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The WD protein Cpc2p is required for repression of Gcn4 protein activity in yeast in the absence of amino-acid starvation

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Summary

The *CPC2* gene of the budding yeast *Saccharomyces cerevisiae* encodes a Gβ-like WD protein which is involved in regulating the activity of the general control activator Gcn4p. The *CPC2* gene encodes a premRNA which is spliced and constitutively expressed in the presence or absence of amino acids. Loss of *CPC2* gene function suppresses a deletion of the *GCN2* gene encoding the general control sensor kinase, but not a deletion in the *GCN4* gene. The resulting phenotype has resistance against amino-acid analogues. The *Neurospora crassa cpc-2* and the rat *RACK1* genes are homologues of *CPC2* that complement the yeast *cpc2* deletion. The *cpc2Δ* mutation leads to increased transcription of Gcn4p-dependent genes under non-starvation conditions without increasing *GCN4* expression or the DNA binding activity of Gcn4p. Cpc2p-mediated transcriptional repression requires the Gcn4p transcriptional activator and a Gcn4p recognition element in the target promoter. Frameshift mutations resulting in a shortened Gβ-like protein cause a different phenotype that has sensitivity against amino-acid analogues similar to a *gcn2* deletion. Cpc2p seems to be part of an additional control of Gcn4p activity, independent of its translational regulation.

Introduction

Starvation of a single amino acid results in increased transcription of genes encoding enzymes for the synthesis of protein precursors in fungi. Gene products include enzymes of different pathways involved in amino-acid biosynthesis (Hinnebusch, 1988; Hinnebusch 1992), in purine biosynthesis (Mösch *et al*., 1991) and in aminoacyl tRNA synthesis (Meussdoerffer and Fink, 1983; Mirande and Waller, 1988). In the yeast *Saccharomyces cerevisiae*, this regulatory network is known as general control of amino-acid biosynthesis (for a model see Hinnebusch, 1997); in filamentous fungi it is often named cross-pathway control (cpc; Sachs, 1996). Amino-acid starvation results in increased synthesis of the transcriptional activator Gcn4p that binds to specific DNA sequences called Gcn4-protein responsive elements (GCRE), which are present upstream of more than 50 target genes of this regulatory network. Expression of the *GCN4* gene is regulated at the level of translation initiation (Hinnebusch, 1994). Four short upstream open reading frames (uORFs), present in the leader sequence of the *GCN4* mRNA upstream of the coding region, prevent efficient translation initiation at the *GCN4* start codon under non-starvation conditions. In the presence of amino acids, ribosomes that have initiated translation at the *GCN4* uORFs dissociate from the mRNA before they are able to reinitiate at the *GCN4* open reading frame. When yeast cells are starved of amino acids, ribosomes that have translated the first uORF are able to reinitiate further downstream at the *GCN4* coding sequences. This regulation depends on the phosphorylation of the translation initiation factor 2α (eIF-2α) by the protein kinase Gcn2p (Dever *et al*., 1992). This protein is the sensor for the degree of uncharged tRNAs in the cell and it depends on the availability of amino acids. Gcn2p consists of an N-terminal protein kinase domain and a C-terminal tRNA synthetase domain (Roussou *et al*., 1988; Wek *et al*., 1989). In cells starved of amino acids, uncharged tRNA molecules bind to the tRNA synthetase domain of Gcn2p, resulting in activation of the neighbouring kinase domain which phosphorylates the α-subunit of eIF-2 (Dever *et al*., 1992; Wek *et al*., 1995). Phosphorylation of eIF-2α inhibits the complex exchange factor eIF-2B, which catalyses the exchange of bound GDP for GTP on eIF-2 after each round of initiation. Because only the GTP-bound form of eIF-2 can deliver initiator $tRNA^{Met}$ to the ribosome, impairing the activity of eIF-2B leads to a reduction in the amount of active eIF-2 that is available for translation initiation

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(Merrick, 1992; Voorma *et al*., 1994). This reduction in the level of active eIF-2 is responsible for allowing ribosomes to scan past the remaining uORFs in the *GCN4* leader without rebinding charged initiator $tRNA^{Met}$. Therefore, reinitiation at these sites is suppressed, and this allows utilization of the *GCN4* start codon instead. At least 12 additional genes are involved in the complete transmission of the amino-acid starvation signal from Gcn2p to *GCN4* translation regulation. They show either a *GCN* (*g*eneral *c*ontrol *n*on-derepressed) or a *GCD* (*g*eneral *c*ontrol *d*erepressed) phenotype (Niederberger *et al*., 1983, 1986). A strain with a deleted *GCN2* gene is unable to initiate the *GCN4* translational regulation. *gcd* mutations are genetically epistatic to a *gcn2* defect, leading to constitutively derepressed translation of *GCN4* mRNA, whereas a deletion of *GCN4* is epistatic to a *gcd* mutation, suggesting a linear signal transduction pathway (Hinnebusch, 1992).

In addition to the translational control of *GCN4* expression, the Gcn4 protein level can be modulated by increasing its half-life under specific amino-acid starvation conditions in auxotrophic mutants (Kornitzer *et al*., 1994). The details of this mechanism remain to be uncovered.

Here, we present evidence that a third regulatory pathway negatively affects gene expression of the Gcn4p-regulated network. Part of this pathway is a Gβ-like protein encoded by the yeast *CPC2* gene, which is also described as *ASC1*, with a deduced involvement of the encoded protein at the level of translation initiation (Chantrel *et al*., 1998). The yeast *CPC2* gene is a homologue of the *cpc-2* gene of the filamentous fungus *Neurospora crassa* and of the human *RACK1* encoding a receptor of activated protein kinase C (Ron *et al*., 1994). A mutation in *cpc-2* has been described previously as affecting the *c*ross-*p*athway *c*ontrol in an unknown manner, resulting in sensitivity of the mutant strain to amino-acid starvation conditions (Krüger *et al*., 1990; Müller *et al*., 1995).

Gβ-like proteins represent a eukaryotic subgroup of WD proteins that are characterized by a conserved repeat structure with a length of ≈36–46 amino acids, generally ending with the amino acids tryptophan (W) and aspartate (D) (Simon *et al*., 1991; Neer *et al*., 1994). Some WD proteins are known to repress transcription of a variety of genes. Well-characterized proteins, such as Tup1p of *S. cerevisiae* and the groucho protein of *Drosophila*, do not directly bind to DNA, but function by interacting with DNA-binding proteins (Keleher *et al*., 1992; Paroush *et al*., 1994).

We show here that a *cpc2Δ* mutation in yeast suppresses a *gcn2Δ* mutation, but not a *gcn4* deletion mutation. Suppression is a result of an increased transcription of Gcn4p-regulated genes. Unlike *gcd* mutations, however, *cpc2Δ* does not lead to increased levels of Gcn4 protein, and therefore *CPC2* does not affect the Gcn2p-mediated translational control of *GCN4* expression. In addition, *CPC2* does not appear to affect the stability of Gcn4p or its ability to bind to its target DNA. It appears instead that *CPC2* negatively regulates the transcriptional activation function of Gcn4p.

Results

ORF YMR116C of Saccharomyces cerevisiae *encodes a Gβ-like protein with high similarity to the* Neurospora crassa cpc-2 *gene*

The *Neurospora crassa cpc-2* gene encodes a Gβ-like protein of unknown function that is involved in the regulation of amino-acid biosynthetic genes. In *S. cerevisiae*, mutation in a *cpc-2*-like gene involved in the regulation of amino-acid biosynthesis has never been isolated in numerous genetic screens for general control mutations (Hinnebusch, 1988, 1992). Therefore, we chose to design degenerate primers deduced from highly conserved amino-acid sequences specific for different Gβ-like proteins to test whether such a gene is present in *Saccharomyces cerevisiae* (Fig. 1). Using the polymerase chain reaction (PCR), we were able to amplify a 762 bp fragment and a nested 186 bp DNA fragment respectively. Southern analysis with total yeast DNA revealed the presence of only one copy of the 762 bp fragment in the genome of *S. cerevisiae*. The PCR product hybridized against chromosome XIII of yeast chromosomes separated by pulsed-field electrophoresis (data not shown). Hybridization against an ordered λ-based library of *S. cerevisiae* located the PCR amplified sequence on the right arm of this chromosome close to the genes *ADE17* and *ILV2*. The entire gene was isolated and the DNA sequence of 1564 bp was determined. An open reading frame of 957 bp could be identified that corresponded to ORF YMR116C of the yeast genome project (Bowman *et al*., 1997). Both sequences were identical in location and sequence, except for the two nucleotides TG instead of AT in our sequence at positions 390 and 391 relative to the first base of the start codon AUG. This resulted in an amino-acid substitution from cysteine to threonine at position 131 in our deduced protein sequence. No evidence was observed for any redundancy of the ORF in the genome sequence. Because ORF YMR116C had not been assigned a function, we renamed it *CPC2*.

