Hap2-3-5-Gln3 determine transcriptional activation of *GDH1* and *ASN1* under repressive nitrogen conditions in the yeast *Saccharomyces cerevisiae*

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The transcriptional activation response relies on a repertoire of transcriptional activators, which decipher regulatory information through their specific binding to cognate sequences, and their capacity to selectively recruit the components that constitute a given transcriptional complex. We have addressed the possibility of achieving novel transcriptional responses by the construction of a new transcriptional regulator – the Hap2-3-5-Gln3 hybrid modulator – harbouring the HAP complex polypeptides that constitute the DNA-binding domain (Hap2-3-5) and the Gln3 activation domain, which usually act in an uncombined fashion. The results presented in this paper show that transcriptional activation of *GDH1* and *ASN1* under repressive nitrogen conditions is achieved through the action of the novel Hap2-3-5-Gln3 transcriptional regulator. We propose that the combination of the Hap DNA-binding and Gln3 activation domains results in a hybrid modulator that elicits a novel transcriptional response not evoked when these modulators act independently.

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INTRODUCTION

The response of *Saccharomyces cerevisiae* to nutrient limitation is a model whose study has amply contributed to our knowledge of the mechanisms determining transcriptional activation (Zaman *et al.*, 2008). Nutrient limitation results in a complex adaptation of the physiology of yeast cells, which allows them to redefine their transcription programme. This leads to the activation of particular sets of genes, whose products are needed to evoke an appropriate physiological response. Transcriptional activators are composed of a DNA-binding domain, which targets these proteins to specialized binding sites, and an activation domain that mediates transcription initiation; these two domains can be contained in single or different polypeptides (Zaman *et al.*, 2008).

When yeast cells are provided with poor nitrogen sources, such as proline, genes coding for enzymes involved in the catabolism of these compounds are highly expressed. Conversely, in the presence of high-quality nitrogen sources such as glutamine, a decrease in the levels of catabolic enzymes is observed. The reduced expression of the genes coding for enzymes involved in the utilization

of poor nitrogen sources is brought about through the action of a regulatory system known as nitrogen catabolite repression (NCR) (Blinder & Magasanik, 1995; Coffman *et al.*, 1995, 1996; Courchesne & Magasanik, 1988; Minehart & Magasanik, 1991). NCR operates through the action of two transcriptional activators, Gln3 and Ntl1/Gat1, and two repressors, Dal80 and Gzf3/Deh1/Ntl2 (Minehart & Magasanik, 1991; Stanbrough *et al.*, 1995; Svetlov & Cooper, 1998; Magasanik & Kaiser, 2002), whose expression is regulated by nitrogen levels. In the presence of repressive nitrogen sources, the TOR signalling pathway promotes the association of the GATA transcription factors Gln3 and Gat1 with the cytoplasmic protein Ure2, thus retaining Gln3 and Gat1 in the cytoplasm (Beck & Hall, 1999; Cardenas *et al.*, 1999; Hardwick *et al.*, 1999). Dissociation of this complex occurs when cells are transferred from a rich medium to one containing a poor nitrogen source, favouring Gln3 and Gat1 nuclear transport and activation of NCR-controlled genes (Beck & Hall, 1999). Thus, under repressive nitrogen conditions, the role of Gln3 and Gat1 as activators is hindered. However, previous work from our laboratory showed that expression of genes pertaining to nitrogen assimilation pathways, such as *GLT1*, encoding glutamate synthase, and *GDH1*, encoding one of the two isoforms of glutamate dehydrogenase, was Gln3-dependent under repressive

Abbreviations: ChIP, chromatin immunoprecipitation; NCR, nitrogen catabolite repression.

nitrogen conditions, suggesting that Gln3 could determine gene expression even under repressive conditions (Riego *et al.*, 2002; Valenzuela *et al.*, 1998). Most interesting was the finding that *GDH1* expression was induced by the HAP complex, whose activator role had been considered to exclusively determine transcription of genes involved in the diauxic shift, mitochondrial biogenesis and energy metabolism, allowing adaptation of yeast from fermentative to respiratory growth (Dang *et al.*, 1994). Although orthodox HAP-dependent gene expression is determined by a heterotetramer constituted by the *HAP2*-, *HAP3*-, *HAP4*- and *HAP5*-encoded proteins (McNabb & Pinto, 2005), it was found that for *GDH1*, the *HAP4*-encoded subunit constituting the activation domain of this complex played no role (Dang *et al.*, 1996a). Thus *GDH1*-HAP complex-dependent activation relied on the action of the Hap2-Hap3-Hap5 heterotrimeric DNA-binding domain in combination with a non-conventional activation subunit, suggesting the possibility of the assembly of a hybrid activator.

Results presented in this paper indicate that expression of *GDH1* and *ASN1* under repressive nitrogen conditions is determined by a hybrid activator in which the Hap2-3-5 DNA-binding domain and the Gln3 activation domain act in a combined fashion, driving selective transcriptional responses not evoked when the parental regulators act independently.

