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The *Aspergillus niger GCN4* homologue, *cpcA*, is transcriptionally regulated and encodes an unusual leucine zipper

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Summary

The general control transcriptional regulator gene *cpcA* of *Aspergillus niger* was cloned by complementation of a *Saccharomyces cerevisiae* Δ*gcn4* mutant strain. The encoded protein conferred resistance to amino acid analogues when expressed in yeast. Disruption of *cpcA* in *A. niger* resulted in a strain which is sensitive towards 3-aminotriazole and fails to respond to amino acid starvation. *cpcA* encodes a transcript of ≈2400 nucleotides in length that includes a 5′ leader region of 900 nucleotides. The 5′ leader region contains two small open reading frames, suggesting translational control of gene expression. Steady-state mRNA levels of *cpcA* increase by a factor of three upon amino acid starvation. The coding region of *cpcA* is interrupted by a 57 bp intron and the deduced amino acid sequence displays an ≈30% overall identity to yeast GCN4p and *Neurospora crassa* cpc-1p. Critical amino acid residues of the transcriptional activation domains of GCN4p are conserved in cpcAp. The basic DNA-binding domain shows up to 70% amino acid sequence identity to other basic zipper (bZIP)-type transcriptional activators. cpcAp binds specifically to a GCN4p recognition element in gel retardation experiments. The C-terminal dimerization domain encodes a leucine zipper with only a single leucine residue.

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Introduction

In fungi, imbalances in the pool of amino acids elicit a co-ordinated derepression of many amino acid biosynthetic enzymes in several unlinked biosynthetic pathways, as well as amino acid tRNA synthetases, and enzymes for nucleotide biosynthesis. This phenomenon, called cross-pathway control in *Neurospora crassa* (Carsiotis and Jones, 1974; Carsiotis *et al*., 1974), and general control of amino acid biosynthesis in *Saccharomyces cerevisiae* (Schürch *et al*., 1974; Wolfner *at al.*, 1975), has also been found in *Aspergillus nidulans* (Piotrowska, 1980). General control has been studied most thoroughly in *S. cerevisiae* (reviewed in Hinnebusch, 1992) leading to the discovery of numerous *GCN* (general control non-derepressed) and *GCD* (general control derepressed) genes. The primary signal in the transduction pathway seems to be uncharged tRNAs which are recognized by the bifunctional GCN2 protein kinase. The cascade finally results in translational derepression of the *GCN4* gene encoding the transcriptional regulator GCN4p which activates transcription of target genes by binding to the palindromic sequence 5′-ATGA(C/G)TCAT-3′ (general control recognition element; GCRE) in their promoters (Hinnebusch, 1984; Thireos *et al*., 1984; Arndt and Fink, 1986). The corresponding gene from *N. crassa*, *cpc-1*, was cloned (Paluh *et al*., 1988) and shown to interact with similar target sequences (Paluh and Yanofsky, 1991).

GCN4 of yeast encodes a transcriptional activator belonging to the so-called basic zipper (bZIP)-type family (Landschulz *et al*., 1988; Vinson *et al*., 1989). It is composed of a DNA-binding domain which contacts target sequences via basic amino acid residues (Agre *et al*., 1989; Talanian *et al*., 1990). The adjacent leucine zipper at the C-terminal end of the protein is characterized by α-helical heptad repeats of leucine residues. The helices of two monomers dimerize by hydrophobic interactions, stabilized by charged amino acid residues, and form a coiled coil (Kouzarides and Ziff, 1989; O′Shea *et al*., 1989; Sellers and Struhl, 1989). These two subdomains are highly conserved in eukaryotes. The corresponding subdomains of yeast GCN4p and human c-Junp can be replaced by each other (Struhl, 1987; 1988; Oliviero *et al*., 1992). While naturally occurring bZIP proteins show a high degree of conservation of the leucine residues

within the dimerization domain, the functional significance of this amino acid has been questioned. It was shown that leucine residues in GCN4p can not only be exchanged for other hydrophobic amino acids, but also for charged amino acid residues without affecting *in vivo* functionality (Hu *et al*., 1990; van Heeckeren *et al*., 1992). Aligned heptad repeats also do not seem to be a prerequisite for dimerization in the *N. crassa* cpc-1 protein (Paluh and Yanofsky, 1991).

The N-terminal part of the GCN4 protein is composed of two distinct transcriptional activation domains (Hope and Struhl, 1986; Hope *et al*., 1988; Drysdale *et al*., 1995). Overlapping these two activation domains, an instability ('PEST') domain has been identified (Kornitzer *et al*., 1994), which is involved in ubiquitin-dependent protein degradation. Alignment of the deduced amino acid sequences of GCN4p and cpc-1p suggests that these domains are principally conserved between yeast and *N. crassa* (Paluh *et al*., 1988; Paluh and Yanofsky, 1991).

Small open reading frames (μORFs) in the 5′ leader sequence of *GCN4* have been identified as being regulatory elements by the fact that they control gene expression at the translational level (Mueller and Hinnebusch, 1986; Hinnebusch, 1994). The presence of μORFs in the *cpc-1* transcript suggested a similar regulatory mechanism in *N. crassa*. Extended 5′ leader sequences containing μORFs have not only been found in lower eukaryotes but also in vertebrates (Kozak, 1991). Generally, these motifs are supposed to confer translational control of gene expression (Geballe and Morris, 1994). While translational control is the only regulatory mechanism described for *GCN4*, other fungal genes containing 5′ μORFs, such as the developmental regulators *brlA* and *stuA* of *A. nidulans* have been shown to be subject to additional transcriptional control mechanisms (Miller *et al*., 1992; Prade and Timberlake, 1993; Han *et al*., 1993).