The *CPC2* open reading frame encodes a putative Gβ-like protein of 319 amino acids exclusively built up of seven WD repeats, and with a calculated molecular weight of 34.8 kDa. The codons of the *CPC2* open reading frame correspond preferentially to the most frequently used yeast codons, suggesting that this gene is highly expressed in *S. cerevisiae* (Sharp *et al*., 1986). This was confirmed by experiments which characterized this open reading frame as one of the most highly transcribed uncharacterized yeast genes (Velculescu *et al*., 1997). The protein shows 73% similarity and 57% identity to the *N. crassa cpc-2*

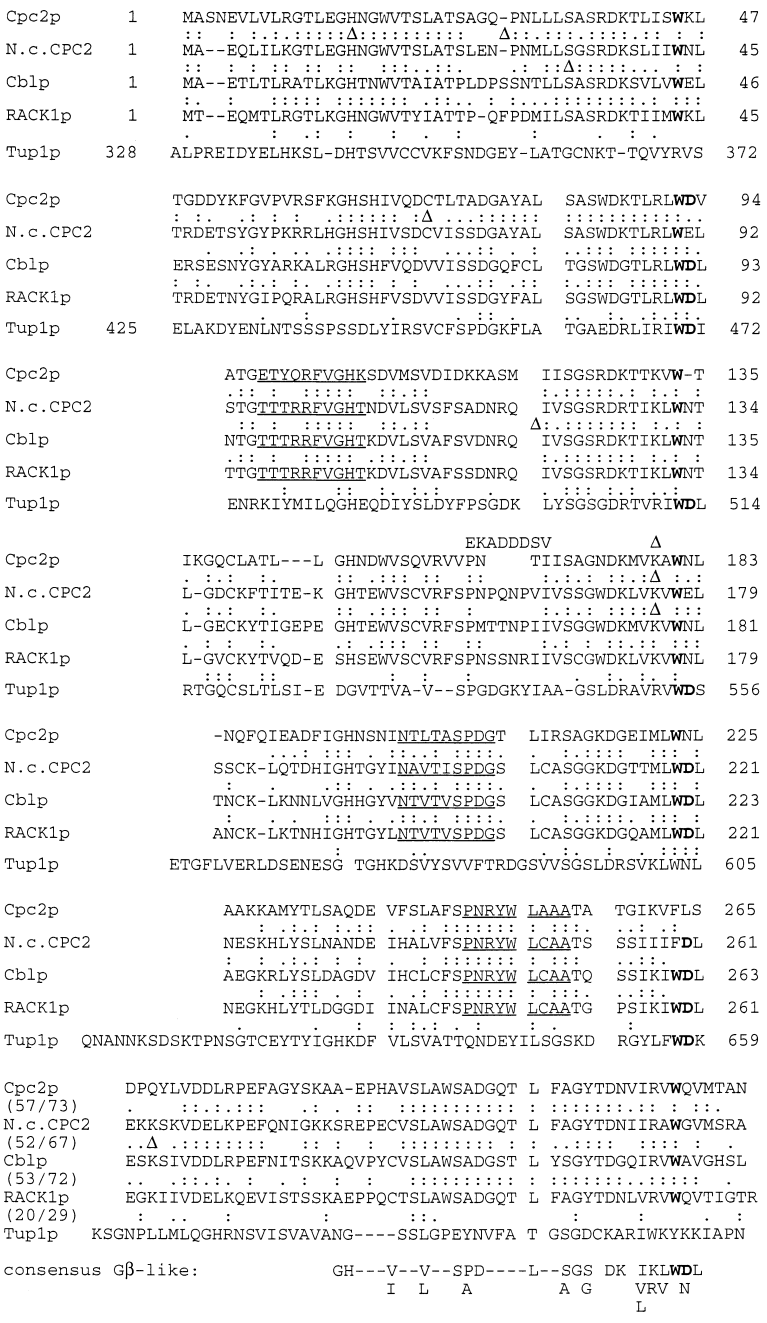

Fig. 1. Amino-acid sequence comparison between yeast Cpc2p, Gβ-like proteins from other organisms and Tup1p. The deduced amino-acid sequence of *Saccharomyces cerevisiae* Cpc2p was compared with other Gβ-like proteins, including the *N. crassa*-CPC2 protein (Müller *et al*., 1995), Cblp of *Chlamydomonas* (Schloss, 1990), RACK1p of rat, which is identical to the human and mouse homologue (Guillemot *et al*., 1989; Imai *et al*., 1994; Ron *et al*., 1994), and Tup1p as examples of other unrelated WD proteins (Keleher *et al*., 1992). The protein alignment was performed with the PROSIS program (Hitachi). Each line represents one of seven repeat units with identical and similar (von Weizsäcker *et al*., 1992) amino-acid residues between yeast Cpc2p and each other WD protein, indicated by double and single dots respectively. Amino acids tryptophan (W) and aspartate (D) are given in bold letters. Underlined letters indicate highly conserved amino-acid sequences of Gβ-like proteins used for construction of degenerate primers for the isolation of a 762 bp or 186 bp DNA fragment of the *CPC2* gene. Deduced intron positions of known genomic sequences are marked by Δ above the amino-acid residues. Numbers in parentheses indicate the overall amino-acid identity and similarity in percentage between each protein and the yeast Cpc2p. A consensus sequence for the Gβ-like proteins is derived. Only those positions are included in the consensus in which identical or equivalent amino acids occur 14 times or more out of 28 possibilities.

encoded protein. Within the deduced Cpc2p sequence, 50–55% of the amino-acid residues are also identical, compared with various Gβ-like proteins from other organisms (Fig. 1). The amino-acid identity of other WD-repeat proteins as true β-subunits of heterotrimeric G-proteins or Tup1p of yeast is in the range of 20–25%.

The spliced CPC2 *mRNA is constitutively expressed under amino-acid starvation conditions*

Transcript levels of the 1.35 kb *CPC2* mRNA were tested by Northern hybridization (Fig. 2A), and were not affected by amino-acid starvation induced by the presence of amino-acid analogues. In addition, the absence of functional *GCN2*, coding for the general control sensor kinase, or functional *GCN4*, encoding the transcriptional activator of general control, had no effect on *CPC2* transcription.

The open reading frame of *CPC2* is interrupted between the corresponding amino-acid residues 179 and 180 by a sequence of 273 nucleotides that shows the 5′-GTAT-GT–TACTAAC–CAG-3′ consensus sequence for introns in yeast (Bruchez *et al*., 1993). This intron lies unusually close to the 3′-end compared with the location of other introns in yeast and has recently been characterized as

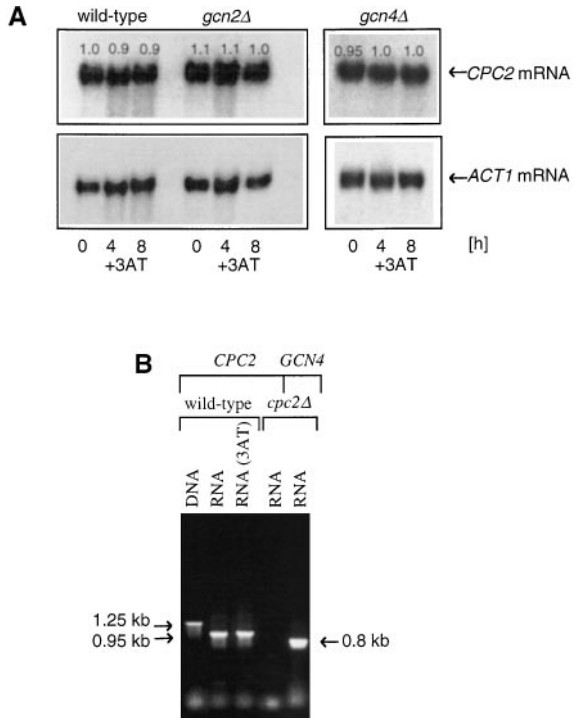

Fig. 2. Analysis of the yeast *CPC2* mRNA.
A. Northern hybridization. Wild-type, *gcn2Δ* and *gcn4Δ* yeast strains were cultured to early exponential growth at 30°C on SD medium. At time 0, the amino-acid analogue 3-aminotriazole (3AT) was added to 10 mM to induce histidine starvation. Cells were harvested for RNA isolation at the indicated times after 0, 4 and 8 h and probed against the radioactively labelled first exon of *CPC2*. RNA amounts per lane were equalized by phosphoimaging using the *Cla*I fragment of *ACT1* as control probe. Numbers above the *CPC2* transcripts refer to relative intensities in comparison with *ACT1* mRNA. 3AT effect was tested by hybridization with a *HIS3*-specific DNA as probe (data not shown).
B. Characterization of splicing of *CPC2* mRNA by analysis of reverse transcriptase-PCR on agarose gels. For reverse transcriptase -PCR experiments, DNA and RNA were isolated from wild-type H1515 and *cpc2Δ* mutant strain H1515-A7 after growth on SD medium for 8 h under non-starvation and amino-acid starvation conditions induced by supplementation with the amino-acid analogue 3-aminotriazole (3AT) to a final concentration of 10 mM. As control for the specificity of the *cpc2Δ* cDNA, the *GCN4* mRNA was reverse transcribed and amplified. Reverse transcription was performed with *CPC2* and *GCN4* specific primers The cDNAs corresponding to fragments of *CPC2* premRNA (1.25 kb), *CPC2* mRNA (0.95 kb) and *GCN4* mRNA (0.8 kb) are indicated.

containing the U24 small nucleolar RNA (snoRNA) coding region (Qu *et al*., 1995), which is required for site-specific 2′-*o*-methylation of 25S rRNA. The last intron of the *cpc-2* gene of *Neurospora crassa* (Müller *et al*., 1995) and the third intron of the *Cblp* gene of *Chlamydomonas* (Schloss, 1990) are located at the same position as in yeast (Fig. 1). Excision of this intron and maturation of the *CPC2* mRNA was verified by reverse transcriptase PCR experiments (Fig. 2B). PCR products with total yeast DNA as template were ≈270 bp larger than PCR products of cDNA from cells cultivated under either amino-acid starvation or non-starvation conditions, suggesting that the *CPC2* precursor mRNA is mainly spliced to result in an mRNA for Cpc2p.

