cpc-3, the *Neurospora crassa* Homologue of Yeast GCN2, Encodes a Polypeptide with Juxtaposed eIF2α Kinase and Histidyl-tRNA Synthetase-related Domains Required for General Amino Acid Control*

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Based on characteristic amino acid sequences of kinases that phosphorylate the α subunit of eukaryotic translation initiation factor 2 (eIF2α kinases), degenerate oligonucleotide primers were constructed and used to polymerase chain reaction-amplify from genomic DNA of *Neurospora crassa* a sequence encoding part of a putative protein kinase. With this sequence an open reading frame was identified encoding a predicted polypeptide with juxtaposed eIF2α kinase and histidyl-tRNA synthetase-related domains. The 1646 amino acid sequence of this gene, called cpc-3, showed 35% positional identity over almost the entire sequence with GCN2 of yeast, which stimulates translation of the transcriptional activator of amino acid biosynthetic genes encoded by GCN4. Strains disrupted for cpc-3 were unable to induce increased transcription and derepression of amino acid biosynthetic enzymes in amino acid-deprived cells. The cpc-3 mutation did not affect the ability to up-regulate mRNA levels of cpc-1, encoding the GCN4 homologue and transcriptional activator of amino acid biosynthetic genes in *N. crassa*, but the mutation abolished the dramatic increase of CPC1 protein level in response to amino acid deprivation. These findings suggest that cpc-3 is the functional homologue of GCN2, being required for increased translation of cpc-1 mRNA in amino acid-starved cells.

In lower eukaryotes, like *Neurospora crassa* and *Saccharomyces cerevisiae*, starvation for any one of a number of amino acids leads to simultaneously induced transcription followed by derepression of the enzymes in many amino acid biosynthetic pathways. The global regulatory mechanism is referred to as general amino acid control (discovered as “cross-pathway control” in *N. crassa*, see Refs. 1 and 2). The ultimate element of the signal transduction pathway, a transcriptional activator protein, is encoded by the homologous genes, cpc-1 of *N. crassa* (3) or GCN4 of *S. cerevisiae* (4), respectively. Recently, homologous proteins were reported also for *Aspergillus niger* and *Cryptonecia parasitica* (5, 6). In yeast, GCN2 plays a crucial role in signal perception and transduction. GCN2 encodes a protein containing an eIF2α1 kinase domain (7–9) that is required for increased GCN4 protein synthesis in amino acid-starved cells.

eIF2α kinases regulate initiation of protein synthesis (10) by phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF2α) on Ser-51. GTP-bound eIF2 is necessary for delivery of charged initiator tRNA<sup>Met</sup> (Met-tRNA<sup>Met</sup>) to the 40 S ribosomal subunits, and after initiation of translation it is released as eIF2-GDP. The phosphorylated form of eIF2 sequesters its own recycling factor eIF2B necessary for exchange of GDP by GTP (11). As only the GTP-bound form of eIF2 is able to initiate translation, sequestering of eIF2B leads to a general reduction of protein synthesis. However, activation of GCN2 in yeast leads to increased translation of one mRNA species, GCN4 mRNA. This gene-specific regulation is mediated by four short upstream open reading frames (uORF) in the 5′ leader of GCN4 mRNA (4).

Extensive genetic analysis of the GCN4 mRNA leader has provided a detailed model for GCN4 translational regulation (4). Irrespective of amino acid availability, the first uORF is translated, and about 50% of the ribosomes resume scanning on the mRNA. Under non-starvation conditions translation of the following three uORFs leads to dissociation of almost all the ribosomes from the mRNA due to specific sequences surrounding the translational stop codons, and therefore translation of GCN4 is prevented. Under amino acid starvation conditions GCN2 becomes activated and phosphorylates eIF2α, leading to low levels of GTP-bound eIF2 and, therefore, reduced concentration of eIF2-GTP-Met-tRNA<sup>Met</sup> ternary complexes. Consequently, after translation of uORF1, ribosomes resume scanning, but the rate at which they rebind ternary complexes is lowered. Thus, ribosomes are less able to re-initiate at any of the translation initiation sites of the following three uORFs, and many re-initiate at GCN4 instead.

So far three eIF2α kinases are known that share extensive homology in the kinase catalytic domain. Apart from the 12 conserved subdomains found in most protein kinases, they have additional characteristic features, including an insert between subdomains IV and VI and subdomains IX and X, re-

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spectively, which distinguishes them from other serine/threo-
nine kinases (10, 12). However, each of these kinases are
activated by distinct stimuli as follows: the heme-regulated
inhibitor (HRI) in rabbit and rat by heme deficiency (13, 14),
the double-stranded RNA-dependent kinase (PKR) in human,
mouse, and rat by the occurrence of double-stranded RNAs
after virus infection (15–17), and GCN2 of S. cerevisiae
by amino acid deprivation. The activation signal and target for the
recently discovered Drosophila melanogaster homologue of
yeast GCN2, DGCN2, are not known (18). In addition to the
kinase catalytic domain, each eIF2α kinase contains unique
sequences that may be responsible for its own characteristic
regulation. For example PKR contains two double-stranded
RNA-binding motifs required for RNA binding (19, 20). Within
the kinase catalytic domain of HRI, two heme regulatory motifs
are known (21, 22). Adjacent to the eIF2 kinase catalytic
domain, GCN2 contains a domain that resembles the histidyl-
RNA synthetases (HisRS), which was postulated to monitor
amino acid availability (8).

Early work by various N. crassa and yeast researchers (23)
demonstrated that uncharged aminoacyl-tRNAs that accumulate in
amino acid-deprived cells are the relevant signal in the mech-
anism of general control. Mutations in the HisRS-like domain
of GCN2 were found to impair phosphorylation of Ser-51 of
eIF2α and the derepression of GCN4 mRNA translation in
amino acid-starved cells. Wek et al. (9) could demonstrate bind-
ing of uncharged tRNAs to the synthetase-related domain.
The exact interaction between the GCN2 regulatory and catalytic
domains upon activation is not yet known. The N-proximal
domain containing a degenerate protein kinase moity (8, 24)
and the C-terminal region beyond the HisRS-like domain are
also required for GCN2 function (25). For the latter, Ramirez et al.
(25) demonstrated a function in ribosome association of the
protein and a role in dimerization was recently elucidated as
well (89).

