetylation and transcriptional stimulation (Grant *et al.* 1998). At present, while all studies converge to determine that subunits of the SAGA complex play different roles, no clear picture emerges for the specific role of Spt3p. Substantial evidence has supported the idea that it functions through interactions with TBP (Eisenmann *et al.* 1989; Eisenmann *et al.* 1992; Madison and Winston 1997; Dudley *et al.* 1999; Bhaumik and Green 2001; Bhaumik and Green 2002), but this has recently been challenged (Sermwittayawong and Tan 2006). Furthermore, Spt3p is also a subunit of a different complex, SLIK/SALSA, which is very similar in composition to SAGA, but lacks Spt8p and the C terminus of Spt7p (Wu and Winston 2002).

In this work, we were interested in determining the specificity and nature of the genetic interaction between Spt3p and Not1p. Our study reveals that the *not1-2* mutation leads mostly to upregulation of transcription at restrictive temperature, and this correlates with increased recruitment of TFIID and SAGA to upregulated promoters. We found a global suppression of *not1-2* mutant phenotypes by deletion of *SPT3* and determined that this global suppression is specific to the Spt3p subunit of the SAGA complex, with some effect of the Spt8p subunit. Finally, we could show that suppression of transcriptional activation in *not1-2* by deletion of *SPT3* occurs in cells lacking a SAGA complex. Taken together,

our results reveal a SAGA-independent function of Spt3p that mediates transcriptional deregulation in a mutant of the Ccr4-Not complex.

MATERIALS AND METHODS

Strains, plasmids, and media: The strains used in this study are listed in Table 1. All media were standard, either YPD for glucose-rich medium or synthetic complete media when the presence of a plasmid was selected for. Single-step deletions were performed by PCR as described by Longtine *et al.* (1998). All of the strains were checked with a PCR reaction performed on genomic DNA extracts using a primer localized in the marker gene and a primer localized at the 5′ noncoding sequence of the target gene. To make strains overexpressing histones H3 and H4, the *HHT1* and *HHF1* genes were amplified by PCR and cloned in pRS426 and pRS425, respectively. All primers used are available upon request.

Microarray experiments: Cells were harvested and total RNA was extracted by the acid phenol method. For microarray experiments, we followed the standard Affymetrix procedure. Description of this procedure and description of the probe sets are presented on their website (http://www.affymetrix.com/index.affx). Briefly, cells were harvested under the appropriate conditions and total RNA was extracted by the acid phenol method. The quality of total cellular RNA was tested on a RNA 6000 Nano Chip (Agilent). Total cellular RNA was next purified with a RNeasy mini handbook kit (QIAGEN, Valencia, CA). Then the synthesis of cDNA, cRNA, the hybridization, and the scan of the chip were performed according to the technical manual of Affimetrix (GeneChip expression analysis). Oligo(dT) was added to 15 μg of purified total RNA. After 10 min of incubation at 70° in first-strand buffer with DTT (10 mm), dNTP (500 μm each), and reverse transcriptase [SuperScript II from Invitrogen (San Diego) at 200 units/μl] were added for the first-strand cDNA synthesis (1 hr at 42°). For the second strand synthesis, dNTP (200 μm each), *Escherichia coli* DNA ligase (10 units), *E. coli* DNA polymerase I (40 units), and *E. coli* RNaseH (2 units) were added to the second-strand buffer and incubated for 2 hr at 16°. Finally, T4 DNA polymerase (10 units) was added to the reaction. The cDNA was purified and, after ethanol precipitation, 15 μg of purified cDNA was incubated for 5 hr at 37° with high yield buffer (Enzo), biotine-coupled ribonucleotides, DTT, an RNase inhibitor mix, and T7 RNA polymerase. The cRNA was then purified in the same way as total RNA and 20 μg of cRNA were fragmented in 5× hybridizing buffer (200 mm Tris–acetate, pH 8.1, 500 mm KOAc, 150 mm MgOAc) and incubated for 35 min at 95°.

Hybridization of cRNA: A total of 15 μg of cRNA was hybridized to the yeast genome S98 chip for 16 hr at 45° with 1× hybridizing buffer [100 mm 2-*N*-morpholino-ethane sulfonic acid (MES), 1 m (Na⁺), 20 mm EDTA, 0.01 Tween 20], oligonucleotide B2 (50 pmol), eukaryotic hybridization controls (20×), herring sperm DNA (0.1 mg/ml), and acetylated BSA (0.5 mg/ml). The chip was washed in the Fluidics station, first with buffer A (6× SSPE, 0.01% Tween 20) and then with buffer B [100 mm MES, 0.1 m (Na⁺), 0.01% Tween 20]. Antibodies were added next [normal goat IgG, 0.1 mg/ml; biotinylated antibodies, 0.5 mg/ml; acetylated BSA 2 mg/ml; 100 mm MES; 1 m (Na⁺); 0.05% Tween 20] followed by the SAPE solution [streptavidine–phycoerythrine 10 μg/ml, acetylated BSA 2 mg/ml, 100 mm MES, 1 m (Na⁺), 0.05% Tween 20]. Finally, the chip was scanned and the scan was analyzed by Affymetrix software (MicroArraySuite, MicroDB, and DataMiningTool).

Results were analyzed using the Iobion Array Assist software provided by Stratagene (La Jolla, CA) at http://www.stratagene.

Strain	Genotype	Source or reference
MY1	*MAT**a** ura3-52 trp1 leu2Δ::PET56 gcn4 gal2* (KY803)	HOPE and STRUHL (1986)
MY2	*MATα ura3-52 trp1 leu2Δ::PET56 gcn4 gal2* (KY804)	HOPE and STRUHL (1986)
MY3	*MAT**a** ura3-52 trp1 leu2Δ::PET56 gcn4 gal2 his3::TRP1*	MAILLET *et al.* (2000)
MY8	Isogenic to MY2 except *not1-2*	LIU *et al.* (1998)
MY896	Isogenic to MY1 except *spt8::LEU2 not1-2*	This study
MY1048	Isogenic to MY2 except *spt3::TRP1*	This study
MY3667	Isogenic to MY1 except *gcn5::TRP1*	This study
MY3712	Isogenic to MY8 except *spt3::TRP1*	This study
MY4136	Isogenic to MY3667 except *MATα not1-2*	This study
MY4260	Isogenic to MY1 except *spt8::TRP1*	This study
MY4058	Isogenic to MY1 except *spt7::SPT7-13MYC-KANMX4*	This study
MY4294	Isogenic to MY4058 except *MATα not1-2*	This study
MY4299	Isogenic to MY4058 except *MATα spt3::TRP1*	This study
MY4330	Isogenic to MY4294 except *spt3::TRP1*	This study
MY4813	Isogenic to MY1 except *ubp8::NATMX*	This study
MY4814	Isogenic to MY4813 except *not1-2*	This study
MY4841	Isogenic to MY1 except *spt3::TRP1 spt7::LEU2*	This study
MY4914	Isogenic to MY4841 except *not1-2*	This study
LY102	Isogenic to MY2 except *not1-2 spt7::LEU2*	This study
LY104	Isogenic to MY3 except *spt7::LEU2*	This study

com/products/showProduct.aspx?pid=731. The stringent analysis consisted of using $P = 0.05$ according to CHP, one of the three proposed methods of analysis. This analysis considers only mRNAs expressed at similar levels in the duplicate samples of each strain, which are also significantly different in the strains that are compared. This analysis certainly underestimates the total number of target genes but nevertheless is useful to globally compare two strains, and the results are presented in supplemental Figure S1 at http://www.genetics.org/supplemental/.