Deletion of yeast CPC2 *suppresses the growth defect of a* gcn2 *mutant strain under amino-acid starvation conditions*

Haploid and diploid yeast strains carrying a full deletion of the *CPC2* open reading frame in the genome were constructed. All resulting *cpc2Δ* mutant strains showed an increased cell size and exhibited a 25% reduction in their growth rate compared with wild-type strains (Table 1). Cultivation on standard minimal media containing either glucose or galactose as single carbon sources at all temperatures tested or on arginine, proline or acrylamide as nitrogen sources showed the same results (data not shown). The same growth phenotype was also observed when cells were cultivated in the presence of the histidine analogue 3-aminotriazole (3AT), which induces the general control system by histidine starvation (Klopotowski and Wiater, 1965) (Table 1).

The *GCN2* encoded protein kinase is the sensor of the signal 'amino-acid starvation', and is necessary for efficient expression of the transcriptional activator Gcn4p under amino-acid starvation conditions to activate more than 50

Table 1. Growth rates of various haploid, isogenic *S. cerevisiae* strains under different growth conditions.

Relevant genotype	YPD	SD	SD + 10 mM 3AT
GCN2, GCN4, CPC2	0.40	0.30	0.18
cpc2Δ	0.29	0.21	0.13
$cpc2\Delta^{1-156}$	0.28	0.22	0.13
gcn2Δ	0.32	0.25	0.04
gcn2Δcpc2Δ	0.39	0.30	0.13
$gcn2\Delta cpc2\Delta^{1-156}$	0.38	0.31	0.14
gcn4Δ	0.38	0.30	0.03
gcn4Δcpc2Δ	0.26	0.20	0.03
cpc2Δ [*CPC2*]	0.39	0.30	0.17
gcn2Δcpc2Δ [*CPC2*]	0.32	0.24	0.04
gcn2Δcpc2Δ [*N. c. cpc-2*][a]	0.35	0.23	0.05
gcn4Δcpc2Δ [*N. c. cpc-2*][a]	0.36	0.28	0.03
gcn2Δcpc2Δ [*RACK1*][a]	0.33	0.25	0.04
gcn4Δcpc2Δ [*RACK1*][a]	0.37	0.29	0.03

Mutants and double mutants were made as described in *Experimental procedures*. All strains were cultivated in liquid, shaking cultures for several times at 30°C over at least 12 h up to an OD_{546} of maximal 2. Growth rates are given in μ, which is defined as $\ln x_1 - \ln x_0 / \Delta t$. The *x*-values characterize the cell density and Δt defines the time between two measurements. Growth rate deviations did not exceed 10%. Identical growth rates were obtained by measuring increasing cell numbers. The plasmid-bearing strains [*CPC2*] contain the yeast *CPC2* gene on the low-copy number plasmid pRS316.
a.The *N. crassa cpc-2* and rat *RACK1* genes ([a]) are driven by the yeast *CPC2* promoter and terminator sequences on the same low-copy number plasmid respectively.

genes in various amino-acid biosynthetic pathways (Hinnebusch, 1988). Mutations in *GCN2* or *GCN4* result in yeast strains unable to induce the general control system, and therefore unable to grow in the presence of amino-acid analogues. Whereas a *gcn2Δ* mutant strain is sensitive for growth on medium containing 3AT or 5-methyltryptophan (5MT), we found that under the same conditions a *gcn2Δcpc2Δ* double-mutant strain grows with a similar rate to a *cpc2Δ* single-mutant strain (Table 1). Thus, a *cpc2Δ* mutation affects the general control system by suppressing a *gcn2Δ* mutation and is genetically epistatic to *gcn2Δ*. The resistance to amino-acid analogues of a *gcn2Δcpc2Δ* double-mutant strain could again be suppressed by introducing the yeast *CPC2* gene on a low copy plasmid. Expression of mutant allele $CPC2\Delta^{1-156}$, lacking the C-terminal three repeats, resulted in the same growth reduction and epistatic effect to a *gcn2* mutation as found for a full deletion of *CPC2* (Table 1).

We also tested whether the *CPC2* gene from *S. cerevisiae*, the *cpc-2* gene of *N. crassa* and the *RACK1* gene of rat are functionally interchangeable. The *cpc-2* and *RACK1* ORFs were expressed under the control of the *S. cerevisiae CPC2* promoter. Both constructs restored the *gcn2Δ* growth phenotype in a *gcn2Δcpc2Δ* yeast mutant strain (Table 1). In addition, the *Neurospora crassa cpc-2* gene product and the *RACK1* protein were able to complement all growth phenotypes caused by the *cpc2Δ* deletion mutation in all genetic backgrounds tested in the same manner as the yeast *CPC2* wild-type gene. Therefore, the genes *CPC2* of *S. cerevisiae, cpc-2* of *N. crassa* and the *RACK1* of rat are not only similar in sequence but also can functionally substitute for *CPC2* in the yeast general control system. Additionally, complementation of *cpc2* mutational phenotypes by cDNA clones of *N. crassa* and rat demonstrated that the defects are caused by the loss of a functional Cpc2 protein and not by the deletion of the snoRNA U24 encoded in the *CPC2* intron (Table 1).

A *cpc2Δgcn4Δ* double-mutant strain was constructed to analyse the position of *CPC2* in the genetic hierarchy of the general control system. A *cpc2* deletion was unable to suppress the growth defect exhibited by a *gcn4* deletion under amino-acid starvation conditions. Expression of *cpc-2* and *RACK1* in a *gcn4* mutant background did not prevent the *gcn4* growth phenotype either (Table 1). Therefore, *CPC2* can be genetically placed between *GCN2* and *GCN4*, or in parallel to *GCN2*, in the general control pathway. Identical results were observed with the $CPC2\Delta^{1-156}$ mutant allele (Table 1).

Expression of CPC2 *negatively affects transcription of the Gcn4p-regulated* HIS3 *gene*

The influence of *CPC2* on the expression of genes that are known to be regulated by the general control system was tested because a deletion of *CPC2* suppresses the growth defect in a *gcn2Δ* mutant strain under amino-acid limitation. Therefore, *HIS3* expression was measured in wild-type, *gcn2Δ*, *cpc2Δ*, *gcn4Δ*, *gcn2Δcpc2Δ* or *gcn4Δcpc2Δ* mutant strains grown in the presence and absence of amino-acid analogues (Fig. 3). The *HIS3* gene encodes the imidazole glycerol phosphate dehydratase [EC 4.2.1.19], and is required for histidine biosynthesis. The gene is regulated by the general control of amino-acid biosynthesis, and contains multiple Gcn4p-responsive elements in its 5′ non-coding region (Struhl and Hill, 1987). Induction of the general control system under amino-acid limitation resulted in an approximately fivefold increase of *HIS3* mRNA transcription shown by Northern analysis and quantification of β-galactosidase activities of a *HIS3–lacZ* fusion protein in wild-type cells (Fig. 3A,B). The induction of *HIS3* gene expression is impaired in a *gcn2Δ* or *gcn4Δ* mutant strain under amino-acid starvation conditions. However, an additional deletion of the *CPC2* gene in the *cpc2Δ* and *gcn2Δcpc2Δ* mutant strains resulted in a 2.5-fold increase of *HIS3* expression under non-starvation conditions compared with the wild-type. *HIS3* expression under amino-acid starvation conditions was increased threefold in a *gcn2Δcpc2Δ* double-mutant strain compared with *gcn2Δ* under the same conditions, and increases in the *cpc2Δ* single mutant to levels similar to the wild-type. In accordance with our genetic data, the increased expression of *HIS3* in a *cpc2Δ* mutant background under non-starvation conditions was found to be independent of an intact *GCN2* gene (Fig. 3A,B), whereas the functional transcriptional activator Gcn4p was required (Fig. 3B).

The same effect on expression of additional genes regulated by Gcn4p, such as the histidine biosynthetic gene *HIS7* or the aromatic amino-acid biosynthetic gene *ARO4*, was observed. Transcription of these two genes is also negatively affected by the presence of the *CPC2* gene, and this effect is independent of the presence of *GCN2* but requires a functional *GCN4* gene (data not shown).

To test whether suppression of the growth defect of a *gcn2Δ* mutant strain by a mutation in *CPC2* is due to an increase in transcription of the *GCN4* gene, we analysed *GCN4* mRNA levels in the presence and absence of *CPC2* under different growth conditions. No difference in *GCN4* mRNA levels was found in a *cpc2Δ* mutant compared with a wild-type strain (Fig. 3A). The specificity of *CPC2* on transcription of *GCN4*-regulated genes was tested by measuring the enzyme activity of isocitrate dehydrogenase [EC 1.1.1.41], which is not regulated by Gcn4p (Hinnebusch, 1992). As seen in Fig. 3, neither the basal activity on glucose medium nor the approximately fourfold increased isocitrate dehydrogenase activity on acetate medium was affected by the presence or absence of a functional *CPC2* gene.