METHODS

Strains. Strains used in this work are described in Table 1. The wild-type strain CLA-1 (*MAT α ura3 leu2*) and the construction of its isogenic *GLN3-myc¹³* derivative have been previously described (Ishida *et al.*, 2006). The isogenic *hap2 Δ ::kanMX* (CLA-501) and *hap4 Δ ::kanMX* (CLA-502) were obtained from strain CLA-1 by gene replacement. A PCR-generated *kanMX4* module was prepared with plasmid pFA6a following the method of Wach *et al.* (1997), using oligonucleotides HAP2KOFw-HAP2KORv and HAP4KOFw-HAP4KORv (Table 2). *HAP2* and *HAP4* were tagged at the carboxy terminus using the *¹³myc-kanMX4* modules described by Longtine *et al.* (1998). Two pairs of deoxyoligonucleotides (HAP2F2-HAP2R1 and HAP4F2-HAP4R1) were designed based on the *HAP2* and *HAP4* coding sequences, and that of the pFA6a-*¹³myc-kanMX4* multiple

cloning site (Table 2). The *hap2 Δ ::kanMX6 GLN3-myc¹³::natMX21* (CLA-508) and *gln3 Δ ::natMX21 HAP2-myc¹³::kanMX4* (CLA-509) double mutants were constructed as follows. The *kanMX4* module from CLA-503 (*GLN3-myc¹³::kanMX4*) was replaced by the *natMX21* cassette, which confers resistance to the antibiotic nourseothricin (Goldstein & McCusker, 1999). The *natMX21* cassette used for transformation was obtained by digesting plasmid p4339 with *EcoRI*. The pertinent strains were transformed according to the method described by Ito *et al.* (1983) and transformants were selected for nourseothricin resistance (100 mg l⁻¹, clonNAT). Strain CLA-302a (*gln3 Δ ::natMX21*) was alternatively tagged at the carboxy terminus of *HAP2* using the *¹³myc-kanMX6* modules described by Longtine *et al.* (1998), as described above (Table 1). Strain CLA-503a (*GLN3-myc¹³::natMX21*) was obtained by gene replacement using the *natMX21* cassette described above. A PCR-generated *kanMX6* module was prepared with plasmid pFA6a following the method of Wach *et al.* (1997) as described above, obtaining strain CLA-508 (*hap2 Δ ::kanMX6 GLN3-myc¹³::natMX21*) (Table 1). *HAP2*-TAP was obtained from the TAP-tagged *Saccharomyces* strain collection and a Hap2-TAP *GLN3-myc¹³* derivative was obtained as previously described (Ishida *et al.*, 2006).

Construction of substitution mutations in the *GDH1* intergenic region. Mutants simultaneously affected in the GATAA⁽³⁾, GATAA⁽⁴⁾ and GATAA⁽⁵⁾ sites present in the *GDH1* promoter (Riego *et al.*, 2002) were constructed by transforming strain CLA-1 (*MAT α ure3 leu2 GLN3-myc¹³NAT*) with a 3.2 kb fragment obtained by PCR amplification of the pCORE plasmid harbouring the *kanMX4*, *KIURA3 CORE* module, using the pCORE/gata Gdh1 Fw and pCORE/gata Gdh1 Rv oligonucleotides described in Table 3 (Storici & Resnick, 2003). Transformation was carried out following the protocol described by Longtine *et al.* (1998). Colonies were isolated on YPD-kanamycin (200 mg ml⁻¹). Correct insertion was verified by PCR amplification. One of the transformants was retransformed with IROs (integrative recombinant oligonucleotides) harbouring mutagenized GATAA⁽³⁾, GATAA⁽⁴⁾ (GcaAAG) sequences (IRO 1-2 gln3 Cm Fw and IRO 1-2 gln3 Cm Rv; Table 3) (Riego *et al.*, 2002). Correct insertion was confirmed by sequencing. The strain obtained was transformed with the above-described *CORE* module and finally with IROs IRO 2-1 gln3 Cm Fw and IRO 2-1 gln3 Cm Rv (Table 3) in order to generate mutants in the third GAATAG box (GATAA⁽⁵⁾). Nine colonies were isolated, purified and sequenced with an Applied Biosystems 3100 Genetic Analyzer.

Growth conditions. Strains were grown on MM containing salts, trace elements and vitamins following the formula of yeast nitrogen base

Table 1. Strains used in this work

Strain	Genotype	Source or reference
CLA-1	<i>MATα ura3 leu2</i>	Valenzuela <i>et al.</i> (1998)
CLA-302 <i>gln3Δ</i>	<i>MATα gln3Δ::kanMX4 ura3 leu2</i>	Valenzuela <i>et al.</i> (1998)
CLA-706 <i>ure2Δ</i>	<i>MATα ure2Δ::kanMX4 ura3 leu2</i>	Sosa <i>et al.</i> (2003)
CLA-501 <i>hap2Δ</i>	<i>MATα hap2Δ::kanMX6 ura3 leu2</i>	This study
CLA-502 <i>hap4</i>	<i>MATα hap4::kanMX6 ura3 leu2</i>	This study
CLA-503 <i>GLN3-myc¹³</i>	<i>MATα GLN3-myc¹³::kanMX4 ura3 leu2</i>	Ishida <i>et al.</i> (2006)
CLA-506 <i>HAP2-myc¹³</i>	<i>MATα HAP2-myc¹³::kanMX4 ura3 leu2</i>	This study
CLA-507 <i>HAP4-myc¹³</i>	<i>MATα HAP4-myc¹³::kanMX4 ura3 leu2</i>	This study
CLA-508 <i>hap2Δ GLN3-myc¹³</i>	<i>MATα hap2Δ::kanMX6 GLN3-myc¹³::nat ura3 leu2</i>	This study
CLA-509 <i>gln3Δ HAP2-myc¹³</i>	<i>MATα gln3Δ::nat HAP2-myc¹³::kanMX4 ura3 leu2</i>	This study
CLA-512 <i>GcaAAG GLN3-myc¹³</i>	<i>MATα GDH1 GATAA::GcaAAG GLN3-myc¹³::nat ura3 leu2</i>	This study
BY-1	<i>BY4741 HAP2-TAP::HIS3 GLN3-myc¹³::kanMX4 ura3 leu2 his3 met51</i>	This study