S1 analysis: Total cellular RNA extraction and S1 analyses were performed as described previously (COLLART 1996). The sequences of the specific oligonucleotides are available upon request.

Western blot analyses: Cells (2.5 OD_{600}) were collected for protein extract preparation by rapid post-alkaline lysis. Briefly, the cell pellet was resuspended in 100 μl of H_2O and then 100 μl of NaOH 0.2 N was added. After a 5-min incubation at room temperature (RT), the samples were spun at 14,000 rpm for 5 min and the supernatant was discarded. The pellet was resuspended in 50 μl of post-alkaline loading buffer (0.06 M Tris, pH 6.8, 5% glycerol, 2% SDS, 4% β-mercapto-ethanol, bromophenol blue). The samples were loaded on a 15% SDS–PAGE gel for Western blot analysis. After transfer of separated proteins to nitrocellulose membranes, these were incubated overnight at 4° with polyclonal antibodies (anti-H3 ab1791 and anti-H4 ab10158-100 from Abcam). The secondary antibody was a polyclonal goat anti-rabbit antibody conjugated either to alkaline phosphatase or to horseradish peroxidase.

Chromatin immunoprecipitation experiments: Chromatin immunoprecipitation (ChIP) experiments were performed as described previously (LENSSEN *et al.* 2005). Briefly, wild-type and mutant cells grown appropriately were fixed with 1% formaldehyde for 20 min at RT, and glycine was added to a final concentration of 125 mM to stop the reaction. The cells were washed three times (20 mM Tris–HCl, pH 7.5, 200 mM NaCl) and broken in FA–lysis buffer (50 mM HEPES–KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, pH 8, 1% Triton, 0.1% sodium de-

oxycholate, 1 mM PMSF) for 30 min and sonicated. A fraction of the extracts (input) was incubated overnight at 4° with specific antibodies (anti-Taf5p, our polyclonal antibody; anti-MYC, monoclonal 9E10; anti-H3-K4-Met3, polyclonal ab8580 from Abcam) and protein G sepharose. The sepharose beads were collected and washed once with FA–lysis buffer, followed by a wash with FA–lysis buffer plus 350 mM NaCl, then buffer III (10 mM Tris–HCl, pH 8, 1 mM EDTA, pH 8, 250 mM LiCl, 1% Igepal, 1% sodium deoxycholate), and finally twice with TE. Immunoprecipitated chromatin was eluted in 1% SDS, 50 mM Tris–HCl, pH 7.5, 10 mM EDTA, pH 8. Then 10 mg/ml of proteinase K was added to the precipitates and to a fraction of the input for 5 hr at 65° to reverse crosslinks. DNA was extracted and precipitated. Specific DNA was measured by real-time PCR using SYBR Green and the amount in the precipitates was expressed relative to the input DNA. The amount of a specific promoter DNA was normalized to the amount of an intergenic sequence. The sequences of specific oligonucleotides are available upon request.

RESULTS

Suppression of *not1-2* is specific to the deletion of the Spt3p SAGA subunit: To address the specificity of the suppression of *not1-2* temperature sensitivity by the deletion of the Spt3p subunit of the SAGA complex (COLLART 1996), we tested whether the deletion of SAGA subunits in addition to Spt3p might suppress *not1-2*. We deleted *SPT8*, *SPT7*, *GCN5*, and *UBP8* in our genetic background and created the *not1-2* double-mutant strains (Table 1). Cells from the single- and double-mutant strains were grown to exponential phase, diluted to an equivalent OD_{600} of 1.0, serially diluted fivefold, and spotted on glucose-rich plates (YPD) that were placed at
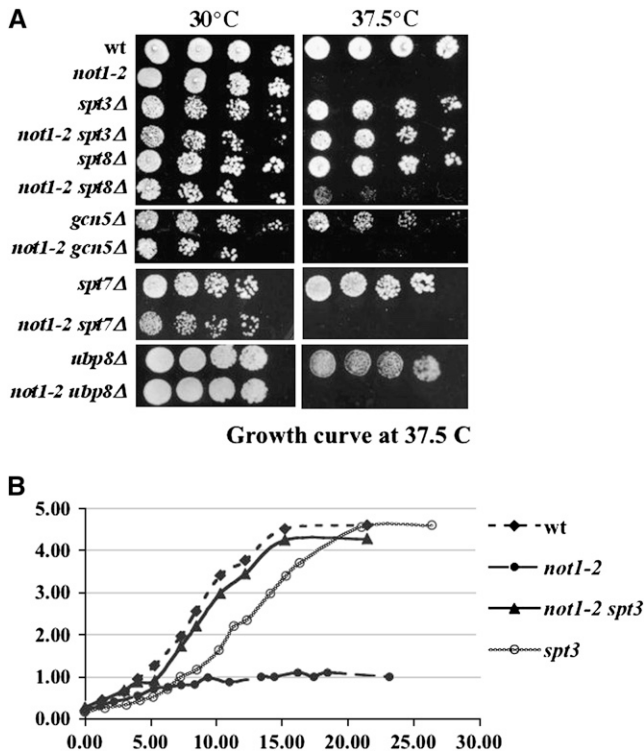
A

Growth curve at 37.5 C

B

FIGURE 1.—*not1-2* temperature sensitivity is suppressed specifically by the deletion of *SPT3*. The indicated strains were grown exponentially in glucose-rich medium for 24 hr and then (A) serially diluted and plated on glucose-rich plates that were placed at 30° or 37.5° for 3 days or (B) were shifted to 37.5° after reaching an OD_{600} of 0.2. Growth of the cells was measured over time.

30° or 37.5° (Figure 1). A suppression of *not1-2* temperature sensitivity was obtained only with the deletion of *SPT3* and, to a lesser extent, with the deletion of *SPT8*, but not with the deletion of other subunits of the SAGA complex. In particular, no suppression was observed upon deletion of *SPT7* that disrupts the SAGA complex. Instead, a slight synthetic growth phenotype was observed (Figure 1A).

Genomewide profiling suggests that suppression of transcriptional deregulation in *not1-2* by deletion of *SPT3* is global: Suppression of *not1-2* temperature sensitivity by the deletion of *SPT3* could be due to the restoration of appropriate expression of one or a select few genes required for growth at high temperature. Alternatively, deletion of *SPT3* might generally restore appropriate gene expression in cells mutant for Not1p. To address this issue, we performed microarray experiments. We grew wild-type, *not1-2*, *spt3Δ*, and *not1-2 spt3Δ* cells for 24 hr exponentially in liquid glucose-rich medium, and then transferred cells at an OD_{600} of 0.2 to 37.5° (Figure 1B). As expected, growth of *not1-2* cells stopped after a couple of divisions at restrictive temperature, whereas double-mutant cells grew like wild-type cells. *spt3Δ* cells grew to the same final density as wild-type

cells, but displayed a slight delay in growth after transfer to high temperature (Figure 1B). Total cellular RNA was extracted for all strains after the shift to restrictive temperature, when the cells were at an OD_{600} of 0.8. Labeled cRNA was prepared for each sample and was hybridized to Affymetrix whole-genome arrays. For each strain, the experiment was performed twice independently, and the results were analyzed using the Array Assist software provided by Iobion (see MATERIALS AND METHODS). We compared each single mutant and double mutant to the wild type to define genes with altered expression, and we also compared the *not1-2* mutant to the *not1-2 spt3Δ* double mutant. Using an analysis that takes into account genes only for which expression is reproducible in the duplicates of the mutant and the wild-type cells, and which display at least a 2-fold difference in expression between the mutant and the wild type, there were 119 genes that displayed increased expression (from 2- to 40-fold) and 33 genes that displayed decreased expression (in general, 2- to 3-fold) in the *not1-2* mutant relative to the wild-type cells.