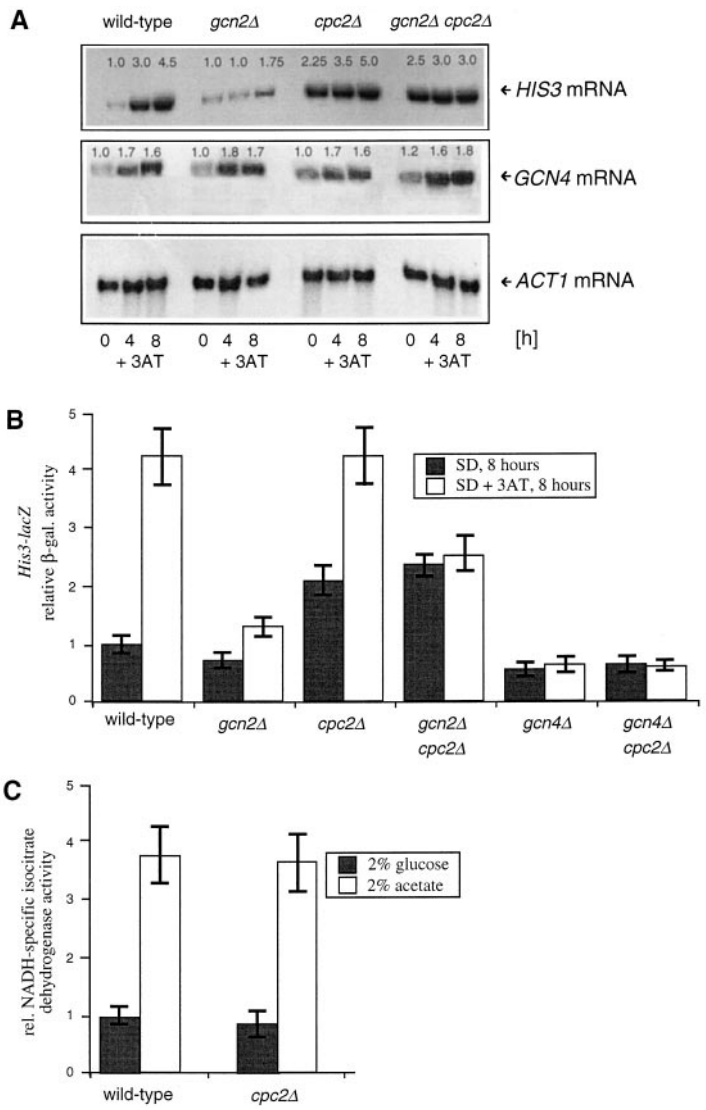

Fig. 3. Influence of the expression of *CPC2* on the transcription and protein activity of general control regulated and unregulated genes.
A. Northern hybridization. mRNA from wild-type, *gcn2Δ*, *cpc2Δ* and *gcn2Δcpc2Δ* strain grown on SD medium or SD medium containing 10 mM 3AT to induce amino-acid limitation were isolated after 0, 4 and 8 h of amino-acid starvation, and probed with a 500 bp fragment of *HIS3* or a 544 bp fragment containing the C-terminus of *GCN4* amplified by PCR. The *ACT1* transcript was used as control for RNA quantification. Numbers above each lane represent the ratios of *HIS3* and *GCN4* mRNA in comparison with *ACT1* mRNA.
B. *HIS3–lacZ*-derived β-galactosidase activities. A *HIS3–lacZ* integrative construct was transformed into various yeast strains with the genotypes indicated. All strains were grown exponentially on SD and SD + 3AT (10 mM) for 8 h. The shown β-galactosidase activity for each strain was measured in four independent cultivations with four probes each time. The standard deviation did not exceed 20%.
C. Influence of *CPC2* expression on NADH-specific isocitrate dehydrogenase activity. Wild-type and *cpc2Δ* mutant strains were grown overnight, diluted in minimal medium containing 2% glucose or 2% acetate as carbon source, respectively, and incubated for an additional 10 h. Isocitrate dehydrogenase activity [EC 1.1.1.41] was measured for each strain in crude extracts in three independent cultivations and a variation in activity of less than 20%.

These results indicate that the *CPC2* product negatively affects transcription of Gcn4p-regulated genes but is not a general repressor of transcription because, for example, the *GCN4* mRNA level and the isocitrate dehydrogenase activity is not derepressed in a *cpc2Δ* mutant.

The Gcn2p-mediated translational control of GCN4 *expression is independent of* CPC2

The absence of the Gβ-like protein encoded by *CPC2* suppressed the growth phenotype of the *gcn2Δ* mutant strain, but not of the *gcn4Δ* mutant. Thus, we wanted to know whether the Cpc2 protein affects the Gcn2p-mediated signal cascade resulting in increased amounts of Gcn4p, or whether Cpc2p affects the Gcn4 protein independent of *GCN4* mRNA translation.

A *GCN4–lacZ* fusion construct containing the wild-type *GCN4* promoter and its untranslated 5′ region was transformed into wild-type strain and into *gcn2Δ*, *cpc2Δ* and *gcn2Δcpc2Δ* mutant strains. Expression of the *GCN4–lacZ* fusion construct correlates with the translational control of *GCN4* mRNA (Hinnebusch, 1985) and was determined for each strain under non-starvation and amino-acid starvation conditions (Fig. 4A). Our results indicate that the translational control of *GCN4* expression is almost completely unaffected by the *CPC2* gene product. Although a small derepression of *GCN4–lacZ* expression (less than twofold) occurred in response to the *cpc2Δ* mutation in the *gcn2Δ* background, none whatsoever was seen in the *GCN2* wild-type background. In contrast, Gcn4p-regulated *HIS3* transcription was derepressed by the *cpc2Δ* mutation in both strains (Fig. 3B).

Possible changes in the amount of Gcn4 protein in the cell in the presence or absence of the Cpc2 protein were

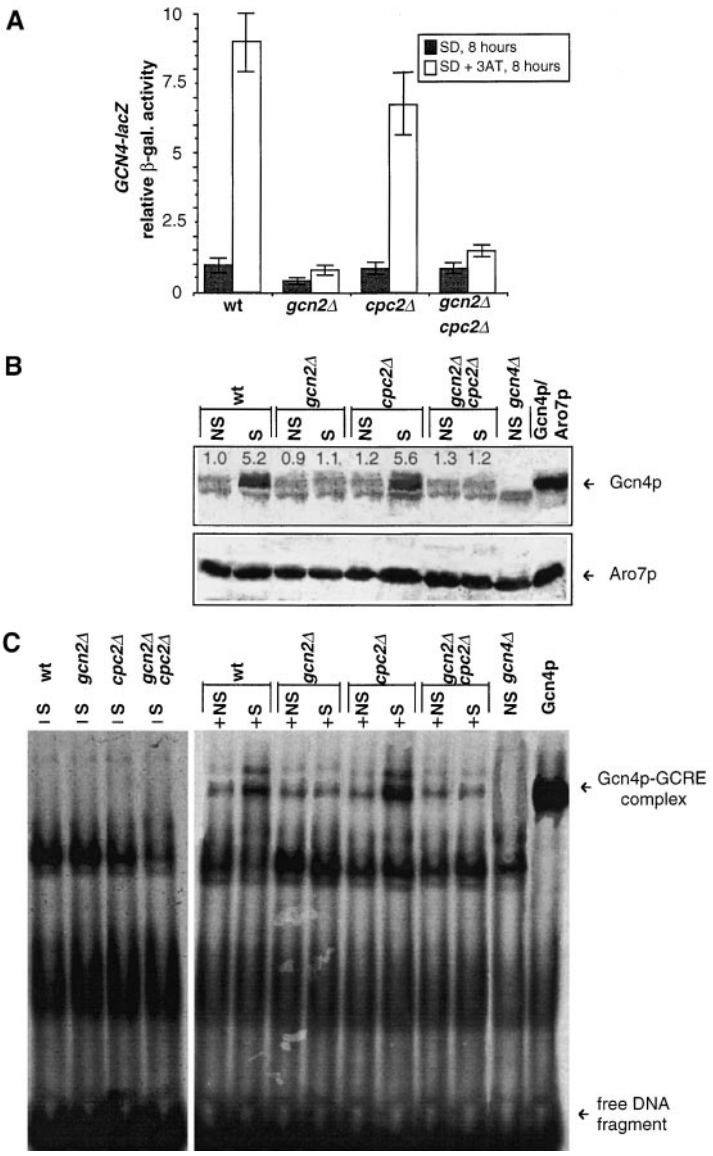

Fig. 4. Influence of *CPC2* expression on Gcn4p protein levels and DNA-binding activity of Gcn4p.
A. *GCN4–lacZ*-derived β-galactosidase activities. *GCN4–lacZ* expression from a low-copy number plasmid was measured in a wild-type and the *gcn2Δ*, *cpc2Δ* and *gcn2Δcpc2Δ* mutant strains. Strains were grown exponentially on SD and SD + 10 mM 3AT for induction of general control. All strains were measured in five independent cultivations in each case with four probes. The β-galactosidase activity of wild-type on SD medium was assigned a relative β-galactosidase activity of 1.
B. Western hybrization of Gcn4p protein and Aro7p protein as control. Gcn4p and Aro7 protein amounts of wild type, *gcn2Δ*, *cpc2Δ*, *gcn2Δcpc2Δ* and *gcn4Δ* strains grown on SD medium under non-starvation (NS) and amino-acid starvation (S) conditions were determined by cross-reaction of 10 μg of crude protein extract of each strain with specific rabbit antibodies. The unspecific low-molecular-weight signal shown in *gcn4Δ* and a polyclonal antibody raised against the purified Aro7 protein of yeast that is not under Gcn4p transcriptional regulation (Schmidheini *et al*., 1990) was used to equalize the probes. The Gcn4p polyclonal antibody was raised against the 60 C-terminal amino acids of Gcn4p and the Aro7p against the whole purified chorismat mutase protein. Purified Aro7p or Gcn4p expressed in *E. coli* were used as control in lane 6. The relative Gcn4p levels are indicated as numbers above each lane after normalization for Aro7p levels.
C. *In vitro* DNA-binding activity of Gcn4p derived from various yeast extracts. A ^{32}P-end-labelled DNA fragment containing a wild-type Gcn4p responsive element (GCRE) (+) of a mutated GCRE (–) for binding of Gcn4p was incubated with 10 μg of protein extracts from identical strains, as in Fig. 4B. Protein–DNA complexes were separated from unbound DNA by native PAGE and visualized by autoradiography. As internal standard, the unspecific DNA–protein complexes also present in the *gcn4Δ* mutant strain were used.

directly compared in wild-type, *gcn2Δ* and *cpc2Δ* strains and *gcn2Δcpc2Δ* double-mutant strains, grown under non-starvation and amino-acid starvation conditions. A specific anti-Gcn4p antibody detected the Gcn4 protein in cell extracts. The amount of Gcn4 protein was low in all strains investigated under non-starvation conditions, even in the absence of *CPC2*. Under amino-acid starvation, increased Gcn4p levels were found in wild-type and *cpc2Δ* mutant strains, whereas in *gcn2Δ* and *gcn2Δcpc2Δ* mutant strains Gcn4 protein levels remained on a low level. The equivalent protein levels in the *gcn2Δcpc2Δ* double-mutant strain compared with the *gcn2Δ* single mutant did not confirm the small increase in *GCN4–lacZ* expression of the corresponding strains. Therefore, we assume that the amount of Gcn4 protein is similar in the *cpc2Δ* and the *gcn2Δcpc2Δ* strain. These results indicate that Cpc2p does not affect the amount of Gcn4 protein in the cell by any mechanism involving stabilization or degradation of Gcn4p (Fig. 4B).