Here, we report the cloning and characterization of *cpcA*, the *GCN4*/*cpc-1* homologue, from *Aspergillus niger*. The *cpcA* gene has been cloned by complementation of a Δ*gcn4* yeast strain using an *A. niger* cDNA expression library. The gene product displays specific DNA-binding activity to a GCRE and mediates resistance against amino acid analogues such as the false feedback inhibitor of histidine biosynthesis, 3-aminotriazole (3AT). In contrast to yeast *GCN4*, *cpcA* contains only a single leucine residue in the presumed dimerization domain and is regulated at the transcriptional level.

Results

An Aspergillus niger *cDNA clone (*cpcA*) confers resistance towards amino acid analogues in an* S. cerevisiae *Δ*gcn4 *mutant strain*

A cDNA expression library of *A. niger* in pEMBLyex4S/S was transformed into the *S. cerevisiae* strain RH1408 lacking the part of the *GCN4* ORF which encodes the 131 C-terminal amino acids (Hinnebusch, 1985; Mösch *et al*., 1990). The yeast strain is therefore unable to carry out the general control response and is sensitive towards the amino acid analogue 3AT, which is a false feedback inhibitor of the histidine biosynthetic enzyme imidazole glycerolphosphate dehydratase. Approximately 20 000 transformants were screened for resistance to the analogue 3AT, on minimal medium containing 10 mM 3AT. One isolated clone additionally displayed resistance towards another amino acid analogue, 5-methyltryptophan, a false feedback inhibitor of anthranilate synthase catalysing the first step in tryptophan biosynthesis. Plasmid DNA isolated from this yeast transformant contained an *A. niger* 1340 bp cDNA insert which was later sequenced. The cloned cDNA was identical to the subsequently cloned genomic sequence except that it was missing the intron present in the chromosomal gene (see Fig. 2 later).

Further evidence that this clone encodes the *A. niger* gene homologous to *GCN4* was obtained by demonstrating the ability of protein extracts isolated from this transformant to specifically retard a DNA fragment containing a GCRE in band-shift analysis (Fig. 1). While protein extracts from neither the untransformed strain nor strain RH1408 harbouring the empty expression plasmid were able to retard a GCRE-containing DNA fragment, extracts from the 3AT-resistant transformant showed a specific binding activity. Compared with the retarded band produced by GCN4p, the shift obtained with protein extracts from the transformed yeast strain shows several retarded bands of lower mobility. This might be the result of either modification (including oligomerization) or degradation of the heterologous *A. niger* cpcA protein during expression within the yeast cell. Additionally, in the presence of a polyclonal antibody raised against the 60 C-terminal amino acids of GCN4p, the intensity of the cpcAp and GCN4p shift is drastically reduced. To a lesser extent, 'supershifts' are visible, presumably consisting of the protein–DNA–antibody complex. Addition of pre-immune serum as a control increases the intensity of the retarded band(s) using either an extract from the transformant or heterologously expressed GCN4p.

Disruption of the chromosomal cpcA *locus abolishes the general control response in* A. niger

Using the isolated cDNA of the 3AT-resistant yeast transformant as a probe, the chromosomal copy of *cpcA* was isolated by screening an *A. niger* λEMBL4 genomic DNA library. Comparison of the cDNA with the genomic sequence revealed an 5′-located intron of 57 bp which interrupts an ORF of 735 bp (Fig. 2). The consensus sequences for the 5′ and 3′ borders of the intron, GTPuNGPy

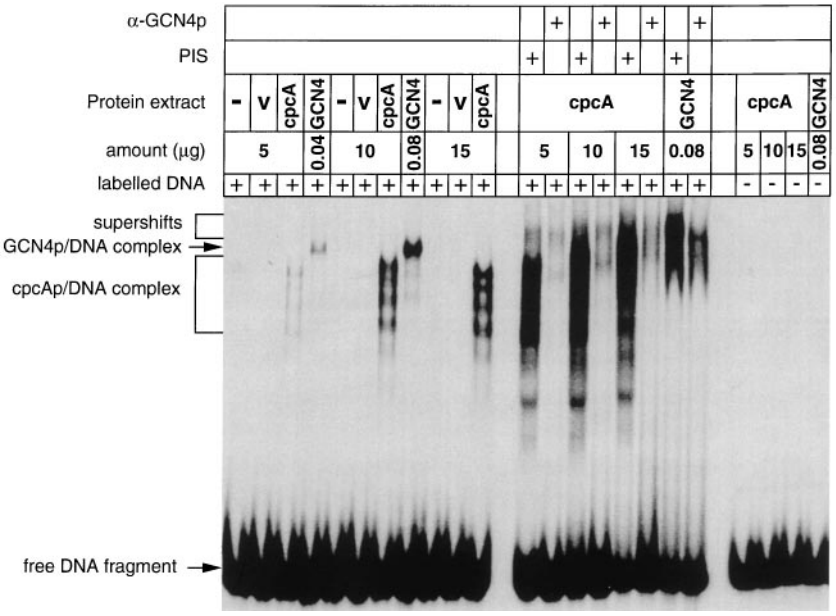


Fig. 1. Gel retardation analysis of protein extracts from the yeast strains RH1408 (–), RH1408 harbouring empty vector pEMBLyex4S/S (v), RH1408 harbouring the cloned *cpcA* cDNA in pEMBLyex4S/S (cpcA), and yeast GCN4p expressed in *E. coli* (GCN4). End-labelled DNA fragments containing either a wild-type GCRE (+) or a mutated GCRE (–) were incubated with different amounts of protein in the presence or absence of pre-immune serum (PIS) or a polyclonal antibody raised against the 60 C-terminal amino acids of GCN4p (α-GCN4p). Protein–DNA complexes were separated from unbound DNA by PAGE and visualized by autoradiography.

and PyAG, respectively, as well as, NNCTPuAPy, the putative internal consensus sequence for lariat formation, are conserved (Unkles, 1992).