Interestingly, 70% of 119 genes that displayed increased expression in *not1-2* were totally (19%) or partially (51%) restored in the double mutant, and 58% of the 33 genes that displayed decreased expression in *not1-2* were totally (18%) or partially (40%) suppressed in the double mutant. In contrast, 49% of the 37 genes that were increased in the *spt3Δ* single mutant were similarly increased in the double mutant, and 40% of the 65 genes with decreased expression in the *spt3Δ* single mutant were similarly decreased in the double mutant (Table 2). The other genes deregulated in the *spt3Δ* single mutant were slightly less deregulated in the double mutant, indicating a low level of suppression of *spt3Δ* by the *not1-2* mutation.

Thus, mutation of *NOT1* leads mostly to an increase in gene expression under the conditions where the growth arrests at high temperature, and the deletion of *SPT3* has a global suppressive effect.

mRNA analysis confirms suppression of *not1-2*-increased gene expression by *spt3Δ*: To obtain confirmation of the results found by the microarray analyses, we analyzed by S1 digestion the mRNA levels of genes induced in *not1-2* and suppressed in the double mutant, such as *SNZ1* and *HSP12*, or decreased in *not1-2* and suppressed in the double mutant, such as *GUA1* and *ADK1* (Figure 2A). We grew cells as above, collected total cellular RNA, and analyzed the levels of specific mRNAs by S1 analysis. Consistently with the microarrays, *HSP12* and *SNZ1* were highly increased and *GUA1* and *ADK1* weakly decreased in *not1-2* (Figure 2B). The deletion of *SPT3* strongly suppressed the increased expression of *HSP12* and *SNZ1*, but only partially suppressed the decreased expression of *ADK1* and not at all that of *GUA1* (Figure 2B). Thus, the transcriptional defects in *not1-2* were most evident for genes upregulated in the mutant, and these defects were well suppressed by the deletion

TABLE 2

Suppression of *not1-2* by *spt3Δ* is global

Mutant	No. of target genes	Expression phenotype	Expression in *not1-2 spt3Δ*
not1-2	119	Increased	23 totally suppressed
			61 partially suppressed
not1-2	33	Decreased	6 totally suppressed
			13 partially suppressed
spt3Δ	37	Increased	18 no suppression
			17 partial suppression
spt3Δ	65	Decreased	26 no suppression
			35 partial suppression

of *SPT3*. In contrast, downregulation of mRNAs in *not1-2* was weak, consistent with the microarray results, but a suppressive effect by deletion of *SPT3* for these mRNA reductions was not very convincing. Hence we focused further analyses on genes activated in *not1-2*.

Total restoration of appropriate transcription in *not1-2* is specific to the deletion of the Spt3p subunit of the SAGA complex: To investigate the nature of the suppression, we chose to concentrate on the *HSP12* and

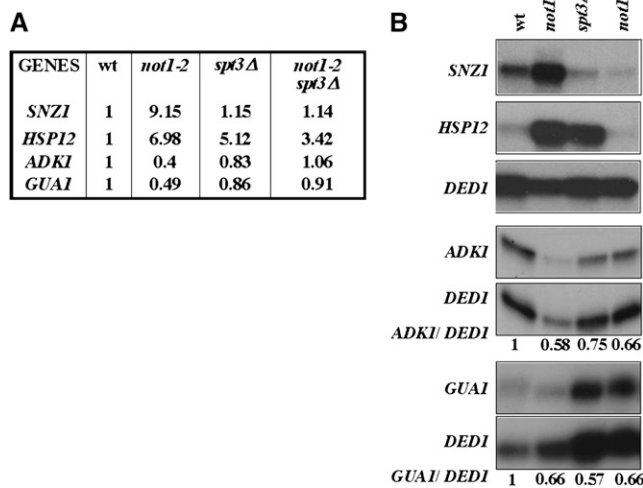| GENES	wt	*not1-2*	*spt3Δ*	*not1-2 spt3Δ*
SNZ1	1	9.15	1.15	1.14
HSP12	1	6.98	5.12	3.42
ADK1	1	0.4	0.83	1.06
GUA1	1	0.49	0.86	0.91

FIGURE 2.—mRNA analysis confirms suppression of transcriptional deregulation in *not1-2* by the deletion of *SPT3*. (A) The results of the microarray experiments for the four indicated transcripts are given. (B) Cells were grown, as in Figure 1, to an OD_{600} of 0.8, and 50 μg of total cellular RNA was analyzed by S1 digestion for the levels of the indicated transcripts. *DED1* was always measured in the same reaction as the other transcripts as an internal control, since the microarray experiments indicated that it was not affected by *not1-2*. The RNA was sampled and analyzed the same day for *SNZ1* and *HSP12*, and the results for *DED1* were the same and are shown only once. The levels of *ADK1* and *GUA1* relative to *DED1* were quantified using phosphoimager analysis and are indicated below the radiographs.

SNZ1 genes. We first tested whether the deletion of other subunits of the SAGA complex that do not suppress the temperature sensitivity of *not1-2* affect expression of *HSP12* and *SNZ1* in *not1-2*. For this, we grew, as above, *not1-2*, *spt3Δ*, *spt8Δ*, *spt7Δ*, and *gcn5Δ* single mutants and *not1-2* double mutants and analyzed equivalent amounts of total cellular RNA for the levels of *HSP12* and *SNZ1* mRNAs. Deletion of *GCN5* or *SPT7* did not reduce *SNZ1* mRNA levels in *not1-2* cells, and, furthermore, deletion of *GCN5* in itself increased *SNZ1* expression. In contrast, the deletion of *SPT3*, and to a lesser extent that of *SPT8*, reduced the level of *SNZ1* mRNA in *not1-2* mutant cells (Figure 3A). Deletion of *SPT3*, *SPT7*, and, to a lesser extent, *SPT8*, but not *GCN5*, reduced the expression of *HSP12* mRNA in *not1-2* (Figure 3A).

Thus, in good correlation with suppression of temperature sensitivity, total restoration of wild-type transcript levels in *not1-2* requires the deletion of *SPT3* specifically, and the deletion of *SPT8* also partially restores wild-type mRNA levels.

The trimethylation mark of histone H3 follows transcription in *not1-2*: It has been previously described that activation of transcription correlates with increased trimethylation of histone H3 ($H3-K4-Me_3$) at the core promoter (SANTOS-ROSA *et al.* 2002; POKHOLOK *et al.* 2005). To determine whether changes in mRNA levels in *not1-2* are due to changes in transcription initiation, we investigated the presence of this histone modification mark at promoters of genes with altered expression in *not1-2* by ChIP experiments. Wild-type, *not1-2*, *spt3Δ*, and *not1-2 spt3Δ* cells were grown as above, cells were crosslinked, and total protein extracts were incubated with antibodies against $H3-K4-Me_3$. For each immunoprecipitation, we first expressed the amount of each DNA in the immunoprecipitate relative to its amount in the total input DNA. Then we normalized the amount of specific DNA immunoprecipitated with the antibody to the amount of an intergenic DNA that was immunoprecipitated. The level of $H3-K4-Me_3$ at the *HSP12* and *SNZ1* promoters was strikingly increased in *not1-2*, and this was suppressed by the deletion of *SPT3*, which by itself had no effect on the levels of $H3-K4-Me_3$ compared to wild-type cells (Figure 4A). In contrast, the presence of $H3-K4-Me_3$ on the *ADK1* promoter was decreased in *not1-2* cells when compared to wild-type cells (Figure 4A), in good correlation with decreased expression from this promoter in *not1-2* (see Figure 2). Thus, the levels of $H3-K4-Me_3$ found at promoters in wild-type and *not1-2* or *not1-2 spt3Δ* cells correlate well with the levels of transcripts measured in these cells, providing support that changes in transcription levels are the basis for the genetic interaction between Spt3p and Not1p.