In contrast to yeast, where GCN2 and several other elements
were identified genetically by abundant mutations that impair
general amino acid control, all but one of the regulation-defi-
cient mutations of N. crassa mapped in the cpc-1 gene (26–28).
The one exception was a mutation that identified the cpc-2 gene
(30, 31); however, cpc-2 of N. crassa showed no relationship with
any of the known yeast genes involved in general amino acid
control. We were interested, therefore, to find out whether
substantial differences exist in the details of the mechanism of
amino acid regulation between these ascomycetes and searched
for a N. crassa gene with homology to yeast GCN2.

Here the molecular identification of the N. crassa cpc-3 gene
and its characterization as a structural homologue of yeast
GCN2 is reported. The molecular engineering of a cpc-3 disrup-
tion allele and the phenotypic consequences of the loss of func-
tion are described. Our results show that the cpc-3 product is a
positive regulator of the general control response of N. crassa
and most likely functions as a translational activator of cpc-1,
alogous to the function of yeast GCN2.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—The N. crassa wild-type strain (St.
Lawrence 74A) and the strains cyh-2A, arg-12a, arg-12b, b were ob-
tained from the Fungal Genetics Stock Center (FGSC, University
of Kansas Medical Center); the cpc-1(2-5) and cpc-2 U142 mutant
strains were from the Barthelmes lab.

N. crassa cultivation on Vogel’s standard medium and crossing tech-
niques followed Davis and de Serres (32). Briefly, for enzyme assays
and DNA, RNA, and protein isolation, exponentially grown mycelium
was obtained by inoculation of 100 ml of liquid medium with 0.5–1 × 10^6
conidia and incubation overnight at 29 °C and 170 rpm. For growth
tests 1 ml of stagnant liquid medium was inoculated with mycelial
slants and incubated at 29 °C.

If required, Vogel’s medium was supplemented with final concen-
trations of 0.5 μg/ml benomyl, 1 μg/ml cycloheximide, 250 or 333 μg/ml
(liquid or solid medium) hygromycin B, 4 or 6 mM (stagnant liquid
or exponential culture) 3-amino-1,2,4-triazole (3AT), and 40 mM
acetate (omitting glucose), respectively. All supplements were prepared
as stock solutions, sterile-filtered, and added to the autoclaved medium.

Plasmids and Libraries—Puriﬁcation of N. crassa genomic DNA
library of Vollmer and Yanofsky (33) was used to screen for cpc-3 se-
quences. Plasmids used in this study were pCPC1-C for cpc-1 (3),
pCPC2-C8 for cpc-2 (31), arg-12 in pUC8 (34), pACTIN for the actin
encoding gene (M. Plamann), pBT6 for the Bml cassette (35), and
pCSN43 for the hph cassette (36).

DNA fragments were cloned, and the cpc-3 disruption allele was
constructed in pbLueskRT. PCR amplification products were cloned in
pUC19. E. coli strains used were DH1 for the cosmid library and
XL-1 blue for all other purposes. Transformation of E. coli was carried
out according to Mandel and Higa (88) or, in case of plasmids larger than
10 kb, via electroporation (37, 38).

Transformation of N. crassa—Spheroplasts obtained from germinat-
ing conidia were used for transformation (33). Transformants were
made homokaryotic via the isolation of microconidia-derived colonies
(39).

Isolation and Analysis of DNA—Isolation of high quality and pure
genomic DNA from N. crassa followed the method of Lee et al. (40). For
PCR analysis of large numbers of genomic DNA samples the methods of
relays (41) and Chow and Chow (42) were employed. N. crassa
was incubated for 2 days in 1 ml of stagnant liquid culture.
Mycelia were squeezed between Whatman paper and transferred to 0.2 ml
of isolation buffer (0.2 M Tris-HCl, 0.5 M NaCl, 0.01 M EDTA, 1%
SDS, pH 7.5). After addition of glass beads (0.3–0.4 mm diameter) and
0.2 ml of 1 M phenol:chloroform the samples were vortexed for 5 min
followed by addition of 0.3 ml of isolation buffer and 0.3 ml of phenol:
chloroform and centrifugation (30 s, 5000 × g). The liquid phase was
again extracted with 0.3 ml of phenol:chloroform. The DNA was
preincubated with 1 μl of ethanol, dissolved in 100 μl of TE buffer
(containing 100 μg/ml RNase) at 37 °C for about 1 h, ethanol-precipitated,
and finally dissolved in 50 μl of TE buffer.

Southern analysis followed standard protocols (43) using nylon mem-
branes (Amersham Pharmacia Biotech). Probes were labeled with DIG-
11-dUTP (DIG-DNA random primed labeling kit, Boehringer Mann-
heim). Labeling of DNA shorter than 700 bp was performed by PCR
reaction (see below, except that 1/3 of dTTP was replaced by DIG-dUTP),
and the labeling reaction was used directly for hybridization. Hybridi-
zation and detection of probes and stripping of probes followed the
manufacturer's protocol (DIG luminescent detection kit,
Boehringer Mannheim).

PCR reaction mixtures consisted of 1× PCR buffer (10 mM Tris-HCl,
1.5 mM MgCl2, 50 mM KCl, pH 8.3, Boehringer Mannheim), 200 μM
of each dNTP, 500 nM each primer, 0.02 units/μl Taq polymerase (Perkin-
Elmer or Boehringer Mannheim), and 5 ng/μl genomic DNA or 5 pg/μl
plasmid/complete DNA. PCR of 10–100 μl volumes were performed in a
Perkin-Elmer DNA thermal cycler TC1; the cycles were 30 s at 95 °C (5
cycles in the first cycle), 1 min at the annealing temperature (5 °C lower
than Tm, for degenerate primers see Table I). Extension time was 1 min
per 1 kb at 72 °C.