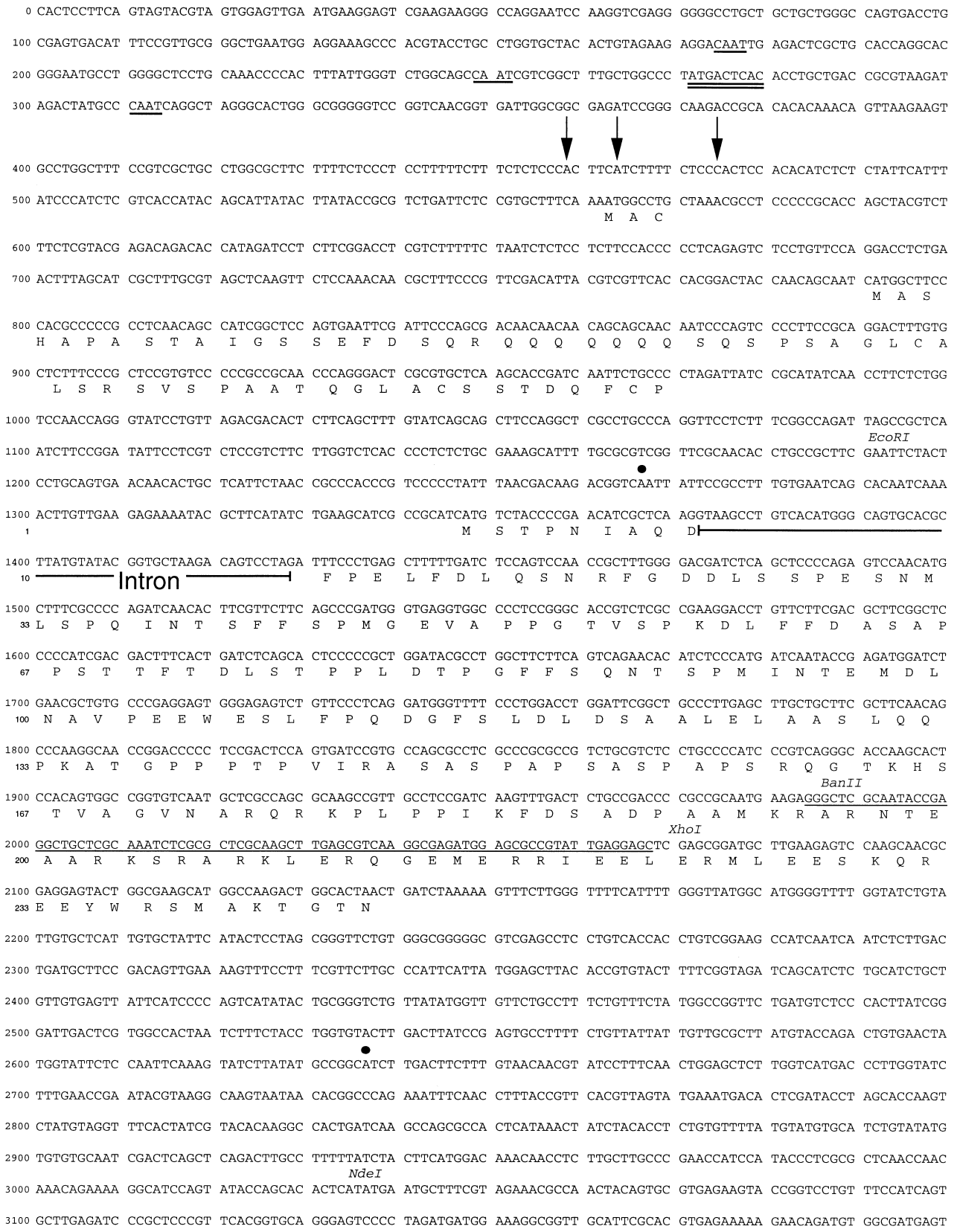
Gene disruption confirms that the gene encoding the isolated cDNA indeed represents the *A. niger GCN4* homologue. The DNA sequence encoding the presumed DNA-binding domain of cpcAp from amino acids 194–222 was deleted by replacing it with a phleomycin-resistance expression cassette (see Fig. 2). Five hundred phleomycin-resistant transformants were replica plated onto minimal medium containing 10 mM 3AT. Transformants which displayed reduced growth in the presence of 3AT compared to the wild-type strain were analysed by Southern hybridization for homologous integration of the phleomycin-resistance expression cassette. Two transformants revealed a single, homologous integration. The two replacement transformants display sensitivity towards 3AT on plates. Growth of the Δ*cpcA* strain on minimal medium is reduced by a factor of approximately two compared with the parental wild-type strain. Growth of the Δ*cpcA* mutant strain becomes abolished in the presence of 30 mM of 3AT, while the *A. niger* wild-type strain is able to grow on 3AT concentrations of up to 50 mM. These relatively high concentrations of inhibitor necessary for growth inhibition might indicate a poor uptake mechanism for the drug in *A. niger*.