Deletion of *SPT3* suppresses increased association of Taf3p with promoters upregulated in *not1-2*: Previous studies have shown that the recruitment of TFIID to promoters was increased or decreased in a promoter-specific manner upon mutation of the Ccr4-Not complex
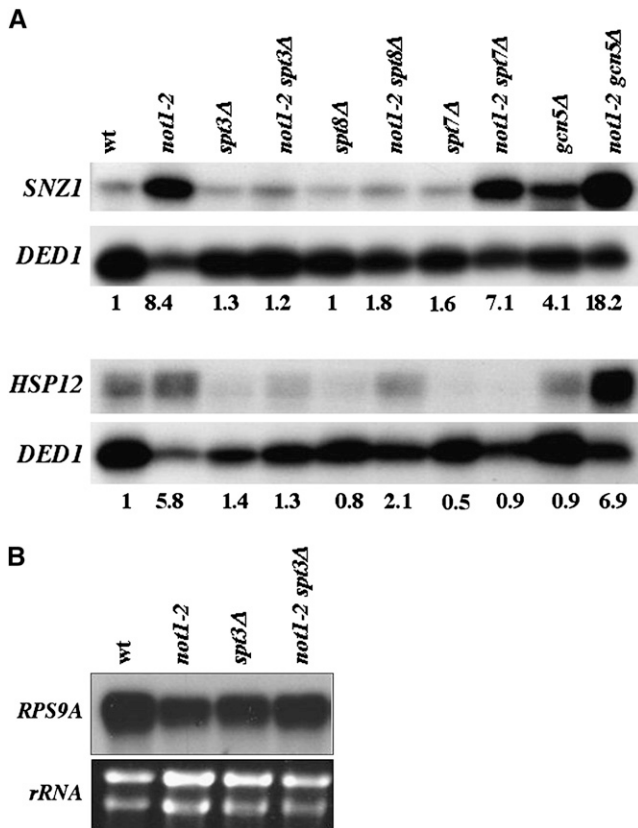
FIGURE 3.—The deletion of *SPT3* specifically reduces increased *HSP12* and *SNZ1* mRNA in *not1-2*. (A) The indicated strains were grown exponentially for 24 hr and then shifted at an OD_{600} of 0.2 to 37.5°. Total cellular RNA was prepared when cells reached an OD_{600} of 0.8 as in Figure 2. The levels of *HSP12* and *SNZ1* mRNAs were measured simultaneously to that of *DED1* in 50 μg of total cellular RNA by S1 analysis. Because of the unequal loading of some lanes, the results were quantified by phosphoimager analysis. (B) The levels of *RPS9A* in 50 μg of total RNA were measured for the indicated strains, and the levels of rRNA in 5 μg of total cellular RNA are indicated as a control.

(DELUEN *et al.* 2002). To determine whether the *not1-2* mutation had such a phenotype, we investigated by ChIP experiments whether the recruitment of Taf3p, a specific subunit of the TFIID complex, was altered in *not1-2* cells at restrictive temperature on induced promoters such as *HSP12* and *SNZ1*. Wild-type, *not1-2, spt3Δ,* and *not1-2 spt3Δ* cells were grown as before, cells were crosslinked, and total protein extracts were incubated with antibodies against Taf3p. The presence of the *HSP12* or *SNZ1* promoters in the immunoprecipitates was investigated by real-time PCR as before. As a control, we looked at the presence of the *RPS9A* promoter in the immunoprecipitates, since *RPS9A* mRNA is roughly similar in all four strains, but slightly decreased in *not1-2* cells relative to wild-type cells, rather than increased as *HSP12* and *SNZ1* (Figure 3B). Taf3p association with the *HSP12* and *SNZ1* promoters was significantly higher in *not1-2* than in wild-type cells, and this was suppressed by the deletion of *SPT3* (Figure 4B). In contrast, there was
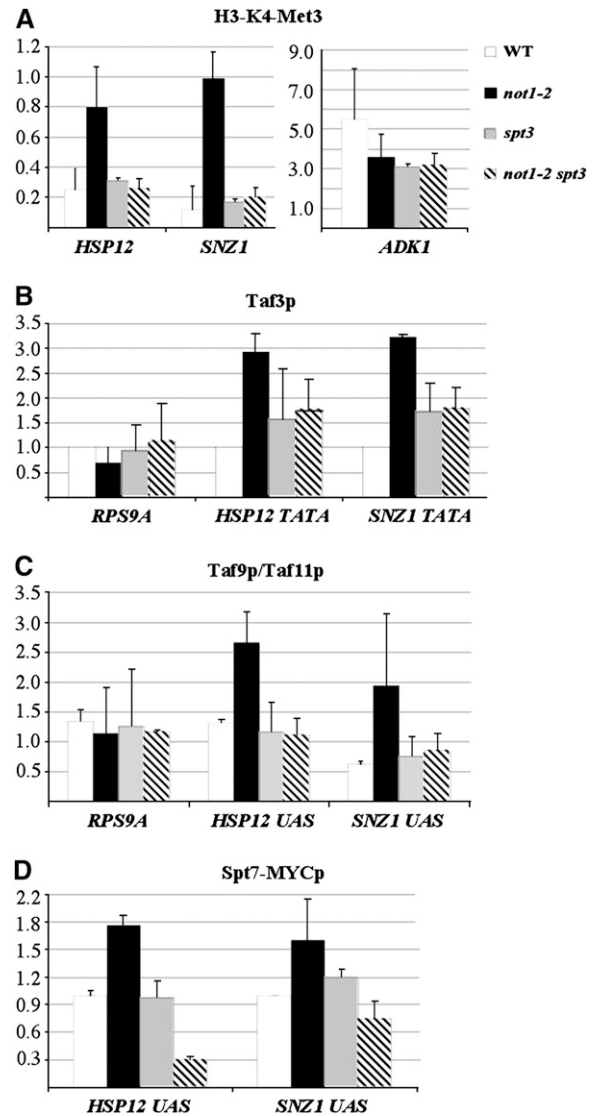

FIGURE 4.—The presence of Taf3p at *HSP12* and *SNZ1* promoters increases in *not1-2* and this is suppressed by the deletion of *SPT3*. The indicated strains were grown as in Figure 3 to an OD_{600} of 0.8 at 37.5°, and the cells were crosslinked prior to total extract preparation and immunoprecipitation with the indicated antibodies. The amount of indicated promoter DNAs in the immunoprecipitates was evaluated by real-time PCR, expressed relative to input DNA, and then normalized to the amount of an intergenic unexpressed DNA. (A) The antibodies were against H3-K4-Me₃ and the results presented are the average of two experiments performed with independent cultures. (B) Antibodies were against Taf3p and the experiment was repeated three times with independent cultures. (C) Antibodies against Taf9p and Taf11p were used and the relative amount of DNA immunoprecipitated with Taf9p to that immunoprecipitated with Taf11p was calculated (Taf9p/Taf11p). The experiment was repeated two times with independent cultures. (D) The indicated strains expressed MYC-tagged Spt7p and antibodies against MYC were used. The experiment was repeated three times with independent cultures.

no increase in the association of Taf3p with the *RPS9A* promoter in *not1-2* relative to wild-type cells, but instead there was a slight decrease that was suppressed by the deletion of *SPT3*. The deletion of *SPT3* in wild-type cells

did not appear to significantly alter the association of Taf3p with any of the promoters (Figure 4B).