Table 2. Oligonucleotides used for protein tagging and construction of null strains

Primer	Oligonucleotide sequence (5'–3')*	Application	Source or reference
Protein tagging			
GLN3F2	AGTTCAGCAATTGCTGACGAATTGGATTGGTTAAAATTTGGTATAcggtatccccgggtaattaa	3'-GLN3 tagging	Ishida <i>et al.</i> (2006)
GLN3R1	AGTAATTATTAACATAATAAGAATAATGATAATGATAATACGCGGgaattcgagctcgtttaaac	3'-GLN3 tagging	Ishida <i>et al.</i> (2006)
HAP2F2	CAGCCGCATGCTACTTCCACCGCAGCTGCAGCAGACAAAAAACAggatccccgggtaattaa	3'-HAP2 tagging	This work
HAP2R1	TAGTTCCTTTAGGAATGATATTAACATTGGAATATTACAAAATTAgattcgagctcgtttaaac	3'-HAP2 tagging	This work
HAP4F2	GACGACCTTGACGAAGATGTCGATTTTTTAAAGGTACAAGTATTTCggatccccgggtaattaa	3'-HAP4 tagging	This work
HAP4R1	CGTGATTTTATGTTGTTTCGTTTTATTGCAACATGCCTATTTCAgaattcgagctcgtttaaac	3'-HAP4 tagging	This work
Null strain construction			
HAP2KOFw	GAAGAGGAACAAGAACGCCATGTCAGCAGACGAAACGGATGCGAgcataggccactagtggatctg	HAP2 null strain	This work
HAP2KORv	CAGATTAATAGGCCATATGGATACCATGTGGTATAAGAGGGCACcagctgaagcttcgtacgc	HAP2 null strain	This work
HAP4KOFw	CTCTCTAAACCCAGTTTATATCGTATATGCTATCTACAGGTCCACgcataggccactagtggatctg	HAP4 null strain	This work
HAP4KORv	GTGTATGTATGAGAATAAATACTAAGTACCAGAAACGCGcagctgaagcttcgtacgc	HAP4 null strain	This work

*Lower-case lettering indicates sequence of the multiple cloning site of plasmid pFA6a-*myc*¹³-*kanMX6* for protein tagging and pUG6 for null strain construction.

(Difco). Filter-sterilized glucose (2%, w/v) or 2% (v/v) ethanol were used as carbon sources and 0.2% (w/v) (NH₄)₂SO₄ or 0.1% (w/v) glutamine or proline were used as nitrogen sources. Amino acids needed to satisfy auxotrophic requirements were added at 0.01% (w/v). Cells were incubated at 30 °C with shaking (250 r.p.m.).

Northern analysis. This was carried out as previously described (González *et al.*, 1992). Total RNA was prepared according to Struhl & Davis (1981) from 200 ml cultures of MM with 0.1% proline or glutamine as nitrogen sources. Prehybridization and hybridization conditions were established as previously described (Valenzuela *et al.*, 1998). Filters were sequentially probed with PCR fragments of 650, 750, 950 and 170 bp from *GDH1*, *ASN1*, *DAL5* and *CYC1*, respectively, and an 870 bp *ACT1* DNA fragment, as loading control. *GDH3* was probed on a separate filter using a 760 bp fragment of *GDH3*. Blots were scanned using the ImageQuant 5.2 (Molecular Dynamics) software.

Chromatin immunoprecipitation (ChIP). Formaldehyde cross-linking and immunoprecipitations were carried out by the procedure described by Hecht *et al.* (1995). Yeast cells (150 ml of OD₆₀₀ 0.5) were incubated on ice for 15 min and cross-linked with 1% formaldehyde for 60 min at room temperature. After addition of 125 mM glycine and incubation for 5 min with gentle agitation, cells were harvested and washed with saline Tris-buffer. Pelleted cells were suspended in lysis buffer (140 mM NaCl, 1 mM EDTA, 50 mM HEPES/KOH, 1% Triton X-100, 0.1% sodium deoxycholate) with protease inhibitor cocktail (Complete Mini, Roche). Cells were lysed with glass beads, and collected by centrifugation. Extracts were sonicated to produce chromatin fragments of <1000 bp, with an average size of <500 bp. ChIP was conducted with 1 µg anti-c-Myc antibody (9E 11, Santa Cruz Biotechnology) for 3 h, washed, suspended in 1 × TE/1% SDS and incubated overnight at 65 °C in order to reverse the formaldehyde cross-linking. The immunoprecipitates were incubated with proteinase