For RT-PCR analysis cDNA was synthesized from 1 μg of total RNA
using Superscript RT RNase H− reverse transcriptase (Life Technolo-
gies, Inc.) according to the manufacturer's protocol. Aliquots of the
reverse transcription reaction mixture (10% v/v) of final PCR reaction
were directly subjected to PCR reactions.

Screening of the Genomic Library—Clones of each microtiter plate of
the N. crassa ordered genomic cosmid library (33) were pooled, and pure
DNA was isolated (plasmid midikit, Qiagen). By using 1 μg DNA of each
pool, a dot blot membrane was generated and screened using cpc-3-spe-
cific sequences as probes (hybridization technique as described for
Southern analysis). To identify the individual positive clones, colonies
of each microtiter plate of interest were transferred to solid medium
with a microtiter replica plater and subjected to colony hybridization
(43).

DNA Isolation and Northern Blot Analysis—Isolation of total cellular
RNA and preparation of Northern blots were done according to
Sokolowsky et al. (44) using 10 μg of RNA of each sample and nylon
membranes (Na +, Amersham Pharmacia Biotech). Probing was done
according to Sambrook et al. (43). DNA probes were radiolabeled with
α-32PdCTP (random-primed labeling kit, Life Technologies, Inc.)
and purified on Sephadex columns (43). Probes were stripped from
membranes by washing with 5% (w/v) SDS at 65 °C for at least 10 min.

Nucleotide Sequence Analysis—By using PCR, DNA sequences were
determined by the Sanger dideoxy sequencing method (fmol sequenc-

20405
Lowercase letters indicate nucleotides added at the 5' end for the construction of restriction sites (underlined). (Numbering of the nucleotide positions refers to the translation start point of cpc-3.) The PCR primers were tested for suitability using the program OLIGO (TIB molbiol). The annealing temperatures used for degenerated primers are indicated. s, sense primer, a, antisense primer. Letters indicating variability are Y (C or T), I (inosine), R (A or G), and H (A or C or T).

**Table I**

| Name   | Position | Restriction enzyme |
|--------|----------|--------------------|
| cpc-3, degenerated and sequence specific oligonucleotides |         |                |
| 2.1 s  | 2414     | SalI              |
| 2.3 a  | 2653     | BamHI             |
| 4.1 s  | 1888     | SphI              |
| 4.2 a  | 2523     | BglII             |
| 5.1 s  | 2586     | SalI              |
| 5.2 a  | 3407     | EcoRI             |
| 6.1 s  | 3301     | KpnI              |
| 6.2 a  | 4155     | PatI              |
| 11.1 s | 511      |                |
| 14.1 s | –220     |                |
| 14.2 a | 612      |                |
| BmalU   |          |                |
| BmalU s |          |                |
| hph    |          |                |
| 11.2 a |          | TTCAATATCATCTTCTGCGACCTC |

**RESULTS**

**Molecular Identification of the N. crassa cpc-3 Gene**—Based on the amino acid sequences in the catalytic domains of eIF2α kinases (8, 13, 15, 16), highly conserved groups of amino acids in the insert region between kinase subdomains IV and VI (characteristic of eIF2α kinases) and in subdomain VII were chosen for the construction of degenerate oligonucleotides (called 2.1 and 2.3, Table I). The oligonucleotides bracketed subdomain VI that contains amino acids characteristic of serine/threonine protein kinases and is surrounded by amino acids typical of eIF2α protein kinases. Knowledge of the sequence of PCR fragments amplified with these primers should be sufficient to determine whether or not they derived from a gene encoding an eIF2α kinase. By using genomic N. crassa DNA as template, a single 1020-bp PCR product, called 2.1–2.3, was obtained using 2.1 and 2.2 as primers (Fig. 1B) that encodes an amino acid sequence with 60% sequence identity to the corresponding yeast GCN2 segment.

The 2.1–2.3 PCR product was used as a probe to isolate two cosmids clones (17.5D and 20.1F) from an ordered genomic library (33). By using cosmid 17.5D as template, and in each case a specific primer constructed from already sequenced areas, and a degenerate primer (see below), adjacent overlapping stretches of DNA were synthesized by PCR. Degenerate primer 4.1 was constructed according to characteristic amino acids of protein kinase subdomain I. Construction of degenerate primers 5.2 and 6.2, respectively, was guided by conserved sequences of HisRS proteins. Sequencing of the PCR-amplified fragments 4.1–4.2, 5.1–5.2, and 6.1–6.2 (Fig. 1B) indicated that N. crassa contains a GCN2-like gene (i.e. encodes a protein characterized by juxtaposed kinase and HisRS-like domains).

From PCR fragments and subcloned restriction fragments of cosmid 17.5D (Fig. 1C), a restriction map was derived (Fig. 1A). Sequencing of PCR fragments and subcloned restriction fragments or direct sequencing of cosmid 17.5 DNA led to the determination of a DNA sequence with coding capacity for a GCN2-like polypeptide (Ref. 50, accession number X91867).

By using 2.1–2.3 (Fig. 1B) for restriction fragment length polymorphism studies (51, 52), the corresponding N. crassa gene, cpc-3, was located on the right arm of linkage group V close to cyh-2 (4.2% linkage). Southern hybridization with the 2.1–2.3 DNA probe or with larger DNA segments (4.1–4.2 and 6.1–6.2, respectively) suggested that cpc-3 represents a single copy sequence (not shown).

By using the 2.1–2.3 fragment, or larger cpc-3 sequences, as a probe in Northern experiments, we observed a faint signal of about 6 kb in total RNA, indicating a low abundance mRNA. However, using RT-PCR methods the expression of cpc-3 was unambiguously demonstrated (not shown). Low expression of cpc-3 is suggested by the codon usage which is typical for low and non-constitutively expressed genes (53).