Specific ornithine transcarbamylase (OTCase; E.C. 2.1.3.3.) activity was measured in mycelial extracts grown in the presence of 3AT to assay the abilility of the Δ*cpcA* strain to activate general control compared to the wild-type strain (Fig. 3). Specific OTCase activity has been shown in *A. nidulans* crude extracts to become derepressed upon amino acid starvation (Piotrowska, 1980) and the promoter of the corresponding gene (*argB*) carries GCREs which are necessary to derepress OTCase activity levels in response to amino acid starvation (Goc and Weglenski, 1988).

In *A. niger* wild-type mycelial extracts, 3AT induces an increase in specific OTCase activity from a basal level of ≈15 nkat mg^{-1} protein by a factor of four within 8 h of exposure to the drug. In the Δ*cpcA* mutant strain the basal level of specific OTCase activity is decreased to approx. 30% of the specific wild-type enzyme activity. Eight hours of exposure of the Δ*cpcA* mutant strain to 3AT does not even result in the basal specific enzyme-activity level of wild-type mycelium under non-starvation conditions. We therefore conclude that *cpcA* encodes the *GCN4* homologue of *A. niger*.

The cpcA *mRNA includes an approx. 900-nucleotide leader region*

While the cloned cDNA has a size of 1340 bp, Northern analysis revealed a hybridizing mRNA of a size of ≈2400 nucleotides (Fig. 4A). Therefore, the position of the transcriptional start site was narrowed down by Northern hybridization analysis. Overlapping DNA fragments generated by the polymerase chain reaction (PCR) which covered the regions upstream of the translational start codon of the *cpcA* ORF were used as labelled probes. The exact transcriptional start sites were determined by primer extension using two different oligonucleotides. Three transcription start sites were determined, located 890, 885, and 874 nucleotides upstream from the translational start codon. Signal intensities varied depending on the primer used (Fig. 4B). The three transcription initiation


Intron
EcoRI
BanII
XhoI
NdeI

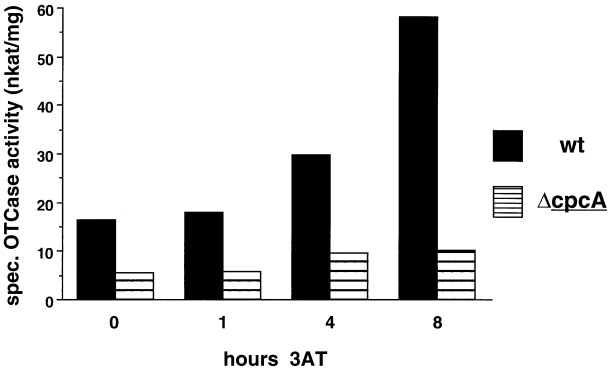


Fig. 3. Comparison of specific OTCase activity levels in *A. niger cpcA* wild-type (wt) and a mutant *A. niger* strain carrying a disrupted *cpcA* allele (Δ*cpcA*). Mycelium was transferred from minimal medium to minimal medium containing 20 mM 3AT for the indicated periods of time, and specific enzyme activities were determined in crude extracts. Bars represent mean values of three independent measurements. Standard deviations did not exceed 15%.

sites are located immediately downstream of a pyrimidine-rich stretch. CT-boxes have been shown in *Aspergillus* to be involved in transcription-start-site selection (Punt *et al*., 1990) as well as in transcription initiation (Hamer and Timberlake, 1987; Adams and Timberlake, 1990; Unkles, 1992).

The mRNA encoded by *cpcA* therefore contains an almost 900-nucleotide 5′ leader sequence that is not present in the isolated cDNA (Fig. 2). Within this sequence there are two small ORFs, capable of encoding peptides of a length of three and 60 amino acids, respectively. Small ORFs in 5′ untranslated regions have also been found in the homologous genes from yeast (Hinnebusch, 1984; Thireos *et al*., 1984) and *N. crassa* (Paluh *et al*., 1988). While the four μORFs in the yeast *GCN4* mRNA have been shown to be involved in translational control of gene expression (Mueller and Hinnebusch, 1986; Hinnebusch, 1992), the function of the two μORFs encoding two and 41 amino acids, respectively, in the 5′ leader of the *Neurospora cpc-1* mRNA (Paluh *et al*., 1988; GenBank Accession Number J03262, sequence update 1993) has not yet been investigated. Interestingly, the deduced amino acid sequences of the first μORF of *cpcA* and of the essential μORF1 of yeast *GCN4* (Mueller *et al*., 1988; Williams *et al*., 1988) are identical and the corresponding codons display only one mismatch at the DNA level. The second ORF in the *cpcA* leader contains 60 codons and is considerably longer than any of the μORFs of *GCN4* which encode peptides of 3, 2, 3, and 3 amino acids in length, respectively. The two μORFs of *cpcA* share no similarities with the two μORFs of *cpc-1*, either at the DNA or amino acid sequence level. However, these data suggest a translational control mechanism of *cpcA* gene expression similar to the control of yeast *GCN4*.

The cpcA *transcript level increases in response to amino acid starvation induced by 3AT*

The promoter region of *cpcA* contains three putative CAAT boxes (see Fig. 2), but lacks obvious TATA elements usually found downstream of CAAT boxes. It contains also one sequence motif which is similar to the GCRE consensus sequence 5′-ATGA(G/C)TCAT-3′ (Arndt and Fink, 1986) ≈190 bp upstream of the transcriptional start sites. While GCRE-like sequences are not found in the promoter of yeast *GCN4*, one such element is present in the *N. crassa cpc-1* promoter ≈300 bp upstream from the transcription-initiation sites (Paluh *et al*., 1988).