Thus, from our analysis of two relevant promoters, we conclude that recruitment of TFIID is increased at promoters that are upregulated in *not1-2* and this is suppressed by the deletion of *SPT3*.

Spt3p mediates recruitment of SAGA to the UAS of genes activated in *not1-2*: To understand how Spt3p might affect recruitment of TFIID in *not1-2*, we investigated the association of SAGA with genes upregulated in *not1-2* by comparing the recruitment of Taf9p, a subunit of both SAGA and TFIID, and of Taf11p, a subunit of TFIID only, to the upstream activating sequence (UAS) of *HSP12* and *SNZ1*. We grew cells as above, crosslinked them, and prepared total protein extracts that we incubated with antibodies against Taf9p or Taf11p. We evaluated, as before, by real-time PCR the presence of the *RPS9A* core promoter and the *HSP12* or *SNZ1* UAS DNAs in the immunoprecipitates. We expressed the amount of each DNA that was immunoprecipitated with Taf9p relative to Taf11p as a tool to measure the contribution of SAGA *vs.* TFIID for each DNA. The association of Taf9p *vs.* Taf11p to the *RPS9A* core promoter was very similar in all strains (ratio of ∼1.0), compatible with TFIID associated with this DNA sequence in all cases (Figure 4C). In contrast, there was a significant increase in the association of Taf9p relative to Taf11p with the UAS sequences of *SNZ1* and *HSP12* in *not1-2* relative to wild-type cells, suggesting an increase of SAGA to this DNA sequence in *not1-2*. Interestingly, this increase was suppressed by the deletion of *SPT3* that had no effect in wild-type cells (Figure 4C).

To confirm these results, we created strains expressing C-terminally MYC-tagged Spt7p (see Table 1) and investigated the association of Spt7p to the UAS of *HSP12* and *SNZ1* by ChIP experiments as before, using antibodies against MYC. In total agreement with the previous experiments, we found that the association of Spt7p was increased at the UAS of both *HSP12* and *SNZ1* in *not1-2* cells compared to wild-type cells (Figure 4D). The deletion of *SPT3* did not have a major impact on the association of Spt7p with these UAS sequences in wild-type cells, but it suppressed the increased association of Spt7p with these sequences in *not1-2* cells (Figure 4D).

Taken together, these results reveal an Spt3p-dependent increase of SAGA association with UAS sequences of genes upregulated in *not1-2*, but they suggest no role of Spt3p for the basal level of SAGA associated with such sequences.

Deletion of *SPT3* suppresses post-transcriptional loss of histone H3 in *not1-2*: Surprisingly, during the course of the experiments presented above, we observed that the level of total H3-K4-Me$_3$ in extracts from *not1-2* cells was reduced when compared to wild-type cells at restrictive temperature (data not shown). We then discovered that the decreased levels of H3-K4-Me$_3$ in *not1-2* correlated with decreased levels of total histone H3 (Figure 5A).

Astonishingly, this loss of histone H3 was suppressed by the deletion of *SPT3* but not by the deletion of *GCN5* or *SPT7* (Figure 5A). Since deletion of *SPT3* suppresses transcriptional deregulation in *not1-2*, we analyzed the levels of the mRNAs encoding histone H3 in wild-type, *not1-2*, *spt3Δ*, *spt8Δ*, *spt7Δ gcn5Δ*, and *not1-2* double mutants by S1 digestion. However, the levels of histone H3 mRNA were roughly similar in all strains (Figure 5B), suggesting that the differences in total levels of histone H3 protein in the different strains described above are due to post-transcriptional control of histone H3 protein.

To determine whether *not1-2* mutant phenotypes might then result from reduced histone H3 levels, we increased histone H3 protein levels in *not1-2* to a level similar to that in the wild type by transforming *not1-2* mutant cells with a multicopy plasmid carrying the *HHT1* gene encoding histone H3 (Figure 5C). Nevertheless, the transformants could not grow at high temperature (Figure 5E), suggesting that restoring histone H3 protein levels alone was not sufficient to suppress *not1-2*. An obvious explanation could be that a decrease of histone H4 might also occur, and indeed, we found a decrease also of histone H4 protein but not mRNA in *not1-2* (Figure 5D). We then created a multicopy plasmid carrying the *HHF1* gene encoding histone H4 that we transformed into wild-type and *not1-2* cells carrying or not carrying the multicopy plasmid expressing histone H3. However, *not1-2* cells carrying the multicopy plasmids expressing histones H3 and H4 still did not grow at high temperature (Figure 5E). Thus, decreased levels of histones H3 and H4 are unlikely to be the cause of temperature sensitivity in *not1-2*.