**Genomic Organization of the cpc-3 Locus**—cpc-3 is 5162 bp in length and consists of 5 exons totaling 4941 bp of cpc-3 coding region. The 4 introns were identified by the conserved splice junctions and lariat sequences (54, 55) and confirmed by RT-PCR reactions using intron flanking primers (not shown). Intron positions did not coincide with the domain structure of the cpc-3-encoded polypeptide. GCN2 lacks introns (8). For DGCN2 only cDNA sequences have been reported (18).

The putative translation start point was narrowed down via the determination of the most 5' in-frame stop codon. Sequences surrounding the first downstream ATG codon showed the best match to the N. crassa Kozak consensus sequence as compared with further downstream ATG codons (54, 55). RT-PCR analysis verified that this putative translational start codon and the sequences upstream of it were part of the cpc-3
mRNA (Fig. 2). This also indicated that the 5' leader sequence is at least 220 bp in length (Fig. 2), which is unusually long for filamentous fungi (53). The sequence (TGTATTA) 77 bp downstream from the TGA codon may represent a polyadenylation signal (AGTATAA, see Refs. 53 and 54). The length of the transcription unit is at least 5238 bp, in agreement with the length of the observed faint transcript (6 kb).

Sequence analysis of the upstream sequences of cpc-3, GCN2, and DGCN2 showed no remarkable homologies, and whereas all three contain uORFs, their positions are not conserved. Located 136 bp upstream of cpc-3 is an uORF coding for 5 amino acids; however, as the whole sequence of the 5' leader is not known its translational start site might be further upstream. Two uORFs also are present upstream of DGCN2 (distance from DGCN2, 38 and 95 bp). uORFs were found that overlap with the translational start points of DGCN2 and GCN2 but not cpc-3. If the uORFs are involved in translational regulation, the expression of the GCN2-like proteins might be differently regulated.

Comparative Analysis between N. crassa CPC3 and the Yeast and Drosophila GCN2 Polypeptides—The deduced amino acid sequence of 1646 amino acids showed the highest overall similarity to GCN2 of yeast (Ref. 8, translation start site according to Ref. 8 and accession number U51030) and DGCN2 of Drosophila (18) (31% identity between GCN2 and DGCN2), with 35 and 32% positional identity, respectively, over almost the entire length of the cpc-3-encoded polypeptide (Fig. 3). Only about 30 amino acids at the N terminus of CPC3 were exempt from the alignment, i.e. CPC3 was found to be longer than Drosophila or yeast GCN2 (8, 18), respectively. The high similarity between the proteins is highlighted when the comparison includes equivalent amino acids (PROSIS); at 58/54% of the positions of GCN2/DGCN2 either identical or equivalent amino acids were found in CPC3 (54% between GCN2 and DGCN2).

The similarity between the proteins allowed us to distinguish for CPC3, as for the GCN2 proteins, four regions/domains with characteristic features: the eIF2α kinase and histidyl-tRNA synthetase-like (HisRS-like) domains and the N- and C-terminal regions (Fig. 1D).

The highest sequence conservation was observed in the kinase domain (amino acids 597–971, Fig. 4), among CPC3, GCN2, and DGCN2 with 46/42/41% (CPC3 versus GCN2/CPC3 versus DGCN2/GCN2 identity and 64/61/60% similarity. This domain in CPC3 contains all the invariant and most of the highly conserved amino acids found in the subdomains of protein kinases (12, 56) (Fig. 4). Based on the sequences of subdomains VI and VIII, it is expected to be a serine/threonine kinase. CPC3 bears a large insert of about 120 amino acids between subdomains IV and VI typical of eIF2α kinases. It also contains most of the signature amino acids of
FIG. 3. Multiple sequence alignment of *N. crassa* CPC3 (accession number X91867), yeast GCN2 (Ref. 6, U51030), and *Drosophila* DGCN2 (Ref. 15, U80223) using GCG program PileUp (gap creation penalty 6, gap extension penalty 2), with some manual adjustment in areas of low similarity. The N- (\(\ldots\)) and C-terminal (\(\ldots\),) ends of the CPC3 N-terminal domain, eIF2\(\alpha\) kinase (PK domain), HisRS-like, and C-terminal domains are indicated. In the N-terminal domain, amino acids written in **bold letters** represent positions matching with kinase-characteristic sequences. Kinase subdomains (roman numerals) and C-/N-terminal boundaries, their characteristic amino acids including tyrosine (\(Y\)) and serine/threonine kinase-specific sequences (\(2\)) are indicated above the alignment of the degenerate kinase region (12, 56, 80). Positions conserving nonpolar residues (\(\S\), FYWIMVA), polar residues (\(\S\), HRKDENQ), small residues with near neutral polarity (\(\S\), PGST), and aromatic residues (\(o\), YFW) are indicated. Lowercase letters represent modest conservation of the corresponding amino acids, **capital letters** high conservation, and **bold capital letters** invariant residues. The consensus sequence marks positions with 100% (**capital letters**) or 67% identity (**small letters**) among the GCN2-like proteins.

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**N-terminal boundary**

\[\text{ FIG. 3 (Continued) }}\]

3. Multiple sequence alignment of *N. crassa* CPC3 (accession number X91867), yeast GCN2 (Ref. 6, U51030), and *Drosophila* DGCN2 (Ref. 15, U80223) using GCG program PileUp (gap creation penalty 6, gap extension penalty 2), with some manual adjustment in areas of low similarity. The N- (\(\ldots\)) and C-terminal (\(\ldots\),) ends of the CPC3 N-terminal domain, eIF2\(\alpha\) kinase (PK domain), HisRS-like, and C-terminal domains are indicated. In the N-terminal domain, amino acids written in **bold letters** represent positions matching with kinase-characteristic sequences. Kinase subdomains (roman numerals) and C-/N-terminal boundaries, their characteristic amino acids including tyrosine (\(Y\)) and serine/threonine kinase-specific sequences (\(2\)) are indicated above the alignment of the degenerate kinase region (12, 56, 80). Positions conserving nonpolar residues (\(\S\), FYWIMVA), polar residues (\(\S\), HRKDENQ), small residues with near neutral polarity (\(\S\), PGST), and aromatic residues (\(o\), YFW) are indicated. Lowercase letters represent modest conservation of the corresponding amino acids, **capital letters** high conservation, and **bold capital letters** invariant residues. The consensus sequence marks positions with 100% (**capital letters**) or 67% identity (**small letters**) among the GCN2-like proteins.