Table 3. Oligonucleotides used for site-directed mutagenesis

Primer	Oligonucleotide sequence (5'–3')*
pCORE/gata Gdh1 Fw	GAACCGGCCCATGCATTTTCAGTGCAGTGGGGCAATAAAAgagctcgttttcga cactgg
pCORE/gata Gdh1 Rv	AGCTTCTCTCTGCTGATTGGACGTGGGGTCGTACTATTTCTTctttaccatta agttgatc
IRO 1-2 gln3 Cm Fw	GTTCCATGACTCCATGGAACGGGCCTGATCTTAATAACctttgcTGACCATCTGATTGGATGGCG ATATTctttgcCAGAACCAATGCGCCG
IRO 1-2 gln3 Cm Rv	AGCTTCTCTCTGCTGATTGGACGTGGGGTCGTACTATTTCTTTTTATTGCCCCACCGCACTGAAAA TGCATGGGCCGGTTCGGCGCATTGGTTCTGC
IRO 2-1gln3 Cm Fw	AACCGGCCCATGCATTTTCAGTGCAGTGGGGCAATAAAAAAGAAATAGTACGACCCACGTCCAAT CAGCAGAGAGAAGCTctttgcAGCTGCTGCGCCG
IRO 2-1 gln3 Cm Rv	TGCTACTTCTTACGCTTCTTCTTCCGCAATTTTTCAGTGCTGATTTTCATTATGGTACCTGATGAAA AAGAAAACGGCGCAGCAGCTgcaagAGCTTC

*All the oligonucleotides were generated in this work. Lower-case lettering indicates amplification sites of *kanMX4* and *KIURA* present in plasmid pCORE used for site-directed mutagenesis. For IRO oligonucleotides, lower-case lettering indicates the mutagenesis site. The *GDH1* promoter wild-type sequence 5'-GATAAG-3' was changed to 5'-GcaAAG-3'.

K (Roche) followed by phenol/chloroform/isoamyl alcohol extraction, precipitated and suspended in $1 \times$ TE buffer. The primer sets used for PCR analysis are listed in Table 4. PCR products were resolved on a 1.5 % agarose gel stained with ethidium bromide.

Immunoprecipitation of Hap2-TAP and Gln3-Myc¹³. Whole-cell extracts were prepared from cells grown to OD₆₀₀ 0.5 in 100 ml MM-Gln. Protein A beads and 15 µl anti-c-Myc antibody were diluted to 5 ml with TNET buffer (50 mM Tris/HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 1 % Triton X-100) and incubated at 4 °C for 1 h. Extract (300 µl) was added and incubated at 4 °C overnight. Beads were washed five times with TNET buffer containing 20 mM Na₂S₂O₈ and 100 mM PMSF and then heated at 65 °C 15 min in 50 µl Laemmli buffer containing 100 mM DTT. Samples of 40 µl were run on 10 % PAGE for Western blotting analysis using anti-TAP antibodies (Thermo Scientific).

Gel electrophoresis and immunoblotting. Protein extracts obtained from the pertinent strains were subjected to SDS-PAGE on 10 % slab gels and transferred to nitrocellulose membranes. Incubation with antibodies was done as described by Towbin *et al.* (1979), with 1 µg anti-c-Myc antibody.

Nucleotide-level alignment. Nucleotide-level multiple alignment of the *GDH1*, *GDH3*, *ASN1* and *ASN2* promoter regions was done using CLUSTALW2 (<http://www.ebi.ac.uk/Tools/clustalw2>). Promoter sequences were obtained from http://www.broad.mit.edu/annotation/fungi/comp_yeasts/downloads.html for *Saccharomyces paradoxus* and *Saccharomyces mikatae* (Kellis *et al.*, 2003), from <http://www.genetics.wustl.edu/saccharomycesgenomes/download.html> for *Saccharomyces kudriavzevii* (Cliften *et al.*, 2001), and from <http://www.yeastgenome.org/> for *S. cerevisiae* (*Saccharomyces* Genome Database).

RESULTS

Ethanol-dependent expression of *GDH1* and *ASN1* is determined by the novel Hap2-3-5-Gln3 transcriptional activator

The observation that strains lacking *HAP2* or *HAP3* grow poorly on ammonium as sole nitrogen source uncovered the fact that expression of *GDH1*, encoding one of the two NADP-dependent glutamate dehydrogenase isozymes

involved in ammonium assimilation and glutamate biosynthesis, was Hap2-3-5-dependent when yeast cells were grown on either glucose or non-fermentable carbon sources, although *HAP4*-independent (Dang *et al.*, 1996a). Analysis of the expression of *GDH3*, encoding a paralogous NADP-dependent glutamate dehydrogenase isozyme, showed that as for *GDH1*, the expression of this gene was *HAP2*-dependent and *HAP4*-independent (Avendaño *et al.*, 2005). Characterization of the expression pattern of the *ASN1* and *ASN2* paralogous pair, encoding two isoforms of asparagine synthetase, showed that the Hap-2-3-5 complex determined the expression of *ASN1* in a Hap4-independent manner, while the expression of *ASN2* was HAP-independent (Dang *et al.*, 1996b). Further analysis of the *GDH1* expression profile showed that it was also determined by the action of the *GLN3*-encoded transcriptional activator. *gln3Δ* and *hap2Δ* mutants displayed similar expression levels, suggesting that both regulators could coordinately determine *GDH1* transcription (Riego *et al.*, 2002).