Specific *in vitro* DNA binding activity of the Gcn4 protein was measured to determine whether the presence of Cpc2p changes the ability of Gcn4p to bind to its specific DNA-binding site. Protein extracts were isolated from wild-type strain, *gcn2Δ*, *cpc2Δ* and *gcn2Δcpc2Δ* mutant strains under non-starvation and amino-acid starvation conditions, and incubated in gel retardation experiments with a radiolabelled DNA fragment containing a wild-type Gcn4p-responsive element (GCRE) or a mutated GCRE (Fig. 4C).

In a wild-type strain, a single specific Gcn4p–DNA complex could be detected under non-starvation conditions. When wild-type cells are starved of amino acids, Gcn4p–DNA complex formation is increased, which corresponds

to the increased amount of Gcn4 protein present in the cell. In a *gcn2Δ* strain, Gcn4p–DNA complex formation was found to be identical to the wild-type strain under non-starvation conditions. However, such a strain can no longer respond to starvation conditions because the Gcn2 protein kinase is missing. In strains carrying a *cpc2* deletion, Gcn4p–DNA complex formation was indistinguishable from a strain with an intact *CPC2* gene, suggesting that the Cpc2 protein does not affect the ability of Gcn4p to bind to DNA *in vitro*. The specificity of the Gcn4p–DNA complex was shown by the loss of complex formation using a mutated GCRE.

In summary, our data suggest that the negative effect of Cpc2p on transcription of genes subject to general control is independent of the translational regulation of *GCN4* mRNA mediated by the central Gcn2p kinase, and affects neither the amount nor the DNA-binding ability of Gcn4p in the cell.

Expression of CPC2 *specifically represses transcription driven by Gcn4p-responsive upstream elements*

Expression of the *CPC2* gene negatively regulates transcription of the *HIS3*, *HIS7* and *ARO4* genes depending on an intact *GCN4* gene. However, *CPC2* does not affect transcriptional and translational expression of *GCN4* or stability and DNA-binding activity of the Gcn4 protein. Therefore, we examined whether Gcn4p-binding promoter elements are sufficient to mediate the negative control of *CPC2* on Gcn4p-regulated genes or whether additional promoter elements are required.

A series of transcriptional *CYC1::lacZ* reporter gene fusions was constructed which are under the control of increasing numbers of synthetic Gcn4p-responsive elements (GCREs), resulting in increased sensitivity to the general control system. These constructs were integrated in single copy into wild-type, *gcn2Δ*, *cpc2Δ* or *gcn2Δcpc2Δ* yeast strains, and expression of the different GCRE::*CYC1::lacZ* reporter constructs was determined in the presence (data not shown) or absence of amino-acid analogues (Fig. 5A).

A *CYC1::lacZ* fusion construct carrying either a minimal promoter without a Gcn4p-responsive upstream element or carrying the authentic *CYC1* upstream activating sequences was not affected by the presence or absence of the *CPC2* gene under any growth conditions tested. Again, this suggests that expression of *CPC2* in yeast does not negatively affect transcription in general. However, expression of the *CYC1::lacZ* reporter construct was negatively affected by *CPC2* when Gcn4p-responsive elements were present in the promoter. Deletion of *CPC2* increased the expression of these GCRE-driven reporter constructs under non-starvation conditions. The most prominent difference was observed when the expression in a *gcn2Δ* mutant strain was compared with a *gcn2Δcpc2Δ* double-mutant strain. Whereas GCRE-dependent transcription in a *gcn2Δ* strain was reduced to about one-half of a *GCN2* wild-type strain, expression in a *gcn2Δcpc2Δ* double-mutant strain was increased by a factor of four in comparison with the wild type. The same relative effects were measured between the *gcn2Δ* and *gcn2Δcpc2Δ* mutant strains under conditions of amino-acid starvation. The variable numbers of GCREs had no influence on the ratio of β-galactosidase activities in the different genetic contexts.

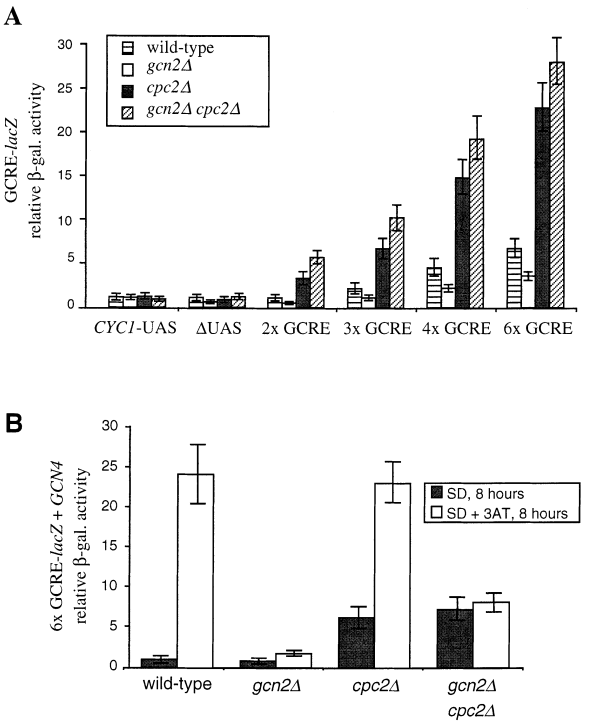

Fig. 5. *CPC2*-mediated negative control on Gcn4p-dependent transcription requires the presence of GCREs and Gcn4p in the cell.

A. Expression of various *GCRE–lacZ* fusions under non-starvation conditions. *CYC1–lacZ* constructs with different numbers of Gcn4p-responsive elements (GCRE) were inserted into a minimal promoter where a 430 bp fragment with *CYC1*-upstream activating sequences (UAS) was deleted. As controls, no GCRE element or the *CYC1-UAS* element was inserted. β-Galactosidase activities of all test constructs were measured in wild-type, *gcn2Δ*, *cpc2* and *gcn2Δcpc2Δ* genetic backgrounds after cultivation on minimal medium. Bars represent β-galactosidase activities normalized to the activity measured in wild type under non-starvation conditions with a value of 1 corresponding to 67 nmol of 4-methylumbelliferyl-β-D-galactoside min^{-1} mg protein.

B. Expression of a 6xGCRE::*CYC1*:: *lacZ* fusion construct under conditions of amino-acid starvation and non-starvation in the presence of high-copy *GCN4*-bearing plasmid. β-Galactosidase activities of an integrated 6xGCRE–*lacZ* artificial test construct in wild-type and *gcn2Δ*, *cpc2Δ* and *gcn2Δcpc2Δ* mutant strains were determined. In all strains, the *GCN4* wild-type gene was simultaneously expressed on a 2 μm plasmid. Strains were cultivated on SD medium under non-starvation and starvation conditions for 8 h. Bars represent β-galactosidase activities normalized to the activity measured in wild-type under non-starvation conditions with a value of 1 corresponding to 438 nmol of 4-methylumbelliferyl-β-D-galactoside min^{-1} mg protein.

The effect of increased Gcn4p levels under non-starvation and starvation conditions was tested with a wild-type *GCN4* on a 2 μm plasmid in *cpc2Δ* and wild-type strains, containing a 6×GCRE::*CYC1*::*lacZ* reporter fusion (Fig. 5B). This led under non-starvation conditions to ≈threefold increased Gcn4 protein levels (data not shown) and drastically increased β-galactosidase activities. Increased Gcn4p levels resulted in an intensified relative difference in β-galactosidase expression between a *cpc2Δ* and wild-type *CPC2* background. These data imply that Cpc2p is still functional in negative control of transactivation activity of increased levels of Gcn4p.