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**C-terminal boundary**

\[\text{ FIG. 3 (Continued) }}\]
FIG. 4. Multiple sequence alignment of all known eIF2α kinases (Domains Program GCG, gap creation penalty 10, gap extension penalty 2) for PKR from rat (rPKR, accession number L29281), mouse (mPKR, accession number Q03963), and human (hPKR, accession number P19525); HRI from rat (rHRI, accession number L27707) and rabbit (kHRI, accession number P33279); GCN2-like proteins from N. crassa (CPC3, accession number X91867), S. cerevisiae (GCN2, accession number U51030), and Drosophila (DGCN2, accession number U80223). Below, the consensus sequence of PKR, HRI, and GCN2-like (GCN2l) proteins and all eIF2α kinases (called consens) are shown, respectively. Conservation of an amino acid of at least 50% in each kinase group is shown in lowercase letters, and absolute identities are indicated with capital letters. The 11 eIF2α kinase-characteristic amino acids pointed out for GCN2 by Ramirez et al. (57) are indicated by *.

In GCN2 between subdomains VII and VIII, the autophosphorylation sites required for kinase activity (Thr-882, Thr-887, Ref. 86) are underlined.
eIF2α kinases diagnosed by Ramirez et al. (57), plus eIF2α kinase-specific conservation in the areas of subdomains IX and X (14) (Fig. 4). These findings suggested that cpc-3 encodes a functional eIF2α serine/threonine kinase.

As in GCN2, CPC3 contains a HisRS-like domain located immediately C-terminal to the kinase domain (amino acids 981–1507, Fig. 5). It is characterized by 34/60% positional identity/similarity with the HisRS-like domain of GCN2 and 30/52% with DGCN2 (30/53% between GCN2 and DGCN2). All three HisRS-like domains are characterized by three motifs above the alignment.

HisRS sequences were taken from human (hsHisRS, accession number P12081), yeast (scHisRS, accession number P07263), and E. coli (ecHisRS, accession number P04804). Below the alignment, consensus sequences are given for HisRS positions (c HisRS), for HisRS-like proteins (c Hlike), and for all proteins (consens). Lowercase letters indicate moderate conservation (at least 60%) and capital letters identity in all considered proteins. Non-equivalent amino acids (#) between consensus sequences of HisRS and HisRS-like domains are marked. HisRS motifs 1–3 are shown above the alignment (81), and the respective amino acids are described as either “small (PGST), § hydrophobic (FYWIMVLA), 1 positive (HRK), 2 negative (DENQ), or * invariant (82). Also the position and the characteristic sequences are shown from motifs histidine A and B (60) and from the patches (R/K)G (62) and VAILGE (62). Positions of mutations in GCN2 mentioned in the text are underlined: Y1119L, R1120L, A1197G, N1295D, and H1308Y.
conserved among class II aminoacyl-tRNA synthetases, plus sequences unique to HisRS proteins (58). However, certain residues conserved in genuine HisRS proteins were absent in CPC3, GCN2, and DGCN2. These include the invariant Arg in motif 3 that contributes to ATP binding (59), the amino acid stretch LVRLDYY (called "histidine A"), and the (R/K)G-patch N-terminal of motif 1 (62). In contrast, the sequence AAGGRYD (called "histidine B") is well conserved. Both histidine A and B motifs were shown to participate in forming the binding pocket for histidine (60). The Arg residue in histidine A plays a catalytic role in histidine activation (59, 61, 62), and the (R/K)G-patch is also vital for full HisRS enzymatic activity (62), but both are missing in the HisRS-like domains of the eIF2α kinases. The lack of conserved sequences listed above suggested that all three HisRS-like domains lack the ability to bind histidine and ATP and, thus, should be enzymatically inactive.

The most similarity between HisRS-like domains and genuine HisRS is found in the region between motifs 1 and 3, especially N-terminal to motif 2. Motif 2 is involved in tRNA binding (63), and the adjacent N-terminal sequences are uniquely conserved among genuine HisRS proteins (64, 65). It is not known which amino acids in authentic HisRS are responsible for the recognition of tRNAHis. However, GCN2 mutations in the HisRS-like domain are known that lead to either inactivation (gcn2 alleles) or constitutive activation (GCN2α alleles) of the kinase domain. Because of their predicted functional importance, CPC3 was inspected for the amino acids residing at the following positions: mutation gcn2-m2 (Y1119L, R1120L) affects amino acids in motif 2 and was shown to impair in vitro binding of tRNAs to GCN2 (8). Both residues are conserved in CPC3 and DGCN2. In contrast, some of the GCN2α mutations affect amino acids that are not conserved in CPC3 or DGCN2 (e.g. GCN2α-N1295D and GCN2α-H1308Y, see Ref. 25) (Fig. 5). Quite the opposite, the Ala residue altered by the GCN2α-A1197G mutation (25) establishes identity with CPC3 at this position. This may indicate that in these areas the tertiary structure of the protein, not the amino acids, is conserved.

For the most C-terminal region of CPC3 (amino acids 1507–1646) the highest similarity found in searching the databases was with GCN2 (32/58% identity/similarity); however, pairwise comparison of CPC3 and DGCN2 showed similarities to almost the same degree (27/51%) (28/49% between GCN2 and DGCN2). No extensive stretches of identical amino acids could be found in this area which was shown for GCN2 to be responsible for interaction with ribosomes (25).