We undertook the analysis of *ASN1*, *GDH1* and *GDH3* expression in order to test the role of Gln3 and the HAP complex in the expression of these three genes. The expression pattern of *GDH1* and *ASN1* in cells grown on glucose or ethanol, under repressive and non-repressive conditions, showed that expression of these genes was Gln3 dependent (Fig. 1a, b). However, the expression pattern did not follow that of a classic NCR response, since transcriptional activation was not completely repressed in the presence of glutamine as nitrogen source on either glucose or ethanol as carbon sources. In addition, in a *ure2Δ* background, expression was similar to that of the wild-type in both repressive and non-repressive conditions, suggesting that restricted transport of Gln3 to the nucleus did not limit *GDH1* and *ASN1* expression (Fig. 1a, b). Since *DAL5* transcriptional activation is determined by the classic control exerted by NCR, we followed its response to the quality of the nitrogen source in the absence of *GLN3* or *URE2*. Fig. 1(a, b) show that as previously reported (Rai

Table 4. Oligonucleotides used for ChIP PCR analysis

Primer	Oligonucleotide sequence (5'–3')	Source or reference
GDH1Fw	TTCTCTAGTGTATCAGGCTCC	This work
GDH1Rv	GCGATAGTACGTATCAAGCAAC	This work
ASN1Fw	GTTCGTGCAACGTGAGCGCA	This work
ASN1Rv	GAGTATGCGATCAGGGTCCA	This work
GDH3Fw	GGCTGCTGACAGATATTCTGC	This work
GDH3Rv	CTGAGTTCGAACGGCTCAAAGTC	This work
DAL5Fw	CAGCGCGCTCCTGCCGCACGC	This work
DAL5Rv	GCGTGCGGCAGGAGCGCGCT	This work
LEU4Fw	CTGACTCCTTGGGTAGAG	Ishida <i>et al.</i> (2006)
LEU4Rv	CTGTACCTTCAATATCGACG	Ishida <i>et al.</i> (2006)
1227	GCGCGCGTCGACCCATAATTC	Ishida <i>et al.</i> (2006)
1508	GCGCGCGGATCCATCACGTTTCGTCGT	Ishida <i>et al.</i> (2006)
HIS4cFw	CCCTAGTTGTGACCAATATG	This work
HIS4cRv	GCATGGAATTTGCGGACG	This work

Fig. 1. *GDH1*, *GDH3* and *ASN1* expression is determined by Gln3 and Hap2 in a Hap4-independent manner. (a, b) Northern analysis was carried out on total RNA samples obtained from wild-type strain CLA-1 and its isogenic *gln3Δ* and *ure2Δ* derivatives, grown on MM with either glucose (a) or ethanol (b) to an OD₆₀₀ of 0.3 on the specified nitrogen sources. (c) Wild-type strain CLA-1 and its *hap2Δ* and *hap4Δ* isogenic derivatives were grown on MM-glucose-glutamine to exponential phase (OD₆₀₀ 0.3), a 50 ml aliquot was collected and total RNA was prepared; the rest of the culture was centrifuged, washed and suspended in MM-ethanol-glutamine, and RNA was prepared from samples collected after 15 or 24 h of incubation. In all panels, filters were sequentially probed with PCR fragments of 650, 750, 950 and 170 bp from *GDH1*, *ASN1*, *DAL5* and *CYC1*, respectively and with an 870 bp *ACT1* DNA fragment, as loading control. *GDH3* was probed on a separate filter using a 760 bp *GDH3* fragment and an 870 bp *ACT1* PCR fragment, as loading control. Representative results from three experiments are shown. Numbers indicate quantitative values for mRNA after normalizing with wild-type actin.

et al., 1987), *DAL5* expression is repressed in glutamine-grown cultures and induced in the presence of proline in glucose- or ethanol-grown cultures or in a *ure2Δ* mutant. As expected, *GDH3* expression was found to be repressed on glucose and induced on ethanol (Avendaño *et al.*, 2005). Under induced conditions (respiratory metabolism) *GDH3* regulation, in contrast to that of *GDH1*, is sensitive to the nitrogen discrimination pathway. Indeed on a repressive nitrogen source *GDH3* expression is induced in a *ure2Δ* genetic background (Fig. 1a, b).

To analyse the role of Hap2 and Hap4 in *GDH1*, *GDH3* and *ASN1* expression, the wild-type strain CLA-1, *hap2Δ* and *hap4Δ* mutants were grown on glucose as sole carbon source. When cultures reached the exponential phase, a sample was taken (G in Fig. 1c) and the rest of the cells were transferred to 2 % ethanol as sole carbon source for 15 or 24 h. In glucose-grown cells, *GDH1* but not *ASN1* expression was HAP2-dependent. However in ethanol-grown cells, *ASN1*, *GDH1* and *GDH3* expression was HAP2-dependent. In a *hap4Δ* strain *GDH1* and *ASN1* expression was maintained on either glucose or ethanol (Fig. 1c, G, E-15 and E-24), and ethanol-induced expression of *GDH3* was observed in a *hap4Δ* genetic

background, indicating that Hap4 was dispensable for transcriptional activation of these genes. Expression of the *CYC1* gene, encoding isoform 1 of cytochrome *c* (Olesen *et al.*, 1987), was monitored as a gene whose transcriptional activation is completely dependent on Hap2-3-5-4. As expected, in ethanol-grown cells *CYC1* expression was abolished in *hap2Δ* and *hap4Δ* mutants.

The above results indicated the existence of a Hap2-3-5-Gln3 transcriptional complex; thus in order to analyse this, co-immunoprecipitation was carried out. As Fig. 2 shows, in an extract prepared from a *HAP2-TAP GLN3-myc¹³* double-tagged strain immunoprecipitated with anti-Myc antibody, the presence of Hap2-TAP was revealed after Western analysis with anti-TAP, indicating that the Hap2-3-5 complex and Gln3 form part of the same transcriptional unit.