Taken together, deletion of *CPC2* in a *gcn2Δ* strain resulted in an 8- to 10-fold increase in GCRE-dependent transcription compared with a *gcn2Δ* single mutant. We, therefore, conclude that the negative effect of *CPC2* on Gcn4p-regulated gene expression requires a functional GCRE but no additional promoter element, and that the

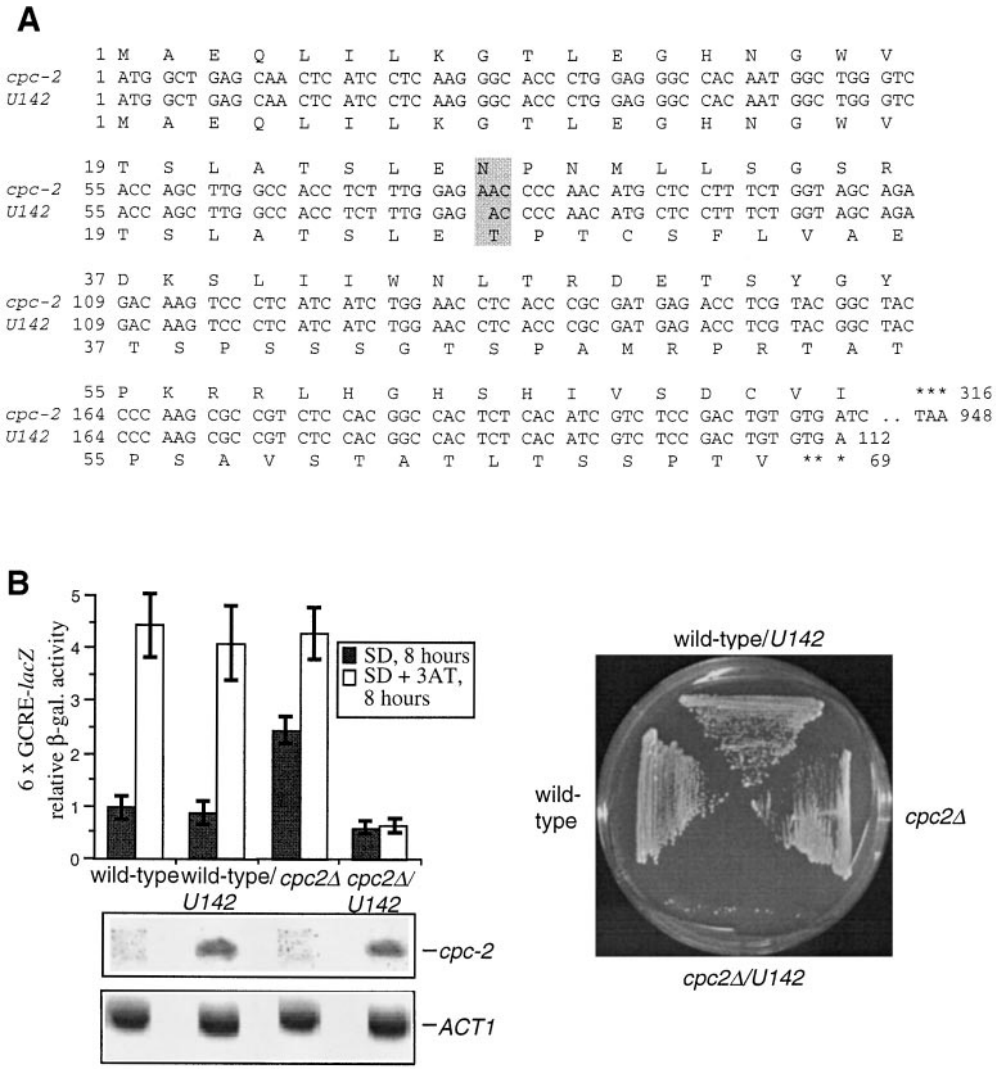

Fig. 6. Expression of the *N. crassa cpc-2* mutant allele in yeast results in sensitivity to amino-acid starvation conditions.
A. Partial nucleotide comparison between the *N. crassa cpc-2* wild-type (Müller *et al*., 1995) and the *cpc-2 U142* cDNA allele. Nucleotide sequence of the mutant allele was obtained by sequencing of two independent cDNA clones. Deduced amino-acid sequences of the mutant allele of *cpc-2* and partially for the *cpc-2* wild-type allele are shown below the DNA sequences. The frameshift mutation is shown by a grey box.
B. Expression of a 6xGCRE::*CYC1::lacZ* fusion construct under non-starvation and amino-acid starvation conditions in the presence and absence of a *cpc-2* mutant allele-bearing plasmid. β-Galactosidase activities of an integrated 6xGCRE–*lacZ* artifical test construct in wild-type and *cpc2Δ* mutant strain were determined. In strain wild-type/*U142* and *cpc2Δ*/*U142* the cDNA of the *cpc-2* mutant allele was simultaneously expressed on a low-copy plasmid under the control of the yeast *CPC2* promoter. Strains were cultivated on SD medium under non-starvation and amino-acid starvation conditions for 8 h. As control for *U142* cDNA expression, mRNA from all strains were isolated after growth on SD medium. mRNA amounts were equalized using the *ACT1* transcript for quantification. *U142 cpc-2* mRNA was probed with the PCR-amplified last exon of the *N. crassa cpc-2*. The growth defect of the *cpc2Δ*/*U142* strain under amino-acid starvation conditions is shown on a plate containing SD medium with 10 mM 3AT. Wild-type, wild-type/*U142*, *cpc2Δ* and *cpc2Δ*/*U142* were incubated for 3 days at 30°C.

negative control of *CPC2* on Gcn4p-regulated genes is specific and not a general effect on yeast transcription.

Expression of the N. crassa cpc-2 *mutant allele in yeast results in 3AT sensitivity*

A deletion of *CPC2* in yeast results in an increased transcription of general control regulated genes. However, the originally characterized *cpc-2 U142* mutation in the *N. crassa* homologue gene *cpc-2* results in an inability to derepress these genes (Krüger *et al*., 1990; Müller *et al*., 1995). To analyse the discrepancy between these two findings, the *cpc-2 U142* allele was isolated. Sequence alignments between the wild-type *cpc-2* and the sequence of the *cpc-2 U142* allele identified a frameshift mutation in the second exon. This results in a deduced truncated open reading frame of 207 nucleotides, encoding 69 amino acids instead of 316 for the wild-type *cpc-2*. The deduced cpc-2 U142 mutant protein contains only two-thirds of the first WD repeat before the frameshift mutation. The frameshift mutation was confirmed by sequencing of two additional cDNA clones of the *cpc-2 U142* mutant allele (Fig. 6A).

The effects of the *cpc-2 U142* mutant allele were investigated by expression of the *U142* cDNA clone under the control of the *S. cerevisiae CPC2* promoter in yeast wild-type and *cpc2Δ* mutant strains containing the 6× GCRE–*lacZ* reporter gene. Expression of the *cpc-2 U142* allele in *S. cerevisiae* was confirmed by Northern analysis (Fig. 6B). Expression of *cpc-2 U142* in yeast containing the wild-type *CPC2* did not affect the growth rates under any condition tested. However, expression of the *cpc-2 U142* allele in yeast strains lacking the endogenous *CPC2* resulted in sensitivity to amino-acid analogues, as found for *N. crassa cpc-2 U142* mutants (Fig. 6B).

To analyse whether the sensitivity to amino-acid starvation conditions is due to a reduced ability of Gcn4p to activate transcription and by expression of *cpc-2 U142* expression of the 6×GCRE–*lacZ* reporter gene was measured. As shown in Fig. 6(B), expression of the *N. crassa cpc-2 U142* allele has no effect on Gcn4p-dependent transcription in *CPC2* wild-type strains, neither under non-starvation nor under amino-acid starvation conditions. However, expression of *cpc-2 U142* in *cpc2Δ* mutant strains drastically decreased the ability of Gcn4p to activate transcription under non-starvation and starvation conditions.

These data indicate that the *cpc-2 U142* allele of *N. crassa* causes a specific, recessive phenotype. Although the wild-type *cpc-2* allele of *N. crassa* is able to complement the *S. cerevisiae cpc2Δ* mutation, expression of *cpc-2 U142* in yeast causes 3AT sensitivity as found in *N. crassa*. However, whereas *CPC2* of yeast represses Gcn4p activity only under non-starvation conditions, expression of the *cpc-2 U142* mutant allele of *N. crassa* constitutively represses Gcn4p-driven transcription, even under amino-acid starvation conditions.

Discussion

The general control of amino-acid biosynthesis is a regulatory network which provides the cell with sufficient amounts of protein precursors under conditions of amino-acid limitation. The final regulator is the transcriptional activator Gcn4p, which activates transcription of more than 50 genes by binding to Gcn4p-responsive elements (GCRE) in the corresponding target promoters. Gcn4p activity is modulated by translational regulation of *GCN4* mRNA and, in specific circumstances, by stabilization of the Gcn4 protein. Translational regulation of *GCN4* expression requires a signal transduction pathway with the sensor kinase Gcn2p, which measures the amount of uncharged tRNA in the cell (Hinnebusch, 1988, 1992). The stabilization mechanism of the Gcn4 protein under amino-acid starvation conditions is unknown (Kornitzer *et al*., 1994).

Here, we show an additional mechanism as to how expression of the general control network can be affected. The absence of the *CPC2* gene leads to increased Gcn4p-mediated transcription. Expression of the *CPC2*-encoded WD protein reduces, in a specific manner, transcription of Gcn4p-regulated genes. The increased transcription in a *cpc2*-deficient mutant strain is sufficient to allow the cells to survive under amino-acid limitation conditions, even in the absence of the sensor kinase encoded by the *GCN2* gene, without affecting the Gcn4 protein level. Therefore, the Cpc2 protein specifically reduces transcription of general control regulated genes without affecting the *GCN4* translational regulation. For other WD repeat proteins such as Tup1p of *S. cerevisiae* or Groucho of *Drosophila*, a function as repressor of transcription of a large set of genes is described (Keleher *et al*., 1992; Paroush *et al*., 1994). Tup1p and Cpc2p contain seven WD repeats, but Tup1p contains additional non-WD repeat sequences and it therefore does not belong to the group of Gβ-like proteins. The complex of Tup1p with Ssn6p and a number of further proteins inhibits transcription (Williams *et al*., 1991). The discussed mechanisms include either the organization of a repressive chromatin structure (Cooper *et al*., 1994), through the direct interaction of the complex with histones (Edmondson *et al*., 1996), or the blocking of activation domains of pathway-specific DNA-binding proteins (Tzamarias and Struhl, 1995; Lutfiyya and Johnston, 1996). Cpc2p does not directly affect the Gcn4p–GCRE interaction, and two-hybrid experiments indicate that Cpc2p does not bind to Gcn4p in this artificial test system (data not shown). Therefore, it is likely that Cpc2p and Tup1p affect transcription by a different mechanism.