In the N-terminal region directly preceding the eIF2α kinase domain cpc-3 encodes a degenerate kinase domain (amino acids 257–550) also found in GCN2 (8, 66) and DGCN2 (18) (Fig. 1D). The degenerate kinase domains in GCN2 and DGCN2 lack certain invariant amino acids characteristic of protein kinase subdomains. In contrast, CPC3 contains all invariant residues;
however, CPC3 lacks the nearly invariant amino acids Phe and Gly in subdomain VII, which participates in ATP binding. It also lacks one nearly invariant amino acid in subdomain I. At those three positions CPC3 contains nonconserved amino acids that are not found in any of the kinases compared by Hanks and Hunter (12). There are additional derivations from highly conserved kinase domain sequences that are unique to each degenerate kinase (for details, see Fig. 3). Two other unusual features shared by all three degenerate kinase domains are: first, the presence of an insertion between subdomains I and II; second, subdomains X and XI are located much closer together than is observed in genuine kinases. Therefore, we presume that all three degenerate kinases are catalytically inactive as protein kinases. However, they may still bind substrates, ATP, and/or regulatory proteins.

The most N-terminal sequence of CPC3 (amino acids 1–260) showed significant similarity to GCN2 and DGCN2 (Fig. 3) but to no other proteins in the data bases available for the on-line BLAST search.

From the extended structural similarities between CPC3, GCN2, and DGCN2 proteins it was concluded that they represent functionally homologous proteins.

Construction of a cpc-3 Mutation, cpc-3::hph, via Homologous Genomic Integration of an In Vitro Constructed Gene Disruption—To study the function of cpc-3 a putative loss of function mutation was engineered via a plasmid-borne cpc-3 disruption construct (Fig. 6). The strategy involved the deletion of about 1 kb of the cpc-3 gene, including the region encoding subdomains VI–XI of the eIF2α kinase domain and part of motif 2 of the HisRS-like domain, and replacing them by the hph cassette as a dominant selectable marker conferring resistance to hygromycin B. A homologous double recombination event was required for replacement of the wild-type cpc-3 allele by the plasmid-borne disruption construct (Fig. 6) which is a rare event in filamentous fungi. To enable a rapid screen of transformants that were likely to contain gene replacements, the Bml cassette was inserted 3’ of the cpc-3::hph allele on the plasmid (Fig. 6). N. crassa Bml encodes a benomyl-resistant β-tubulin, providing a dominant marker which should be lost in the course of homologous recombination (67) (Fig. 7). However, since ectopic integration of parts of the plasmid could equally result in benomyl-sensitive transformants, molecular proof for correct gene replacement was required. By using one primer (11.2) complementary to hph sequences and another (11.1) complementary to sequences 5’ of the cpc-3 disrupted region (present in the host genome but missing in the transforming plasmid), a PCR amplification product of 2.1 kb should be produced if genomic cpc-3 is replaced by cpc-3::hph via homologous recombination (Fig. 7). Homokaryotic cpc-3::hph strains should not yield amplification of the 302-bp PCR fragment with primers 2.1 and 2.3, where 2.3 is complementary to the deleted region of cpc-3 (Fig. 7). Further confirmation for site-specific and unique integration was obtained by Southern analysis and genetic linkage studies (see below).

The cycloheximide-resistant strain cyh-2 was transformed with plasmid pESXI3100 bearing the cpc-3::hph;Bml disruption construct (Fig. 7). Of 595 hygromycin-resistant transformants that were isolated, 322 were sensitive to benomyl, and of these 52% carried an unstable hygromycin resistance and were discarded. PCR analysis of genomic DNA of the remaining 166 candidates with primer pair 11.1/11.2 yielded the anticipated 2.1-kb fragment (Fig. 7) from two strains, S1/148 and S1/152. From both primary transformants 10 potentially homokaryotic microconidial subcultures where isolated, and their DNA was subjected to PCR reactions using primer pairs 11.1/11.2 or 2.1/2.3, respectively. Out of the 10 S1/152-derived cultures 8 allowed amplification of 11.1–11.2 fragments only (not shown) indicating homokaryosity for the cpc-3::hph allele. Persistent heterozygosity in all microconidial subcultures from transformant S1/148 was indicative of a lethal event in the transformed nucleus.

Southern analysis of genomic DNA of S1/148, S1/152, and the S1/152-derived subcultures was conducted to confirm the results of the PCR analysis. The data in Fig. 8 verified that strains S1/152M1-M5 and M7-M9 were homokaryotic for the cpc-3::hph allele. The primary transformant S1/148 showed correct fragment sizes in the Southern hybridization (Fig. 8), and no ectopic integration of the transformant plasmid was found which might have accounted for disruption of an essential gene. The lethal event could have occurred due to the mutagenic nature of the transformation procedure itself. Viable hygromycin-resistant segregants of a cross between S1/148 and wild-type provided evidence that the postulated lethality was not correlated with the cpc-3::hph gene disruption. In any event, the difference between primary transformants S1/148 and S1/152 documented that two independent transformants with site-specific integration events were obtained.

Disruption of the correct gene was supported by linkage
studies. In crosses between homokaryotic derivatives of S1/152 (putative genotype cpc-3::hph, cyh-2) and wild-type, a total of 58 segregants was tested for hygromycin and cycloheximide resistance. Each locus segregated in a 1:1 ratio, but only three of the segregants differed from the parental allele combinations (not shown) in agreement with 4.2% recombination found in the restriction fragment length polymorphism mapping studies between a molecular marker for cpc-3 and the cyh-2 locus (see above).

**cpc-3 Disruption Interferes with the Regulation of Amino Acid Biosyntheses**—Since the homokaryotic cpc-3::hph mutant strains were not only viable but grew and reproduced vegetatively and sexually like the wild type, we concluded that cpc-3 does not provide an essential cellular function. The structural homology between CPC3 and GCN2 called for a closer examination of amino acid regulation in cpc-3::hph mutants (abbreviated cpc-3). Starvation for histidine was achieved by supplementing the medium with 3AT, a competitive inhibitor of imidazole glycerophosphate dehydrogenase in histidine biosynthesis (68). *N. crassa* wild type with intact amino acid regulation can grow on certain 3AT concentrations; however, regulation deficient mutants like *N. crassa* cpc-1 and cpc-2, unable to counteract enzyme inhibition via derepression of amino acid biosynthetic enzymes, are 3AT-sensitive (27, 30).