Evidence for additional transcriptional regulation of fungal genes that contain small ORFs in their 5′ leaders (Miller *et al*., 1992; Han *et al*., 1993; Prade and Timberlake, 1993) prompted us to monitor *cpcA* mRNA levels after general control was turned on (Fig. 5). In the presence of 3AT, which induces starvation for histidine, *cpcA* mRNA steady-state levels increase up to a factor of three after 8 h. After 1 h exposure to 3AT, a transient peak in the accumulation of *cpcA* transcript was observed. This peak in transcript levels is followed by a slow and continuous increase. Control of expression of *cpcA* is therefore composed of a transcriptional component and presumably also of an additional translational component, suggested by the presence of the two μORFs in the 5′ leader sequence.

The protein encoded by cpcA *contains only a single leucine residue in its bZIP domain*

cpcA encodes a bZIP-type protein of 245 amino acids with a calculated molecular weight of 26 811. The deduced amino acid sequence displays an identity of ≈35% and a similarity of ≈50% to GCN4p of yeast (Hinnebusch, 1984) and cpc-1p of *Neurospora* (Paluh *et al*., 1988), respectively. The putative basic DNA-binding domain at the C-terminus (amino acids 193–211) shows up to 70% identity at the amino acid level to other bZIP-type transcriptional activators including GCN4p, cpc-1p, and the related human proto-oncoprotein c-Jun (Bohmann *et al*., 1987), indicating binding affinity to similar target sequences (Fig. 6A).

Fig. 2. DNA sequence and deduced amino acid sequence of *cpcA* and flanking regions. Transcriptional start sites are indicated by arrows, putative CAAT boxes are underlined, and the putative GCRE is double underlined. The DNA sequence in the coding region of *cpcA* which has been replaced by a phleomycin-resistance expression cassette is also underlined. Restriction sites used for the disruption/deletion construct are indicated. The beginning and the polyadenylation site of the cloned cDNA are headed by a dot.

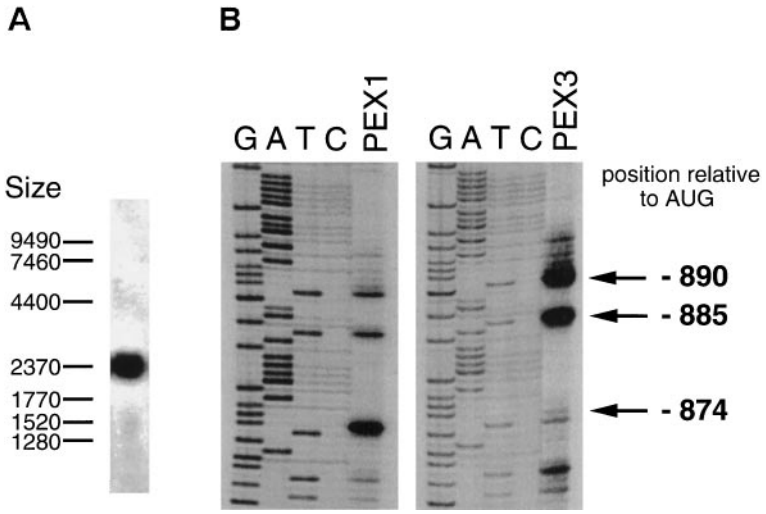


Fig. 4. Determination of transcript length and mRNA 5′ ends.
A. Northern blot analysis of total RNA of *A. niger* separated on an agarose/formaldehyde gel hybridized with the cloned cDNA as probe. RNA size markers (in nucleotides) are indicated on the left.
B. Primer-extension analysis using the primers PEX1 and PEX3 as described in the *Experimental procedures*. Lanes containing sequencing reactions using the same primers are headed by G, A, T, and C. The relative position of transcriptional start sites with respect to the AUG start codon of the ORF encoding cpcAp are indicated on the right.

Functional analysis of yeast GCN4p suggests the presence of two independent, multipartite transcriptional activation domains (Drysdale *et al*., 1995). Hydrophobic amino acid residues in the central acidic activation domain (CAAD) (originally identified by Hope and Struhl, 1986; Hope *et al*., 1988) as well as in an N-terminal activation domain (NTAD) play an important role in transactivation potency. Both domains, especially the critical hydrophobic amino acid residues, are conserved in cpcAp (Fig. 6B), again suggesting a similar function of both proteins within the cell.

Besides the basic DNA-binding domain, bZIP-type proteins are further characterized by a C-terminal leucine-zipper motif immediately adjacent to the DNA-binding domain, which enables dimerization (Harrison, 1991). Dimeric α-helical coiled coils comprise three to four repeats of seven amino acid residues (amino acid positions are designated from 'a' to 'g') in which the 'a' and 'd' positions are occupied by hydrophobic, and the 'e' and 'g' positions are occupied by charged amino acid residues (Alber, 1992). In most bZIP proteins, the 'd' position harbours a leucine residue, giving this motif its name. The corresponding region in cpcAp deviates from the classical leucine zipper in bearing only one leucine residue at the 'd' position (L222). Nevertheless, most 'a' and 'd' positions are occupied by other hydrophobic amino acid residues, and the 'e' and 'g' positions harbour mainly charged amino acid residues (Fig. 6A). The only exception is represented by S229, a hydrophilic residue at the 'd' position. However, the bZIP protein yAP-1 from yeast also carries an asparagine residue at the 'd' position in its leucine zipper (Moye-Rowley *et al*., 1989). Since cpcAp is similar to GCN4p, which has been shown to be active as a dimer (Hope and Struhl, 1987) and additionally is able to substitute for GCN4p *in vivo*, we suggest that the atypical leucine zipper of cpcAp also promotes dimerization.