Gln3 binding to the *GDH1* promoter is Hap2-dependent

Hap2, Hap4 and Gln3 promoter occupancy was analysed by chromatin immunoprecipitation (ChIP) experiments. Immunoprecipitates were obtained from *Gln3-myc¹³*,

Fig. 2. Hap2 and Gln3 form part of the same transcriptional complex. Whole-cell extracts were prepared from *HAP2-TAP GLN3-myc¹³* cells grown to OD₆₀₀ 0.5 in 100 ml MM-glucose-glutamine and treated as described in Methods. Immunoprecipitation was performed independently with anti-Myc (9E11 Santa Cruz Biotechnology) or anti-TAP (Thermo Scientific) antibodies as indicated by '+' or '-' at the top of the lane. Samples (40 µl) were run on a 10% SDS-PAGE gel and Western blotting analysis was done with anti-TAP antibodies. A 40 µl sample of whole-cell extract (WCE) was used as Hap2-TAP control.

Hap2-myc¹³ and Hap4-myc¹³ epitope-tagged strains with anti-Myc antibody. Functional characterization of these tagged mutants showing that they maintain their regulatory properties is presented in Supplementary Fig. S1, available with the online version of this paper. Amplification of *LEU4* or *HIS4* coding sequences was used as control; amplifications were performed from the same immunoprecipitates. As shown in Fig. 3(a–d), in a wild-type background, Hap2 and Gln3 were recruited to the *GDH1* and *ASN1* promoters in either glucose- or ethanol-grown cells. However, in a *hap2Δ* mutant, Gln3 was not recruited to these promoters in either glucose- or ethanol-grown cells (Fig. 3a–d). Gln3 recruitment to the *UGA3-GLT1* promoter (Ishida *et al.*, 2006) was followed as an additional control. It was found that Gln3 bound this promoter in a *hap2Δ* mutant (Fig. 3a, b), indicating that for the *UGA3-GLT1* promoter Gln3 recruitment was Hap2-independent. In order to confirm that in a *hap2Δ* mutant, the amount of Gln3-Myc13 was equivalent to that found in a *HAP2* strain, Western analysis was performed in extracts obtained from the *HAP2 GLN3-myc¹³* and *hap2Δ GLN3-myc¹³* strains (Supplementary Fig. S2). Conversely, Hap2 recruitment was not Gln3-dependent, since in a *gln3Δ HAP2-myc¹³* strain, the *GDH1* and *ASN1* promoters were amplified (Fig. 3a–d). These results indicate that Gln3-Hap2-dependent recruitment is required for activation under fermentative and respiratory conditions. As expected, Hap4 only bound these promoters when cells were grown on ethanol as carbon source (Fig. 3a–f), meaning that although *GDH1* and *ASN1* expression under respiratory conditions is *HAP4*-independent, Hap2-3-4-5 complexes bind these promoters. Neither Gln3-myc¹³, Hap2-myc¹³ nor Hap4-myc¹³ was recruited to the *GDH1* promoter in glucose-grown cells (Fig. 3e, f), since under

this condition, this promoter shows a closed chromatin configuration, which hampers activator binding (Avendaño *et al.*, 2005). However, in ethanol-grown cultures, the three activators were recruited to the *GDH1* promoter, although Gln3 recruitment was not Hap2-dependent.

CLUSTALW2-aligned promoter sequences of the *GDH1*, *GDH3*, *ASN1* and *ASN2* promoters show that *GDH1* bears four perfectly conserved GATAAG(C) motifs and two HAP-binding sites; *GDH3* harbours two non-conserved HAP sites and a single conserved GATAAG site; *ASN1* has one GATAAG and one HAP conserved site, while *ASN2* has no evident HAP sites and two imperfectly conserved GATTAG motifs (Fig. 4). Multiple alignments of four *Saccharomyces 'sensu stricto'* promoter sequences of *GDH1*, *GDH3*, *ASN1* and *ASN2* are shown in Supplementary Figs S3 and S4.

To analyse whether the GATAAG(C) *cis*-acting elements present in the *GDH1* promoter (Riego *et al.*, 2002) played a role in the Hap2-3-5-Gln3-mediated response, a mutant impaired in the GATAAG(C) (3, 4 and 5) sites was constructed and *GDH1* transcriptional activation and Gln3 promoter occupancy were analysed.

Conserved GATAAG(C) sites present in the *GDH1* promoter are indispensable for Hap2-3-5-Gln3-dependent transcriptional activation