Evidence for SAGA-independent functions of Spt3p: Since our experiments so far show that suppression of *not1-2* is obtained specifically by deleting the Spt3p subunit of the SAGA complex, we investigated whether Spt3p might have a function outside of the SAGA complex. We created an *spt3Δ spt7Δ* double-mutant strain by crossing the single mutants, sporulating the diploids, and dissecting the tetrads. The double *spt3Δ spt7Δ* grew slower than either single mutant at both 30° and 37.5°, indicating a synthetic phenotype between the two deletions. The deletion of *SPT7* in *not1-2* also led to a synthetic slow-growth phenotype as mentioned above (Figure 1A), but interestingly, the triple *not1-2 spt3Δ spt7Δ* grew better than the *not1-2 spt7Δ* double mutant, indicating that the deletion of *SPT3* suppressed *not1-2* in the absence of *SPT7*. Suppression of *not1-2* by the deletion of *SPT3* nevertheless did not restore growth at high temperature in the context of *spt7Δ* (Figure 6A). The synthetic phenotype between *spt3Δ* and *spt7Δ* and the suppression of *not1-2* in *spt7Δ* suggested a function for Spt3p in the absence of a SAGA complex. This idea was confirmed when we analyzed total histone H3 levels by Western blot analysis of total protein extracts. Indeed, the *spt3Δ spt7Δ* double mutant, but neither single mutant, displayed reduced levels of histone H3 protein when
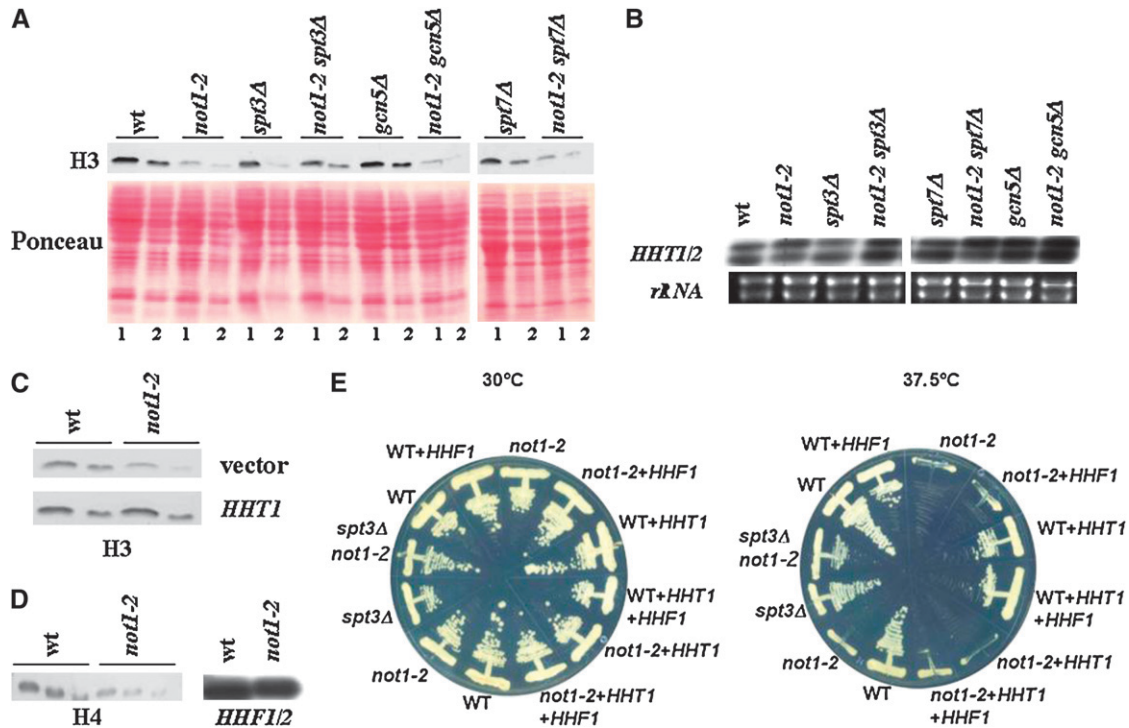
FIGURE 5.—The level of total histone H3 and H4 protein but not mRNA is decreased in *not1-2* and this is suppressed by deletion of *SPT3*. The indicated strains were grown as in Figure 3 to an OD_{600} of 0.8 at 37.5°. (A) Total protein extracts were prepared by post-alkaline lysis. Proteins were separated on 15% SDS–PAGE and analyzed by Western blot for the levels of histone H3. Lane 1, 14 μl of post-alkaline lysis corresponding to 0.7 OD_{600}; lane 2, 7 μl of post-alkaline lysis corresponding to 0.35 OD_{600}. (B) Total cellular RNA was extracted and 50 μg were analyzed by S1 digestion for the levels of *HHT1/2* mRNAs. The probe cannot distinguish between the two different mRNAs that encode histone H3. The amount of rRNA is shown as the loading control. (C) Total protein extracts were prepared by post-alkaline lysis, as indicated in A, but also from wild-type or *not1-2* cells carrying either an empty vector or a multicopy plasmid expressing histone H3 as indicated. (D) Total protein extracts were prepared by post-alkaline lysis. Proteins were separated on 15% SDS–PAGE and analyzed by Western blot for the levels of histone H4, using 28, 14 or 7 μl of post-alkaline lysis for each strain (left), total cellular RNA was extracted, and 50 μg was analyzed by S1 digestion for the levels of *HHF1/2* mRNAs (right). (E) The indicated strains overexpressing or not overexpressing histone H3 and/or H4 as indicated were streaked on glucose-rich plates and placed at 30° or 37.5° as indicated.

compared to the wild type, indicating once again a synthetic phenotype (Figure 6B).

To determine whether the SAGA-independent function of Spt3p suggested by the experiments presented above might be responsible for mutant phenotypes in *not1-2*, we looked at the expression of *SNZ1* mRNA. Indeed, our first experiments (see Figure 3A) revealed that *SNZ1* mRNA was upregulated by *not1-2* in the absence of SAGA, namely in *spt7Δ*. We grew wild type, *not1-2*, *spt3Δ*, *spt7Δ*, *spt3Δ spt7Δ*, and the *not1-2* double or triple mutants as before and prepared total cellular RNA that we analyzed by S1 digestion for the levels of *SNZ1* mRNA. The levels of *SNZ1* mRNA were increased by the *not1-2* mutation in *spt7Δ* mutant cells, but not in *spt3Δ* cells or in *spt7Δ spt3Δ* double-mutant cells (Figure 6C). In other words, in the absence of *SPT7*, the deletion of *SPT3* suppressed the *not1-2*-dependent induction of *SNZ1* mRNA.

Taken together, these results show that Spt3p has a function outside of the SAGA complex and that the *not1-2* mutation leads to an Spt3p-dependent increase in *SNZ1* mRNA even in the absence of a SAGA complex.

Spt3 mediates transcription activation in *not1-2* at permissive temperature: The experiments presented so far demonstrate Spt3p-dependent transcriptional activation in *not1-2* at restrictive temperature. To make sure that the observed effects might not be indirectly due to loss of viability or growth arrest at high temperature, we analyzed the levels of *SNZ1* and *HSP12* mRNAs in wild type, *not1-2*, *spt3Δ*, and *not1-2 spt3Δ* at permissive temperature. Both mRNAs were induced in *not1-2*, and this was suppressed by the deletion of *SPT3* (Figure 7A). Thus, Spt3p-dependent activation of genes in *not1-2* occurs at permissive temperature already.

To extend our findings, we analyzed at permissive temperature the expression of other genes identified in the microarrays as targets for Spt3-dependent transcriptional activation in *not1-2* at restrictive temperature, namely *SPG4*, *GPG1*, *XBP1*, and *JLP1* (see supplemental Figure S1 at http://www.genetics.org/supplemental/). All four mRNAs were induced in *not1-2* at permissive temperature, and this was suppressed by the deletion of *SPT3* (Figure 7A).
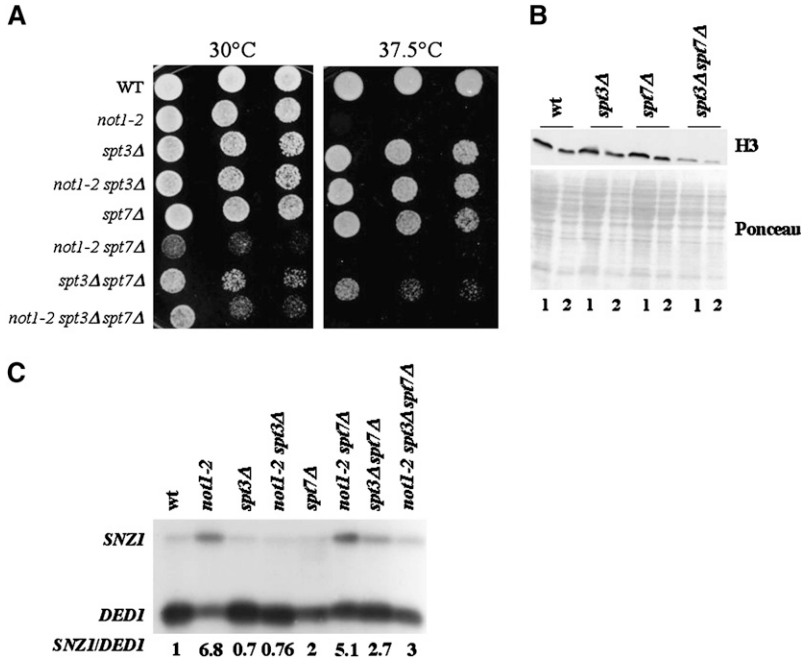
FIGURE 6.—*spt3*Δ and *spt7*Δ display a synthetic phenotype. The indicated strains were grown exponentially for 24 hr and then (A) serially diluted and plated on glucose-rich plates that were placed at 30° or 37.5° as indicated for 3 days. (B) Strains shifted to high temperature, as in Figure 1, to an OD_{600} of 0.8. Total extracts were made by post-alkaline lysis and analyzed by Western blot for the levels of histone H3. The Ponceau staining is shown for loading control. Lane 1, 14 μl of post-alkaline lysis corresponding to 0.7 OD_{600}; lane 2, 7 μl of post-alkaline lysis corresponding to 0.35 OD_{600}. (C) Strains shifted to high temperature, as in Figure 1, to an OD_{600} of 0.8. Total cellular RNA was extracted and 50 μg were analyzed by S1 digestion for the levels of *SNZ1* mRNAs and *DED1* as an internal control. Results were quantified by phosphoimager analysis.