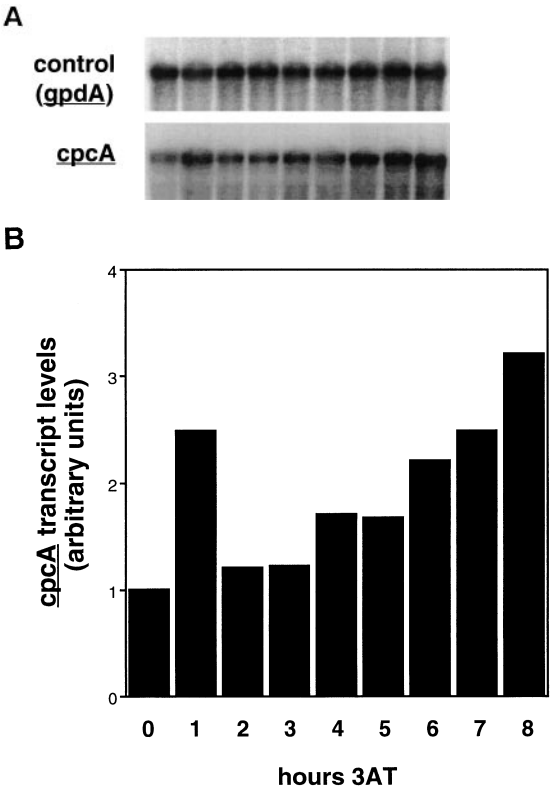


Fig. 5. Northern analysis of poly(A) RNA of *A. niger* grown in minimal medium and transferred to minimal medium containing 10 mM 3AT for the indicated periods of time.
A. mRNAs were separated on an agarose/formaldehyde gel, blotted onto a membrane filter, then hybridized with *gpdA* (as the internal, constitutive standard) and with the cloned *cpcA* cDNA as probes.
B. Quantification of *cpcA* mRNA levels normalized with respect to *gpdA* levels using a Phosphoimager. Bars represent mean values of three independent experiments. Standard deviations did not exceed 20%.

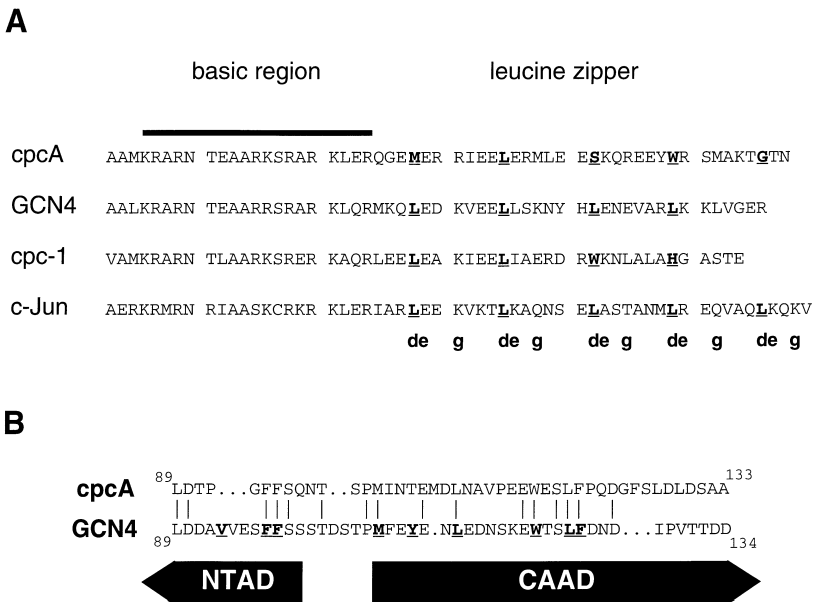


Fig. 6. A. Alignment of the deduced amino acid sequences of the bZIP motif from *cpcA* (*A. niger*), *GCN4* (yeast), *cpc-1* (*N. crassa*), and *c-Jun* (human). The basic DNA-binding domain is indicated by a horizontal bar. Amino acid residues at heptad positions ('d') in the leucine zipper region are in bold and underlined. Amino acid residues at the 'e' and 'g' positions are supposed to participate in dimer stabilization by electrostatic interactions. B. Alignment of the deduced amino acid sequences of *cpcA* and *GCN4* between the C-terminal end of the N-terminal activation domain (NTAD) and the N-terminal part of the central acidic activation domain (CAAD). Amino acid residues identified in GCN4p to be essential for transactivation potency (Drysdale *et al*., 1995) are in bold and underlined. Numbers indicate the positions in the deduced amino acid sequences.

Discussion

The phenomenon of general control of amino acid biosynthesis is ubiquitously found in lower eukaryotes, including yeast and filamentous fungi, to provide sufficient amounts of protein precursors under various environmental conditions. Two central components have been described which appear to be characteristic for this mechanism. One protein is the bifunctional protein kinase GCN2p which has been described in yeast (Wek *et al*., 1989; Dever *et al*., 1992) and recently also in *N. crassa* (Sattlegger, 1996). GCN2p seems to be the essential sensor for the level of uncharged tRNAs in the cell. The other protein is the ultimate transcriptional activator which increases the initiation of transcription of all the target genes in the general control network.