Previous work from our laboratory indicated that four GATAAG(C) *cis*-acting elements present in the *GDH1* promoter (Fig. 4) could play a role in Gln3-dependent transcriptional activation from this region (Riego *et al.*, 2002). A mutant was constructed in which three conserved *GDH1* GATAAG(C) sequences were simultaneously substituted for GcaAAG(C). The wild-type, the *gln3Δ* mutant and the *cis*-GcaAAG(C) triple mutant strains were grown on media containing glucose or ethanol as carbon source and proline as nitrogen source. When cultures reached exponential phase, a sample was taken from each culture (G in Fig. 5a); the rest of the culture was centrifuged and suspended in ethanol-proline medium, and a sample was taken after 24 h incubation. As shown in Fig. 5(a), *GDH1* expression was abolished in both *gln3Δ* and promoter mutants, in glucose or ethanol cultures, while that of *DAL5* was only hindered in the *gln3Δ* mutant, indicating that the GATAAG(C) (3, 4 and 5) sites of the *GDH1* promoter were indispensable to sustain *GDH1* expression on both carbon sources, and as expected did not affect *DAL5* expression. ChIP experiments carried out on the *GLN3-myc¹³* strain harbouring the GATAAG(C) *cis* mutations showed that Gln3-Myc did not bind the *GDH1* promoter, while it was able to bind the *DAL5* promoter (Fig. 5b). This indicates that the GATAAG(C) (3, 4 and 5) sites of the *GDH1* promoter were indispensable to sustain *GDH1* expression in either glucose- or ethanol-grown cultures, and as expected did not affect *DAL5* expression, indicating that Hap2-3-5-

Fig. 3. Gln3 binding to the *GDH1* and *ASN1* promoters is Hap2-dependent. ChIP assays were performed using anti-Myc antibody (9E 11, Santa Cruz Biotechnology) on wild-type strains containing *myc*¹³ epitope-tagged *GLN3*, *HAP2* or *HAP4*. As a control, equivalent samples were treated with anti-HA antibody. Strains were grown on MM-glutamine with 2 % (w/v) glucose to mid-exponential phase, the culture was divided and half of it was used to perform ChIP assays (a, c, e); the rest of the culture was centrifuged, washed, suspended in MM-glutamine with 2 % (v/v) ethanol and incubated for 24 h, after which cells were centrifuged, collected and used to prepare samples for ChIP (b, d, f). PCR was performed with the oligonucleotides described in Table 4. Results are representative of three independent experiments.

Gln3-dependent *GDH1* transcriptional activation relied on Gln3 binding to this promoter's cognate sites.

DISCUSSION

Adaptation to new environments and nutrient utilization result in intricate changes in the physiology of cells, allowing them to turn on starvation response pathways involved in the transport, biosynthesis and catabolism of nutrients. Although all organisms possess a vast array of DNA-binding proteins which play crucial roles in the specificity of these transcriptional responses, the possibility of enlarging the repertoire of activators through the combined action of DNA-binding and activation domains could result in novel and more complex responses.

Gln3 plays a role as HAP-complex co-activator

Our results show the occurrence of heterodox HAP complexes in which the activation role of Hap4 is delegated to a non-conventional partner, which does not exactly mimic the Hap4 role, since Gln3 bears a DNA-binding domain, while Hap4 does not. As shown in this paper, under repressive nitrogen conditions, ethanol-dependent

expression of *ASN1* and glucose- and ethanol-dependent expression of *GDH1* rely on a heterodox HAP complex in which Hap4's activator role is delegated to Gln3, unveiling a new role for this well-known *GLN3*-encoded transcriptional activator. The role of Gln3 as transcriptional activator is determined by its nuclear localization, which occurs under non-repressive nitrogen conditions, upon rapamycin treatment or in a *ure2Δ* mutant background (Beck & Hall, 1999; Cardenas *et al.*, 1999). Under repressive conditions, the nuclear pool of Gln3 is small and thus its role in transcriptional activation is hindered. Results presented in this paper show that under these conditions Gln3 can drive expression in a Hap-dependent fashion. This suggests that Gln3 can borrow the DNA-binding domain of a regulator whose nuclear localization is not limited under repressive nitrogen conditions, and which recruits Gln3 to the relevant promoters. Under these conditions, the nuclear pool of Gln3 is not a limiting factor, since a *ure2Δ* mutant does not enhance either *GDH1* or *ASN1* expression in the presence of glutamine (Fig. 1). Once recruited to the *GDH1* promoter, Gln3 binds to its cognate binding sites (UAS_{NTR} GATAAG), probably contributing to the stabilization of the complex, displacing HAP-Hap4 complexes, and constituting a novel transcriptional complex. As mentioned earlier, under respiratory

Fig. 4. The *GDH1* and *ASN1* promoters have conserved Gln3- and Hap2-3-5-binding sites. Full promoters are depicted as rectangles with *cis*-acting binding sites for Gln3 (GATA) (arrowheads) and the HAP complex (bars). Conserved binding sites are shaded in black while non-conserved sites are depicted as white symbols, according to the multiple alignment of four yeast species performed for each promoter region (Supplementary Figs S2 and S3). Binding sites are numbered starting from the most 5' and the number is placed at the 3' end of each site.

conditions, Hap4 was able to bind the *GDH1* and *ASN1* promoters; since expression of these genes is not affected in a *hap4Δ* mutant it can be concluded that formation of Hap2-3-5-4 complexes does not hamper Hap2-3-5-Gln3-driven expression. It is possible that Hap4 could bind to the HAP complex at a different site from that occupied by Gln3.