Next, we wanted to determine whether Spt3p-dependent, SAGA-independent transcriptional activation in *not1-2* might be relevant to more genes than just *SNZ1*, and whether this was also visible at permissive temperature. Indeed, we found that *XBP1*, *JLP1*, and *GPG1* mRNAs were induced in *not1-2* at permissive temperature in the absence of *SPT7* and that this was Spt3p dependent (Figure 7B).

Finally, for *HSP12* and *SNZ1*, we investigated whether Spt3p-dependent mRNA induction in *not1-2* at permissive temperature correlated with an Spt3p-dependent increased presence of SAGA and/or TFIID at the promoters, as observed before at restrictive temperature. We performed ChIP experiments as above, but grew the cells at permissive temperature to exponential phase and used antibodies to Taf9p, a subunit of both
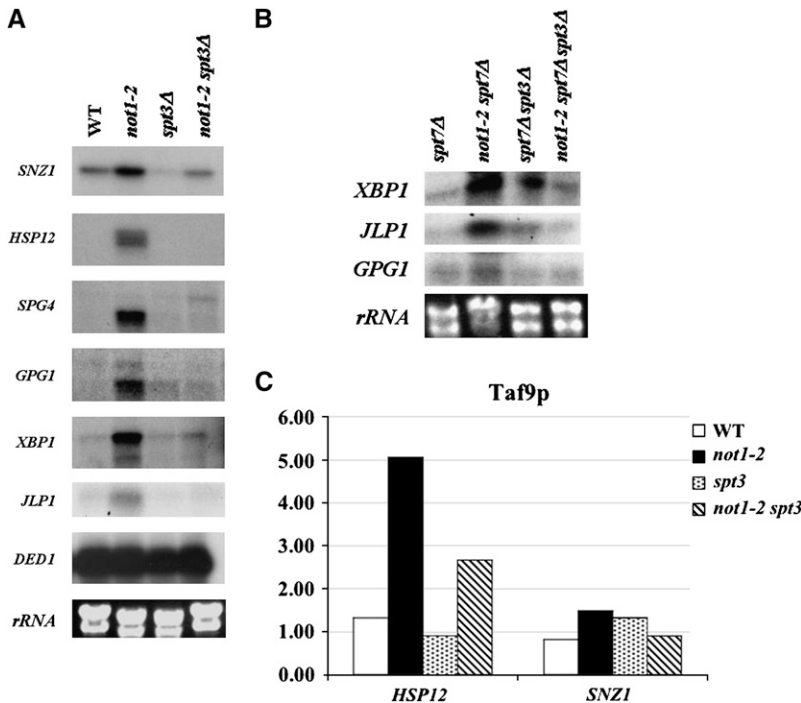

FIGURE 7.—Spt3p-dependent activation in *not1-2* occurs at permissive temperature. The indicated strains were grown in glucose-rich medium to exponential phase and (A and B) total cellular RNA was extracted for analysis by S1 digestion of the indicated mRNAs. *DED1* mRNA and rRNAs are shown as the loading control. (C) The cells were crosslinked prior to total extract preparation and immunoprecipitation with Taf9p polyclonal antibodies. The amount of indicated promoter DNAs in the immunoprecipitates was evaluated by real-time PCR, expressed relative to input DNA, and then normalized to the amount of an intergenic unexpressed DNA.

TFIID and SAGA. We observed that, indeed, even at permissive temperature, the presence of Taf9p at the promoters of *HSP12* and *SNZ1* was increased in *not1-2* and this was suppressed by the deletion of *SPT3* (Figure 7C).

DISCUSSION

Spt3p mediates mutant phenotypes in *not1-2*: In this work, we have used genetic and molecular tools to investigate the interaction between Not1p, the essential and scaffold subunit of the Ccr4-Not complex, and Spt3p, a subunit of one functional module of the SAGA complex. Indeed, a previous study demonstrated that the thermo-sensitivity associated with a *not1-2* mutation could be suppressed by deletion of *SPT3* (COLLART 1996). *not1-2* is a nonsense mutation in which a C-terminally truncated form of Not1p accumulates and only low levels of full-length protein containing the essential C-terminal domain are expressed. The mutant phenotypes of *not1-2* most certainly result from reduced levels of Not1p, and thus of the Ccr4-Not complex, as the overexpression of the N-terminal portion of Not1p has no observable mutant phenotype (our unpublished observations) and all Not1p protein in the cell is associated in Ccr4-Not complexes (MAILLET *et al.* 2000). We found that the mutant phenotypes associated with *not1-2* were entirely mediated by Spt3p. Indeed, microarray analyses revealed that transcriptional deregulation in *not1-2* was generally dependent upon Spt3p and consisted mostly of increased gene expression. Furthermore, by ChIP experiments we could show that the Spt3p-dependent increased transcription in *not1-2* correlated with Spt3p-dependent increased recruitment of SAGA and TFIID to upregulated promoters. Finally, we observed that transcriptional deregulation and temperature sensitivity in *not1-2* correlated with an Spt3p-dependent post-transcriptional decrease of total histone H3 and H4 protein levels in the cell. Taken together, our results suggest that the Ccr4-Not complex regulates an Spt3p-dependent function to maintain growth at high temperature, as well as appropriate histone H3 and H4 protein levels and transcriptional repression at this temperature.

We were able to show that Spt3p-dependent transcriptional deregulation in *not1-2* already occurs at permissive temperature when *not1-2* cells grow as well as wild-type cells. At this temperature, in contrast, histone H3 and H4 levels are not affected in *not1-2* compared to the wild type (see supplemental Figure S2 at http://www.genetics.org/supplemental/). Furthermore, overexpression of histones H3 and H4 in *not1-2* leading to histone levels similar to the wild type does not restore growth at high temperature. This would suggest that decreased histone levels are not the cause of Spt3p-dependent transcriptional upregulation in *not1-2*, nor a cause for growth arrest at restrictive temperature, but instead might be a consequence, for instance, of growth arrest at high temperature.

A function of Spt3p independent of SAGA is controlled by Not1p: Spt3p is a subunit of the SAGA complex and of its very similar SLIK/SALSA complex. Its precise function in these complexes has not been clearly established, and no SAGA- or SLIK/SALSA-independent role for Spt3 has been described. Generally, studies of Spt3p have been global, and they have shown that it plays roles in both activation and repression of transcription. While original experiments suggested that Spt3p functions through interactions with TBP (EISENMANN *et al.* 1989, 1992), it has not been demonstrated that TBP co-immunoprecipitates with Spt3p, and in fact interaction of SAGA with TBP clearly requires Spt8p but not Spt3p (SERMWITTAYAWONG and TAN 2006). Interactions between Spt3p and Spt8p might explain the confusion about the interaction between Spt3p and TBP. Indeed, genetic studies suggest that the functions of Spt3p and Spt8p are very similar (INGVARSDOTIR *et al.* 2005). However, Spt3p, but not Spt8p, is a subunit of the SLIK/SALSA complex, suggesting that the functions of the two proteins are certainly not identical. Our study shows that deletion of SAGA subunits other than Spt3p does not suppress the *not1-2* mutation, except for the deletion of *SPT8* that also suppresses *not1-2* but to a lesser extent than the deletion of *SPT3*. These results are in agreement with all previous studies showing that the function of Spt3p is closest to that of Spt8p within the SAGA complex, but is different from that of the other SAGA subunits. The results also show that the roles of Spt3p and Spt8p are not exactly identical. The partial suppression of *not1-2* obtained by deletion of *SPT8* might suggest that Spt8p contributes toward a function(s) of Spt3p that is independent of the SAGA complex.