Homokaryotic cpc-3 mutants derived from either S1/152 or S1/148 were found to be 3AT-sensitive (simultaneous supplementation with histidine-restored growth). 3AT sensitivity was recessive in cpc-3/cpc-3 heterokaryons. In crosses between cpc-3 mutants and wild type, the 3AT sensitivity and hygromycin resistance phenotypes were tightly linked and did not separate (not shown), indicating a causal relationship between the cpc-3 disruption and the defect in the regulation of histidine biosynthesis. Any combination of forced heterokaryons carrying two different nuclei with mutations in cpc-3, cpc-1, or cpc-2, respectively, showed complementation of the 3AT sensitivity (not shown), confirming that these mutations identify different functions.

To obtain evidence that the cpc-3 mutant had a “cross-pathway” defect, we investigated the regulation of the arginine biosynthetic enzyme L-ornithine carbamoyltransferase (encoded for by arg-12 in *N. crassa*) in response to histidine deprivation imposed by 3AT supplementation. Fig. 9 shows that a 5-fold induction of enzyme activity (derepression) occurred in the wild-type, the cyh-2 recipient, and the cpc-3::hph/cpc-3+ heterokaryotic strains. However, a complete lack of enzyme derepression was found in all homokaryotic cpc-3::hph subcultures in response to growth on 3AT. The remaining enzyme level in the mutants was similar to the induced wild-type activity, comparable to the phenotype of cpc-2 mutants (30), whereas cpc-1 mutants cause a further reduction in basal enzyme level (27) (Fig. 9). Functional consequences of the observed basal enzyme activity were investigated by introducing the regulatory mutations into the arg-12+ background. The bradytrophic arg-12+ allele encodes for an enzyme with drastically reduced OCT activity (47). An arg-12+;cpc-3 double mutant was found to grow almost at the wild-type rate without arginine supplementation (like arg-12+;cpc-2, see Ref. 30), whereas an arg-12+;cpc-1 strain is an arginine auxotroph (26, 27) (data not shown). This suggested that in a cpc-3 mutant the basal level of cpc-1 function provides sufficient induction of arg-12+ transcript for arginine prototrophy and that the cpc-3::hph mutation does not decrease the basal enzyme activity of enzymes under general amino acid control.

To show that the cpc-3 mutation specifically impairs the expression of amino acid biosynthetic enzymes, an enzyme belonging to the citric acid cycle, citrate synthetase, was investigated on different carbon sources. Its regulation was not affected by the cpc-3 mutation (not shown) demonstrating that the cpc-3 mutation did not abolish derepression in general.

Since the general amino acid control activates transcription of the biosynthetic target genes in amino acid-starved cells (23), the arg-12 transcript was investigated in the homokaryotic cpc-3 isolates by Northern analysis. In contrast to the increased arg-12 transcript level found in the wild-type grown on 3AT (Fig. 10A) only a very low transcript level was observed in cpc-3 mutant strains irrespective of amino acid sufficiency, *i.e.* the arg-12 transcript induction appeared completely dependent on a functional cpc-3 allele. These results allowed us to conclude that cpc-3, like GCN2, supplies a positive function critically required for transcriptional derepression of genes subject to general amino acid control.

In *N. crassa* amino acid deprivation elicits a strong increase in the mRNA level of the cpc-1 transcriptional activator (3, 30, 69). The cpc-3 mutation did not prevent the substantial up-regulation of cpc-1 transcript level in response to amino acid deprivation (Fig. 10A), with the mRNA increased to 58% of the wild-type level (Fig. 10B). Previous investigations of cpc-1 mutants (29, 34) had shown that derepression of the arg-12 transcript depends completely on a functional cpc-1 gene. The finding that no derepression of arg-12 mRNA occurred in the cpc-3 mutant despite a substantial increase in cpc-1 transcription (see above) is consistent with a function of cpc-3 in stimulating cpc-1 translation.

With respect to cpc-2 it was found that the cpc-3 mutation abolished the down-regulation of cpc-2 mRNA in amino acid-deprived cells, as described previously for cpc-1 mutations (31). On the other hand, in *cpc-2*cpc-3 and *cpc-2cpc-1* double mutants the cpc-3 and cpc-1 mutations did not mask the phenotypes characteristic for a cpc-2 mutation, *i.e.* reduced growth rate (50%) and female infertility (data not shown), thereby indicating a broader function of cpc-2 operating outside the mechanism of general control.

**cpc-3 Is a Posttranscriptional Activator of cpc-1 Expression**—In cpc-3 mutants we found that amino acid starvation
**DISCUSSION**

The conservation of the polypeptide sequence found between *N. crassa* CPC3, yeast GCN2, and *Drosophila* GCN2 argues that these are homologous proteins. The most notable structural similarity is the juxtaposition of a protein kinase and HisRS-related domain. In addition, GCN2 and CPC3 share extensive similarity in degenerate kinase domains located N-terminal to their conventional kinase domains. The CPC5 kinase domain consists of many of the conserved features observed previously for the eIF2\(\alpha\) kinases GCN2, HRI, and PKR (57). The only continuous amino acid stretches uniquely conserved in the GCN2-like proteins are "WRLFRKXEXL" in subdomain VIA and "VVKY" in subdomain IV. The CPC3 HisRS-like domain lacks sequences essential for binding both histidine and ATP, supporting the model that the HisRS-like domains in the GCN2-related kinases lack HisRS activity and function as sensors of multiple uncharged tRNAs (8). It was shown that the HisRS-related domain of yeast GCN2 binds uncharged tRNA (9) and that the HisRS-like sequences are required for *in vitro* activation of GCN2 kinase function (66); however, it remains to be shown that discrimination between tRNA species is lacking. Consistent with nonspecific binding of tRNAs, as noted for GCN2 (57), motif 2 sequences for the class II enzyme AspRS that interact with the 3′ end of the acceptor stem of yeast tRNA\(^{\text{Asp}}\) (63) are conserved in genuine HisRS but only partially conserved in CPC3 and DGCN2. In addition, the C-terminal domain of *E. coli* HisRS was shown to be responsible for recognition of the tRNA\(^{\text{His}}\) anticodon (70), and the only HisRS-characteristic motif in this region (Ref. 62 and Fig. 5), is poorly conserved in the HisRS-like domains of the GCN2-related kinases.