The ability of the *A. niger* cpcAp to substitute for GCN4p in yeast *in vivo* and to confer resistance to inhibitors of amino acid biosynthetic enzymes, indicates that general-control transcriptional activators such as yeast GCN4p, *N. crassa* cpc-1p, and *A. niger* cpcAp are functionally conserved throughout these organisms.

The three proteins share subdomains which display different degrees of amino acid conservation resulting in an ≈30% overall identity. A classification can be deduced which apparently reflects the strategic variability of different organisms in order to conserve individual functions of subdomains, ranging from conservation of primary amino acid sequence up to conservation of secondary, tertiary and quaternary structural motifs.

The deduced amino acid sequence of the DNA-binding domain is not only highly conserved between GCN4p, cpc-1p, and cpcAp, but also displays significant identity to other bZIP-type transcriptional activators such as human c-Junp. This high degree of conservation is demonstrated by the apparent identical DNA-binding specificity of GCN4p and cpcAp (Fig. 1), reflecting the affinity of this family of transcription factors for similar *cis*-acting target sequences, and by the ability of a polyclonal antibody raised against the bZIP domain of GCN4p to specifically recognize cpcAp expressed in yeast (data not shown).

The transcriptional activation domains appear to be less conserved at the amino acid sequence level. Acidic and hydrophobic amino acid residues critical for transactivation potency of GCN4p (Hope and Struhl, 1986; Hope *et al*., 1988; Drysdale *et al*., 1995) are conserved in cpcAp and cpc-1p (see Fig. 6B; Paluh *et al*., 1988). However, structural motifs of higher order seem to be important in addition to identical primary amino acid sequences.

The C-terminal leucine zipper of *cpcA* represents the most degenerate of the known domains comparing the deduced primary amino acid sequences (Fig. 6A). The 'leucine zipper' of cpcAp contains only a single leucine residue at the 'd' position. Methionine, tryptophan, and serine residues occupy the other 'd' positions. The respective positions in *Neurospora* cpc-1p show only two leucine, a tryptophan, and a histidine residue compared to the 'classical' leucine zippers of GCN4p and c-Junp. These two leucine residues have been shown to be non-essential for functionality of cpc-1p, by the fact that they can be replaced by other hydrophobic amino acids (Paluh and Yanofsky, 1991). Additionally, mutational analysis of leucine residues in GCN4p (Hu *et al*., 1990; van Heeckeren *et al*., 1992) indicates that these can be substituted by not only hydrophobic but even hydrophilic amino acid residues without affecting the protein functionality. Therefore,

leucine residues at the 'd' positions are not a prerequisite for coiled-coil formation, but seem, in concert with residues in the 'a' position, to influence the oligomerization status (two, three, or four helix bundles) of the protein complex (Harbury *et al*., 1993). Whether the atypical leucine zipper of cpcAp promotes oligomerization, as one might speculate from the multiple DNA–protein complexes of the gel retardation assay (Fig. 1), remains to be shown. Degradation of cpcAp when expressed in yeast was not observed in Western blot analysis using an antibody against the bZIP domain of GCN4p (data not shown) and therefore does not seem to contribute to the appearance of multiple retarded bands in gel shift analysis (Fig. 1). The leucine zipper of cpcAp represents a highly degenerate, naturally occurring, and apparently functional oligomerization motif. Similar degenerate leucine zippers have, as yet, only been obtained by *in vitro* mutagenesis.

In yeast, regulation of *GCN4* expression has been shown to occur at the translational level mediated by the μORFs present in the ≈600-nucleotide 5′ leader sequence (Mueller and Hinnebusch, 1986; Miller and Hinnebusch, 1989). A similar role has been suggested for the ≈700-nucleotide *Neurospora cpc-1* 5′ leader containing two μORFs (Paluh *et al*., 1988). In the case of *cpcA*, the presence of μORFs strongly suggests that regulation also takes place at the translational level. The similarity between the first μORF of *cpcA* compared to the regulatory essential μORF1 of yeast *GCN4* supports this view.

In addition to the supposed translational control we provide evidence for transcriptional control in *cpcA* gene expression. The time-course of steady-state *cpcA* transcript levels in response to amino acid starvation appears to be composed of two phases. Within 2 h, *cpcA* transcript levels increase transiently and resume a level similar to that found under non-starvation conditions. Afterwards, *cpcA* mRNA levels increase in a continuous manner. This continuous increase over 8 h is accompanied with the physiological response to amino acid starvation by derepressing biosynthetic enzymes such as OTCase (Fig. 3). In contrast, the transient increase of *cpcA* mRNA, which has been also described in *Neurospora* for *cpc-1* (Paluh *et al*., 1988), does not affect OTCase activity levels in *A. niger*. While the significance of this transient increase remains to be elucidated, it is obvious that transcriptional control mechanisms govern *cpcA* expression in addition to the supposed translational control suggested by the presence of μORFs.

The presence of a GCRE-like sequence in the promoters of *cpcA* and *cpc-1* (Paluh *et al*., 1988) leads to the speculatation that an autoregulatory component is involved in their expression. A similar combinatorial mechanism of transcriptional and translational control has been shown for the developmental regulators *stuA* and *brlA* of *A. nidulans* (Miller *et al*., 1992; Han *et al*., 1993; Prade and Timberlake, 1993). While yeast *GCN4* obviously lacks such transcriptional control elements, the sister genes of the *Jun* family are regulated at the transcriptional level, including autoregulation (Angel and Karin, 1991; Berger and Shaul, 1994). It appears that regulation of gene expression within this class of transcriptional activators ranges from translational control (yeast *GCN4*), to a presumable combination of translational and transcriptional mechanisms (*Aspergillus cpcA*; *Neurospora cpc-1*), to transcriptional regulation (mammalian *Jun*).