We were able to determine that the deletion of *SPT3* suppresses transcriptional induction in *not1-2* cells lacking Spt7p, and thus SAGA or SLIK/SALSA complexes. These observations suggest that Spt3p has a function(s) outside of the SAGA complex and that this function(s) of Spt3p mediates transcriptional deregulation in *not1-2*. Compatible with this idea is the observation that the deletion of *SPT3* displays a synthetic phenotype when combined with a deletion of *SPT7*. Indeed, this synthetic phenotype supports a function for Spt3p in the absence of a SAGA complex. Furthermore, the deletion of *SPT3* was observed to suppress the synthetic growth phenotype obtained by combining a deletion of *SPT7* that results in the loss of SAGA and *not1-2*. Again, such an observation is compatible with a function of Spt3p outside of SAGA.

The Ccr4-Not complex regulates the capacity of Spt3p to contribute to recruit SAGA and TFIID to certain promoters: The *not1-2* mutant leads to increases in transcription initiation, since increases in mRNA levels correlate with increased association of TFIID and SAGA, as well as with increased presence of the H3-K4-Me$_3$ mark, at promoters of upregulated genes in *not1-2*. In the *not1-2* mutant cells, Spt3p mediates the recruitment

and/or stabilization of SAGA and TFIID to promoters of upregulated genes, but Spt3p is not required for the basal levels of either TFIID or SAGA associated with these same promoters in wild-type cells. Thus, in wild-type cells the capacity of Spt3p to recruit and/or stabilize SAGA and TFIID to these promoters is under the control of the Ccr4-Not complex.

So far, contradictory results concerning the role of Spt3p in the recruitment of SAGA to promoters have been published. It was generally accepted that Spt3p is not required for the recruitment of the SAGA complex at SAGA-dependent promoters (Larschan and Winston 2001), but a recent study (Qiu *et al.* 2005) showed that high-level recruitment of Tra1p and other SAGA subunits requires Spt3p. Our results suggest that if the Ccr4-Not complex is not completely wild type in some strains, this could explain the different results obtained in different studies.

Our studies also raise the possibility that TFIID and SAGA co-occupy promoters, since Spt3p mediates recruitment and/or stabilization of both complexes to the same promoters of genes upregulated in *not1-2*. This finding is in agreement with a previous study that has suggested that Spt3p, like Gcn5p, might help to recruit Taf1p (Van Oevelen *et al.* 2005).

Spt3p mediates activation of transcription in *not1-2* even in the absence of a SAGA complex, suggesting that it can lead to the recruitment of TFIID in the absence of a SAGA complex. Thus, Spt3p is likely to be capable of recruiting and/or stabilizing TFIID at the core promoter from within the SAGA complex at the UAS or without being associated with the SAGA complex.

How might Spt3p mediate recruitment of SAGA and TFIID in *not1-2*? Spt3p might be able to provide a contact surface for transcriptional activators and connect them to TFIID. Alternatively, it could be that Spt3p interacts with complexes such as mediator that can have a stimulatory effect on SAGA recruitment as suggested previously (Qiu *et al.* 2005). Spt3p has also been shown to be important for nucleosome remodeling by cooperating with Mot1p (Madison and Winston 1997; Topalidou *et al.* 2004; Van Oevelen *et al.* 2005). A possible connection between Spt3p and chromatin is also suggested by our work since we observe an Spt3p-dependent decrease of cellular histone H3 and H4 levels in *not1-2* that correlates with an Spt3p-dependent increased recruitment and/or stabilization of SAGA and TFIID to promoters of genes upregulated in *not1-2*. In other studies, evidence connecting Spt3p and Spt8p to TFIIA has been provided. Indeed, overexpression of TFIIA suppresses *spt3* mutations (Madison and Winston 1997) and a recent study has shown that Spt8p reduces affinity of SAGA for the other general transcription factors because of competition with TFIIA (Warfield *et al.* 2004). However, overexpression of TFIIA does not suppress *not1-2* or alter suppression of *not1-2* by the deletion of *SPT3* (data not shown), suggesting that interactions between TFIIA and Spt3p are not involved in the *not1-2* mutant phenotypes.

Mechanism of transcriptional deregulation in *not1-2*: The identification of Spt3p as the protein that specifically mediates mutant phenotypes in *not1-2* leads to the important question of how the Ccr4-Not complex controls Spt3p-dependent transcriptional activation. A relevant question in this regard is whether the Ccr4-Not complex controls Spt3p directly or whether Spt3p happens to be required for transcriptional activation of all the genes induced in *not1-2*. We were not able to show any co-immunoprecipitation of SAGA subunits with Ccr4-Not subunits (data not shown), yet it is possible that the Ccr4-Not complex regulates post-translational modification of SAGA subunits. Indeed, we have preliminary data suggesting subtle changes in the association of Spt3p with Spt7p when compared to that of Taf5p with Spt7p in *not1-2* (data not shown). These findings are consistent with some direct effect of the Ccr4-Not complex on Spt3p and/or the SAGA complex. Alternatively, the Ccr4-Not complex might regulate Spt3p-dependent recruitment of SAGA to promoters by regulating the 19S proteasomal subunits that contribute to SAGA recruitment as recently shown (Lee *et al.* 2005) or by regulating transcription factors that recruit SAGA to activate transcription. We addressed a possible role of the proteasome in *not1-2* by analyzing induction of *HSP12* and *SNZ1* mRNAs in *not1-2* cells carrying the temperature-sensitive proteasomal mutation *sug1-25*. However, induction of both genes was similar in *not1-2* or *not1-2 sug1-25* mutants at restrictive temperature (data not shown), suggesting that the proteasome is not required for upregulation of these genes in *not1-2*. In contrast, we already know that the Ccr4-Not complex controls the activity of at least one transcription factor, Msn2p, through control of its post-translational modification (Lenssen *et al.* 2005). Thus, one could imagine that the Ccr4-Not complex controls a subset of transcription factors that act to recruit SAGA to genes through the Spt3p subunit. We previously determined that the alterations in TFIID distribution to promoters in mutants of the Ccr4-Not complex were independent of Msn2p (Lenssen *et al.* 2005). Nevertheless, the Ccr4-Not complex may control more than one transcription factor such that the deletion of one individual transcription factor would indeed not act as a general suppressor. In contrast, the deletion of a protein that connects all of these transcription factors to the general transcription machinery should act as a general suppressor. Spt3p could be such a connecting protein. If this latter model is correct—namely if the primary target of the Ccr4-Not complex is a number of Spt3p-dependent transcription factors—it is at present unclear why the *not1-2* mutation would lead to an Spt3p-dependent global reduction of histone H3 and H4 levels. It could be that this latter phenotype is completely independent or, as mentioned above, is really only a consequence of growth arrest at high temperature. We are

currently undertaking a biochemical study of Spt3p and SAGA in wild-type and *not1-2* cells with the hope of providing answers to the remaining questions.

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