HAP-Gln3-dependent *GDH1* and *ASN1* expression ensures enzyme provision in the presence of glucose and ethanol even under repressive nitrogen conditions. These results support previous observations and suggest that in addition to its classic role in the NCR, Gln3 determines expression of genes pertaining to the ammonium assimilatory pathways such as *GDH1* and *GLT1*, whose expression does not

Fig. 5. A *GDH1* promoter mutant of the GATAAG(C) 3, 4 and 5 conserved sites shows impaired *GDH1* expression and Gln3 promoter binding. (a) Northern analysis was carried out on total RNA samples obtained from wild-type strain CLA-1 and its isogenic *gln3Δ* and *cis-GcaAAG*(C) mutants. Strains were grown on MM-glucose-proline to exponential phase (OD₆₀₀ 0.3), a 50 ml aliquot was collected and total RNA was prepared; the rest of the culture was centrifuged, washed and suspended in MM-ethanol-proline, grown for 24 h, collected and used to prepare total RNA. Filters were sequentially probed with 650 and 950 bp PCR fragments of *GDH1* and *DAL5* respectively, and an 870 bp *ACT1* DNA fragment, as loading control. (b) Samples for ChIP were simultaneously collected. ChIP was performed as described in Fig. 3.

respond to the quality of the nitrogen source (Riego *et al.*, 2002; Valenzuela *et al.*, 1998) and is highest under repressive nitrogen conditions. It can thus be concluded that a low nuclear concentration of Gln3 can be bypassed by recruiting Gln3 to the pertinent promoters by an additional regulator. This does not exclude the possibility of Gln3-assisted recruitment even under non-repressive conditions. In this regard, Gln3-assisted promoter binding has been observed in nitrogen non-repressive conditions, since Gat1 is required to promote Gln3 binding to the *GAP1* promoter in proline-grown cultures (Georis *et al.*, 2009), suggesting that even under conditions favouring Gln3 nuclear localization, its promoter-binding capacity is fostered by additional activators. In addition, the auxiliary modulator must have binding sites on the promoter, probably arranged in a precise combinatorial fashion with the cognate Gln3-binding GATAAG elements. In this regard, it has been shown that the HAP⁽¹⁾ and HAP⁽³⁾ sites present in the *GDH1* promoter (Fig. 4) are needed to drive *GDH1*-HAP-dependent expression (Dang *et al.*, 1996a). Thus for this promoter, action of the Hap2-3-5-Gln3 regulator could involve the HAP⁽¹⁾/HAP⁽³⁾ sites and a single or double combination of the three conserved GATAAG(C) sites (Fig. 4). The *ASN1* promoter has a GATA and a HAP element separated by 10–17 bp, which are conserved in five *Saccharomyces* ‘*sensu stricto*’ species, and which could constitute a composite binding site required for HAP-assisted Gln3 promoter binding. In contrast to *GDH1* expression, *GDH3* is regulated by Gln3 in response to the quality of the nitrogen source (Avendaño *et al.*, 2005); thus for this gene, Gln3 plays its

classic NCR role. Although *GDH3*-ethanol-induced expression is dependent upon a hybrid activator composed of a non-conventional HAP complex, results presented in this paper indicate that for the *GDH3* promoter, the Hap2-3-5 complex does not determine Gln3 binding, suggesting that Hap4’s activating function has been delegated to a hitherto unidentified co-activator. The *GDH3* promoter does not show the GATA-HAP *cis*-element arrangement observed in *GDH1* and *ASN1* promoters (Fig. 4). With regard to *GLT1* transcriptional regulation, results presented in this paper show that Gln3 recruitment to this promoter is Hap2-independent, indicating that although this gene is highly expressed in the presence of glutamine (Valenzuela *et al.*, 1998), as for *GDH3*, an alternative regulator could participate in Gln3 recruitment to this promoter. No analysis has been performed to determine whether *GLT1* expression is HAP-dependent.

Our results indicate that Hap4 could be functionally substituted by alternative activation domains that could determine the role of the HAP complex as a transcriptional activator responding to metabolic signals, not directly involved in respiratory metabolism. In fact, the orthologous HAP complex in mammals (CBF) has a wide range of target genes (Maity & de Crombrughe, 1998). In fungi the HAP complex has been implicated in regulation of genes involved in the catabolism of carbon and nitrogen sources and secondary metabolism (Litzka *et al.*, 1996). It is thus possible that different activator subunits can be associated with the core complex (‘domain borrowing’), providing specificity for particular sets of genes.

Hap2-3-5-Gln3 interaction determines a novel transcriptional response

Gln3 is considered as the key regulator of the NCR network. However, a number of examples have been found showing that as for *GDH1* and *ASN1* expression, Gln3 participates in transcriptional activation, showing profiles that do not fit the classic NCR (Riego *et al.*, 2002; Valenzuela *et al.*, 1998). Most striking was the finding that the response of *ENA1* to salt stress was Gln3-dependent (Crespo *et al.*, 2001). These examples underscore the possibility that Gln3 plays additional roles in the NCR response, when combined with other regulators. Since *ASN1* and *GDH1* expression is not completely abolished in *gln3Δ* mutants, it is possible that residual expression could be Gat1 dependent and that in a *gln3Δ* genetic background Gat1 could also be recruited to the *GDH1* and *ASN1* promoters in a HAP-dependent manner.

The results presented in this work open the possibility that hybrid transcriptional activators could be built up by borrowing domains from non-conventional partners. This could overcome restrictions such as limited nuclear localization of transcriptional modulators or binding specificity, giving rise to novel regulators triggering specific and refined transcriptional responses, to subtle or combined variations of the environment. Further research is needed in order to identify the complete network of genes whose expression is determined through the combined action of Hap2-3-5-Gln3. Additionally, the identification of interactors determining Gln3 recruitment to promoters such as *GDH3* and *GLT1* could contribute to the understanding of the NCR-independent role played by Gln3.

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