Experimental procedures

Aspergillus *techniques*

A. niger N402 (*csp*A1; Bos *et al*., 1988) was used as the wild-type strain and cultivated on minimal medium (Bennet and Lasure, 1991) at 30°C. Transformation was carried out as described (Punt and van den Hondel, 1992) and transformants were selected on minimal medium containing 10 μg ml^{-1} phleomycin (Cayla). The chromosomal library of *A. niger* N402 contained *Sau*3AI partially digested, size-selected DNA in λEMBL4 (Stratagene).

Screening of the A. niger *pEMBLyex4S/S cDNA expression library*

Messenger RNA obtained from *A. niger* grown on minimal medium containing 0.5% yeast extract and 0.1% casamino acids was transcribed into cDNA and cloned into the *Sma*I site flanked by *Sfi*I linkers of the 2μ yeast/*E. coli* shuttle vector pEMBLyex4S/S, which is a derivative of pEMBLyex4 (Goldman *et al*., 1992). The expression library driven by the yeast *GAL10* promoter, provided by Dr R. Contreras (Gent University, Belgium), was transformed (Schiestl and Gietz, 1989) into the *S. cerevisiae* strain RH1408 (MAT**a**, Δ*gcn4-103*, *ura3-52*, *gal2*) lacking the 131 C-terminal codons of the *GCN4* ORF (Hinnebusch, 1985; Mösch *et al*., 1990). Transformants were screened for growth on MV medium (Miorazzi *et al*., 1983) containing 5% galactose and 0.2% sucrose as the carbon source, 40 mg ml^{-1} arginine, and 10 mM 3AT.

Protein methods

Protein contents were estimated according to Bradford (1976). OTCase (E.C. 2.1.3.3.) activity in crude extracts of *A. niger* was assayed as described (Davis, 1962) at 30°C. Yeast crude extracts for gel retardation and Western blot analysis were prepared as described (Arndt *et al*., 1987). *S. cerevisiae* strain RH1408 harbouring the *cpcA* cDNA in pEMBLyex4S/S was grown in MV medium containing 5% galactose and 0.2% sucrose as the carbon source. Yeast strain RH1408, either untransformed or harbouring the empty expression vector, pEMBLyex4S/S, was grown in the same medium containing 40 mg l^{-1} arginine. Cultures were harvested at an OD_{546} of 2.0.

GCN4p expressed in *E. coli* was purified as described (Arndt and Fink, 1986; Braus *et al*., 1989).

Recombinant DNA techniques

Unless otherwise stated, standard procedures were used (Sambrook *et al*., 1989).

DNA was sequenced using the dideoxy chain-terminating method (Sanger *et al*., 1977) utilizing custom oligonucleotides (MWG Biotech) and the T7 sequencing kit (Pharmacia).

The *cpcA* disruption/deletion cassette (see also Fig. 2) was constructed in pAN8-1 (Punt and van den Hondel, 1992). An 800 bp *Eco*RI–*Ban*II (with the *Ban*II site blunted) fragment, containing the *cpcA* 5′ region, was inserted into *Eco*RI/*Bgl*II (with the *Bgl*II site blunted)-digested pAN8-1. The 3′ region of *cpcA* was cloned as a 1 kb *Xho*I–*Nde*I (with the *Xho*I site blunted) fragment into *Xba*I/*Nde*I (*Xba*I site blunted)-digested pAN8-1 containing the *cpcA* 5′ region. The resulting 5.1 kb *Eco*RI–*Nde*I linear DNA fragment was used for transformation.

Northern blot and primer-extension analyses

Total RNA from *A. niger* was isolated using the TRIzol reagent (Gibco BRL Life Sciences), and mRNA was purified using Oligotex (Qiagen). RNA was separated on denaturing agarose gels and electroblotted (Egli *et al*., 1995) onto Biodyne B membranes (Pall) and hybridized at 42°C according to the manufacturer's instructions. DNA probes were labelled by random priming (Feinberg and Vogelstein, 1984). Hybridizing signals were quantified with a BAS-1500 Bio-imaging analyser (Fuji).

Primer-extension analysis was performed on mRNA isolated from *A. niger* growing on minimal medium using the primers PEX1 (5′-GAAGAGGATCTATGGTGTCTGTCTCG-TACG-3′) and PEX3 (5′-GCTGGTGCGGGGGAGGCGTT-TAGCAGGCC-3′). Phosphorylated primer (*c*. 17 kBq) and 5 μg of mRNA were ethanol precipitated, resuspended in 8 μl H_2O, annealed for 3 min at 70°C, and placed on ice. Next, 12 μl of PE mix (150 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$, 30 mM dithiothreitol (DTT), 0.8 mM dNTPs, 30 U RNAguard (Pharmacia), 17 U AMV reverse transcriptase (Pharmacia)) were added and incubated for 10 min at room temperature, followed by 1 h at 42°C. Products were loaded next to sequencing reactions using the same phosphorylated oligonucleotide as primer, and separated on polyacrylamide gels (6%) containing 8 M urea.

Gel retardation analysis

Protein was incubated in the presence of an 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). Alternatively, before adding the labelled DNA fragment, protein was pre-incubated in the presence of pre-immune or immune serum that had been raised in a rabbit against a synthetic peptide identical to the 60 C-terminal amino acids of GCN4p.

Nucleotide sequence accession number

The DNA sequence reported in this article has been submitted to the EMBL Nucleotide Sequence Database (Accession Number X99215).

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