WD proteins consist of 4–8 WD repeats, each with a

length of 36–46 amino acids and typically ending with tryptophan (W) and aspartate (D). These repeats were also named β-transducin repeats (Duronio *et al*., 1992). They were originally found in the β-subunits of heterotrimeric GTP-binding proteins, which transduce signals across the plasma membrane. Several WD proteins in various organisms are involved in different functions like signal transduction (Whiteway *et al*., 1989), RNA processing (Icho and Wickner, 1988), gene regulation (Keleher *et al*., 1992) or cell cycle control (Choi *et al*., 1990). Whether these functions are caused by the WD repeats or additional flanking sequences present in many WD proteins is mostly unknown. One general function of WD repeats seems to be the mediation of protein–protein interactions. Crystal structures have shown that β-subunits of heterotrimeric G-proteins form a seven-bladed β-propeller (Wall *et al*., 1995; Sondek *et al*., 1996). This β-propeller, which contains seven structurally similar WD repeats, defines the stereochemistry of the WD repeat and the probable architecture of all WD-repeat-containing domains. Single WD repeats do not correspond to a stable folded domain, and a small shift in the folding could produce a large change in the conformation of the exposed surface and an alteration in function or binding partners (Neer *et al*., 1994; Neer and Smith, 1996). Therefore, it seems likely that Cpc2p forms also a β-propeller and acts as a scaffold for other proteins.

The *Neurospora crassa cpc-2* gene has been described previously and encodes a WD protein which is similar to the yeast *CPC2* gene product (Krüger *et al*., 1990; Müller *et al*., 1995). Although a *cpc-2 U142* mutation is involved in regulation of amino-acid biosynthesis in *N. crassa* with a contrary phenotype as found for the yeast *cpc2* deletion, the *cpc-2* gene complements the yeast *cpc2Δ* mutation, suggesting that *N. crassa cpc-2* and yeast *CPC2* are functionally homologues. Isolation of the *cpc-2 U142* mutant allele identified a frameshift mutation at amino acid 27, resulting in a deduced shortened protein of 69 amino acids with only two-thirds of the first WD repeat. In yeast, expression of this allele also results in sensitivity to amino-acid analogues, caused by a drastically decreased ability of Gcn4p to activate transcription. In *S. cerevisiae*, as in *N. crassa*, expression of the *cpc-2 U142* allele is recessive. In both cases, this could be caused by the low-mRNA level. It is still unclear whether the first unaffected amino acids of the *cpc-2* protein or whether the 43 amino acids behind the frameshift are responsible for this specific phenotype. Nevertheless, the *N. crassa* mutant allele argues for the possibility that the cpc-2 protein of *N. crassa* and the Cpc2p of yeast are able to affect the activity of Gcn4p in a negative manner. The Cpc2p homologue in rat, the RACK1 protein, is 53% identical to Cpc2p and also able to complement the yeast *cpc2Δ* phenotypes. RACK1p is involved in the localization of activated protein kinase C (PKC). Binding between these proteins results in translocation of PKC from the cytoplasm to the cell particulate fraction (Ron *et al*., 1994; Ron and Mochly Rosen, 1995). The increased transcription of Gcn4p-regulated genes under non-starvation conditions seems to be overcome in a *cpc2Δ* mutant strain, but not in the *gcn2Δcpc2Δ* double mutant under starvation conditions. Activation of Gcn2p by uncharged tRNA molecules could be important for a partial Cpc2p repression in strains grown under amino-acid starvation conditions. Therefore, the function of Cpc2p in the general control system seems to be independent of the Gcn2p-mediated translational regulation of *GCN4* mRNA, but might require Gcn2p itself. Gcn2p could be required to antagonize Cpc2p-mediated repression in starved cells. This would explain the expression levels of Gcn4p-regulated genes in the *cpc2Δ* mutant strain, which are similar to wild-type levels under amino-acid starvation conditions, but the expression levels of the *gcn2Δcpc2Δ* double mutant remain increased in comparison with the *gcn2Δ* single mutant in starved cells.

The observation that Gβ-like proteins isolated from the phylogenetically distant eukaryotes *S. cerevisiae*, *N. crassa* and rat are able to complement the yeast *cpc2Δ* phenotype indicates a highly conserved function of this protein family that is not yet fully understood. A connection between cpc-2p, RACK1p and Cpc2p function could be the involvement in kinase signal transduction pathways.

There are different possibilities as to how *CPC2* expression negatively affects Gcn4p-dependent transcription. For the yeast Gβ-like protein, it was also shown that deletion leads to an accumulation of halfmer polyribosomes (Chantrel *et al*., 1998). These data and cofractionation of the protein with 40S ribosomal subunits argued for one possibility of Gβ-like function in regulation of translational efficiency. Although these data were not consistent with the unaffected translation of Gcn4p in the *cpc2Δ* mutant strains, further experiments should show the connection between all results found for the Gβ-like protein of yeast. In case *CPC2* is part of a pathway that acts on the Gcn4-protein in the cell, it has to be determined as to whether Gcn4p action is reduced by a direct modification, for example by phosphorylation as found for c-*Jun*, which is another member of the basic zipper type transcriptional activator family, or whether Cpc2p prevents correct localization of Gcn4p. Such a function described for RACK1p regulation by localization was also identified for the *S. cerevisiae* transcription factor yAP-1, which is also a member of the basic zipper type transcriptional activator family with an identical DNA binding element to Gcn4p (Coleman *et al*., 1997). After induction by oxidative stress, yAP-1 relocalizes from the cytoplasm to the nucleus (Kuge *et al*., 1997). Similarly, it has to be determined whether Cpc2p can prevent translocation of Gcn4p from the cytosol to the nucleus under non-starvation conditions, and thus

negatively affect transcription of general control regulated genes.

In summary, the WD protein Cpc2p negatively affects transcription of genes regulated by the general control network without an involvement in translational regulation of *GCN4* mRNA. Therefore, this network, which is activated by a linear signal cascade starting with the sensing of uncharged tRNAs and resulting in an increased transcription of numerous genes mediated by the transcriptional activator Gcn4p, seems to be integrated with an additional pathway that reduces the activation function of Gcn4p under non-starvation conditions. One possibility is that this is part of an emergency system for an over-reaction of general control under conditions in which this system is not really required. Alternatively, or in combination, it could also reduce Gcn4p-regulated transcription under non-starvation conditions in which expression of all regulated genes is not required. *CPC2* expression acts behind or beside the Gcn2p-mediated signal transduction pathway. A specific mutation in Gβ-like proteins causes a gain of function, resulting in a switch from a *gcn*- to a *GCD*-like phenotype. *CPC2* is the first member of this negative control, and additional components, their function and possible regulation remain to be elucidated.

Experimental procedures

Strains, medias and genetic techniques

All yeast strains used were derivatives of the *S. cerevisiae* strain S288C. The *cpc2* deletion mutant strains are derivatives of strains H1515 (*MAT*a *leu2–3,-112 ura3–52 trp1–63*), H1894 (*MAT*a *leu2–3,-112 ura3–52 trp1–63 gcn2Δ*) and RH1885 (*MAT*a *leu2–3,-112 ura3–52 his3-Δ200 trp1-Δ901 ade2–101 gcn4Δ::TRP1*). H1515, H1894 and resulting *cpc2Δ* mutants are isogenic yeast strains. Cultivation of yeast was performed at 30°C in SD medium with recommended amounts of supplements (Rose *et al*., 1990). 3-Aminotriazole (3AT) was added to the media at a final concentration of 10 mM; 5-methyltryptophan (5MT) was used at a final concentration of 2 mM. Yeast cells were made competent for transformation by treatment with lithium acetate (Ito *et al*., 1983). For *N. crassa*, wild-type St. Lawrence strain K93–5a was used. *Escherichia coli* strain DH5α was used for plasmid propagation.

Isolation of CPC2

To isolate the *CPC2* gene of *S. cerevisiae*, known deduced Gβ-like proteins were aligned to identify specific and highly conserved amino-acid regions. GTTTRRFVGH in the third, NAVTISPDG in the fifth and PNRYWLCA in the sixth repeat were chosen to design primers B.H.4 (5′-TAATCTAGACTACTACTAGWAGWTTTGTTGGICAYAC-3′), B.H.5 (5′-TAAGCATGCRGCGCATAGCCAGTAWCKGTTIGG-3′) and B.H.6 (5′-AATGCNGTTACTATHTCICCIGATGG-3′) respectively. DNA fragments of *CPC2* were amplified by PCR and cloned into pUC19 after digestion with *Xba*I and *Sph*I. PCR fragment B.H.4/B.H.5 was ^{32}P-labelled and used as a probe for isolation of the genomic *CPC2* clone. DNA of positive λ-clones carrying the genomic *CPC2* gene was isolated as described previously (Sambrook *et al*., 1989) and directly used for PCR and sequencing reactions.