Is there an explanation for the choice of HisRS to be linked in evolution to the eIF2\(\alpha\) kinase domains of the GCN2-like proteins? The unique ability of HisRS to recognize acceptor stem base pairs both in the context of full-length tRNA and in mini- or microhelices (73–76) might single out this enzyme as the best candidate for diversification of tRNA binding specificity. Monitoring uncharged tRNAs in general would require that the HisRS-like domains ignore the unique identity element of tRNA\(^{\text{His}}\) species, the extra nucleotide G**, at their 5′ end (77).

Since the disruption mutation of *cpc-3* destroyed essential parts of the kinase and HisRS-like domains, a complete loss of function was assumed. The phenotypes of the *cpc-3::hph* mutant proved that the gene is required for the function of general amino acid control. *cpc-3* mutations were probably not detected in searches for *N. crassa* regulatory mutations since most of these relied on the postulated arginine auxotrophy of *cpc* mutants (27) or 4 h after supplementation of 6 mM (final) 3AT (A). Aliquots of each sample containing 50 \(\mu\)g of protein were separated in an SDS-polyacrylamide gradient gel (4–12%) and transferred to nitrocellulose membranes (NOVEX). The anti-CPC1-antiserum from rabbit (49) was not affinity purified prior to use, in contrast to previously published work (49). The arrow indicates the position of CPC1. The apparent mass of CPC1 based on its electrophoretic mobility is higher than predicted from its nucleotide sequence (30 kDa), even higher than observed previously (3), which perhaps can be explained by the use of different gel systems. A large discrepancy between predicted and apparent molecular mass has been noted also for GCN4, the homologue protein in yeast (87). The nonspecific signals were used as internal controls for equal loading of proteins. The position of the molecular mass markers are indicated by **98, 64, 50, 36 and 30 kDa**.
uORFs are sufficient for almost wild-type regulation (4). GCN4 translational control requires that the first uORF does not promote dissociation of ribosomes after termination of translation, and the last codon and 10 bases 3′ to the translational stop codon are decisive for this property (72). As mentioned by Luo et al. (71), the nucleotide composition and C/G content around the stop codons of cpc-1 uORF1 and uORF2 are similar to those at GCN4 uORF1 and uORF4, respectively, suggesting a common translational mechanism for GCN4 and cpc-1. In agreement with the idea that CPC3 is a translational activator of cpc-1, we found that amino acid deprivation in a cpc-3 mutant did not lead to any detectable increase in CPC1 protein level, despite a remarkable increase in cpc-1 mRNA levels. From this, we propose that CPC3 stimulates translation of cpc-1 mRNA by the same mechanism elucidated for GCN4 mRNA in yeast, involving down-regulation of eIF2-GTP-tRNA\(^{Met}\) ternary complex formation by phosphorylation of eIF2\(\alpha\).

If CPC3 stimulates cpc-1 mRNA translation by the same mechanism elucidated for GCN2/GCN4 mRNA in yeast (4), we would expect to observe increased phosphorylation of eIF2\(\alpha\) in amino acid-starved \textit{N. crassa} cells. By using isoelectric focusing gels, increased phosphorylation of eIF2\(\alpha\) under amino acid deprivation was shown in yeast (83). Because antibodies against \textit{N. crassa} eIF2\(\alpha\) are not available, and the yeast eIF2\(\alpha\) antibodies do not appear to cross-react with the \textit{N. crassa} protein (data not shown), we could not test this prediction. The sequences surrounding Ser-51 in yeast eIF2\(\alpha\), the phosphorylation site recognized by GCN2, PKR, and HRI (84), are highly conserved between yeast, mammals, and \textit{Drosophila} (85); however, the sequence of \textit{N. crassa} eIF2\(\alpha\) is not known. For the eIF2\(\alpha\) kinase domains of PKR and GCN2, it was found that phosphorylation of two Thr residues in the activation loop are required for high level kinase activity (86). In CPC3 these Thr residues are conserved (Fig. 4) suggesting a similar activation/regulation mechanism as for GCN2 and PKR.

Thus far the investigation of \textit{N. crassa} cpc-3 does not point out distinct differences in the mechanism of general control between \textit{N. crassa} or yeast. Comparable to the yeast system (78, 79), induction of cpc-1 mRNA in response to amino acid limitation occurred not only in the presence of the cpc-3 mutation (this investigation) but also in the presence of various cpc-1 alleles (3, 29). This argues that an independent second mechanism must exist that can register amino acid deprivation and stimulate cpc-1 transcription.

A search in the EST data base identified sequence fragments of mouse and human covering a stretch from protein kinase subdomain XI to the N-terminal part of HisRS sequences (up to motif 2 for mouse EST accession number AA016507; human EST accession number AA216651) suggesting that mammals possess an eIF2\(\alpha\) kinase linked to a HisRS-like domain. This might represent a general metabolic requirement for eIF2\(\alpha\) kinases activable by uncharged tRNA, providing the means to down-regulate general translation and induce a starvation response protein like CPC1 or GCN4. \textit{N. crassa} cpc-3 or yeast \textit{gcn}2\(\Delta\) mutants do not show any restriction in vegetative growth or sexual reproduction under non-starvation conditions, indicating that CPC3 and GCN2 are not critically involved in these processes. The developmentally regulated expression of DGCN2 and, in later stages, restricted expression in a few cells of the central nervous system (18) suggest the exciting possibility of additional functions for this interesting protein kinase in higher organisms.

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N. crassa cpc-3 Encodes an eIF2α Kinase

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