Plasmid construction

For construction of the *CPC2* deletion plasmid pRSBH1–14, 200 bp 5′-flanking and 230 bp 3′-flanking sequences of *CPC2* were amplified by PCR using primers B.H.16 and B.H.17 (16, 5′-TAATCTAGACTATGTACGGATGTTAGG-3′; 17, 5′-TAAGAGCTCAAGAGAGAGAGAACGAGT-3′) and B.H.18 and B.H.19 (18, 5′-TTTCTCGAGAGTTTGGCAA GTTATGA-3′; 19, 5′-TAACTGCAGCAAACAGAAAGCATAGT-3′), respectively, and λ-DNA containing the entire *CPC2* gene as template. The 5′-amplificate was digested with *Xba*I and *Sac*I and ligated into the vector pRS306 (Sikorsky and Hieter, 1989) containing the *LEU2* marker. In a second ligation, the *Xho*I- and *Pst*I-digested 3′-amplificate was ligated into the latter plasmid, resulting in the plasmid pRSBH1–14. The *Bam*HI restriction site was used to linearize the plasmid for integration. The same method was used for construction of the partial *CPC2* disruption plasmid pCPC2Δ1–156. This plasmid disrupted the *CPC2* open reading frame at position 467 relative to the AUG start codon, resulting in an exactly bisected *CPC2* gene. Artificial test plasmids containing different numbers of GCREs for assaying the Gcn4p transactivation activity were constructed using the yeast integration vector pLI4 containing the *CYC1–lacZ* reporter gene (Sengstag and Hinnen, 1988). Replacement of the upstream activation site (UAS) of the *CYC1–lacZ* by GCREs was achieved by first removing the *Xho*I fragment containing the UAS. Religation of the *Xho*I site resulted in pME1108. Then, two synthetic oligonucleotides (GAC1, 5′-GATCGGATGACTCATTTTTT-3′; and GAC2, 5′-GATCAAAAAATGAGTCATCC 3′) were annealed to form an optimal double-stranded GCRE (Hope and Struhl, 1985). T4 DNA ligase was added to get oligomeric GCRE–DNA probes. The products were then blunt ended with Klenow and inserted into the blunt-ended *Xho*I site of pME1108. The resulting plasmids pME1109–pME1112 contain two, three, four and six GCRE sites respectively. Ectopic integration of these plasmids was performed by linearizing the plasmids with *Stu*I. The plasmid p180 containing the *GCN4–lacZ* construct was described previously (Hinnebusch, 1985; Mueller *et al*., 1987). Plasmid B1574 of Kevin Struhl containing the *HIS3–lacZ* construct was integrated by cutting with *Bam*HI. To create an overexpression plasmid of *GCN4*, a *Sal*I/*Eco*RI *GCN4* fragment from p180 was cloned into plasmids pRS424 and pRS426 (Sikorsky and Hieter, 1989), resulting in plasmids pME1450 and pME1451. For creation of plasmids pME1452 and pME1453, the whole *CPC2* gene was amplified in independent PCR reactions and cloned into plasmids pRS314 and pRS316. For complementation analysis, the promoter and terminator sequences of *CPC2* and the coding region of the cDNA clone 20C of *N. crassa cpc-2* (Müller *et al*., 1995) were amplified in different PCR reactions and inserted into the plasmids pRS424 and pRS426, resulting in plasmids pME1454 and pME1455. Correct DNA sequences of all fragments amplified by PCR were verified by sequencing analysis. Kidney cell line of rat (established by N. Duc-Ngugen, unpublished) was used and cultivated

in DMEM cell media (ICN) supplemented with 5% bovine serum albumin to isolate the cDNA of the rat *RACK1*. RNA was isolated with Trizol reagent (Gibco BRL), and RACK1 cDNA was synthesized using the *RACK1* specific primer B.H.105 (5′-TTAGTCGACCCCGGGATGACCGAGCAAATGACCC-TTCG-3′) and Superscript™ reverse transcriptase (Gibco BRL). *RACK1* cDNA was amplified by polymerase chain reaction using Pfu polymerase (Stratagene) and primers B.H.105 and B.H.106 (5′-TTAGTCGACGAGCTCTTAGCGGGTACC-AATAGTCACCTG-3′). *RACK1* cDNA was inserted into plasmids pRS314 and pRS316. In the plasmids pME1456 and pME1457, the open reading frame is driven by the yeast *CPC2* promoter and terminator sequences. For isolation of a genomic clone of the *cpc-2 U142* mutant allele, genomic DNA of *N. crassa* was digested with *Hin*dIII and fractionated on an agarose gel. The PCR-amplified last exon of the *N. crassa cpc-2* wild-type gene was used as radiolabelled probe and hybridized to a 3 kb *Hin*dIII DNA fragment. *Hin*dIII-digested DNA in the size range 2.5–3.5 kb was subcloned into pBluescript SK^+ and positive clones were isolated. Low levels of *cpc-2* mRNA in the *cpc-2 U142* mutant strain (Krüger *et al*., 1990) were used to construct cDNA clones of the *cpc-2* mutant allele and performed with *cpc-2* specific primers as described for *RACK1*. In the resulting plasmid pME1458, the *cpc-2* cDNA is driven by the yeast *CPC2* promoter and terminator sequences on a low-copy plasmid.

Construction of strains carrying CPC2 *mutant alleles*

Plasmid pRSBH1–14 carrying the *CPC2* deletion cassette was cut with *Bam*HI and transformed into yeast strains H1515, H1894 and RH1885 to obtain strains H1515-A7 (*MAT*a *leu2–3,-112 ura3–52 trp1–63 cpc2Δ::LEU2*), H1894-E2 (*MAT*a *leu2–3,-112 ura3–52 trp1–63 gcn2Δ cpc2Δ::LEU2*) and RH1885-D2 (*MAT*a *leu2–3,-112 ura3–52 his3-Δ200 trp1-Δ901 ade2–101 gcn4Δ::TRP1 cpc2Δ::LEU2*), carrying a complete deletion of *CPC2*. A partial disruption of *CPC2* was performed in strain H1515 and H1894 to analyse the function of the first 156 amino acids of the Cpc2 protein. Therefore, plasmid pRSBH2–6 was linearized with *Bam*HI and transformed to create strains H1515-$CPC2^{1-156}$ (*MAT*a *leu2–3, -112 ura3–52 trp1–63* $CPC2^{1-156}$*::LEU2*) and H1894-$CPC2^{1-156}$ (*MAT*a *leu2–3,-112 ura3–52 trp1–63 gcn2Δ* $CPC2^{1-156}$*::LEU2*). Correct deletion and disruption of *CPC2* in these strains was verified by PCR and Southern blot analysis.

Recombinant DNA techniques

For recombinant DNA techniques, standard procedures were used (Sambrook *et al*., 1989). DNA sequencing was performed by the dideoxynucleotide chain termination method (Sanger *et al*., 1977), using custom oligonucleotides (Gibco BRL) and the T7 Sequencing kit (Pharmacia).

Protein assays

β-Galactosidase activities were determined using permeabilized yeast cells and the fluorogenic substrate 4-methylumbelliferyl-β-D-galactoside, as described previously (Künzler *et al*., 1993). Routinely, yeast cells were cultivated in minimal medium overnight, diluted to an optical density of ≈0.2 at 546 nm (OD_{546}) in minimal medium (SD) with and without 10 mM 3AT, and cultivated for between another 4 and 8 h before assaying. One unit of β-galactosidase activity is defined as 1 nmol of 4-methylumbelliferone $h^{-1} ml^{-1} OD_{546}^{-1}$. The given values are means from at least four independent cultures, each measured four times. For investigation of isocitrate dehydrogenase activity, the wild type and *cpc2Δ* mutant strains were grown overnight in SD medium and diluted to an OD_{546} of 0.1 in minimal medium containing 2% glucose or 2% acetate as carbon source respectively. Strains were harvested after an additional cultivation of 10 h. Isocitrate dehydrogenase activity was measured in crude cell extracts as described previously (Keys and McAlister-Henn, 1990). Protein concentrations were determined using the Bradford method (Bradford, 1976).

Northern analysis

Strains were cultivated as described for β-galactosidase assays. Total RNA was isolated as described previously (Zitomer and Hall, 1976). For Northern hybridization, 20 μg total RNA per lane was separated on a formaldehyde agarose gel, electroblotted onto a nylon membrane (Amersham) and hybridized with DNA fragments that were ^{32}P-labelled according to the oligolabelling technique described by Feinberg and Vogelstein (1984).

Western analysis

Strains were cultivated as described for β-galactosidase assays up to an OD_{546} of 1. Crude protein extracts were isolated in the presence of a specific protease inhibitory mix (Drysdale *et al*., 1995). Protein (10 μg) of each strain was separated by polyacrylamide gel electrophoresis (PAGE), and Gcn4p was visualized using a polyclonal rabbit antibody raised against a synthetic peptide containing the 60 C-terminal amino acids of Gcn4p (Drysdale *et al*., 1995). As second antibody, a peroxidase-labelled anti-rabbit antibody (Amersham) was used in a dilution of 1: 5000. The protein band identified in the *gcn4Δ* mutant strain and with lower molecular weight than Gcn4p in all other lanes represents an unspecific antibody signal, and was still observable after purification of the Gcn4p antibody by dialysis. Protein amounts were equalized using the unspecific Gcn4p-antibody signal and a polyclonal rabbit antibody raised against the whole purified chorismat mutase protein (Aro7p) of yeast (Schnappauf *et al*., 1997).

Gel retardation assay

Ten micrograms of crude protein extract isolated from each strain was used in the gel retardation assay. Protein was incubated in the presence of a ^{32}P-end-labelled 53 bp *Mlu*I-*Hha*I yeast *TRP4* promoter fragment (Mösch *et al*., 1990), which contained either a wild-type GCRE (5′-ATGACTAAT-3′) or a mutated GCRE (5′-ATcACTAgT-3′), and then separated on a polyacrylamide gel as described previously (Braus *et al*., 1989). Unspecific DNA–protein complexes were identified in *gcn4Δ* as well as for all other strains and used as an internal standard.

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