THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 273, No. 32, Issue of August 7, pp. 20404–20416, 1998
Printed in U.S.A.

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*

(Received for publication, January 29, 1998, and in revised form, May 1, 1998)

Evelyn Sattlegger‡§¶, Alan G. Hinnebusch§, and Ilse B. Barthelmess‡

From the ‡Institute of Applied Genetics, University of Hannover, Herrenhäuser Strasse 2, D-30419 Hannover, Germany and the §Laboratory of Eukaryotic Gene Regulation, NICHD, National Institutes of Health, Bethesda, Maryland 20892-2716

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 *Cryptonecria parasitica* (5, 6). In yeast, *GCN2* plays a crucial role in signal perception and transduction. *GCN2* encodes a protein containing an eIF2α[1] 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 delivering charged initiator $tRNA^{Met}$ (Met-$tRNA_i^{Met}$) 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-$tRNA_i^{Met}$ 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-

* The work was supported in part by a grant from the Deutsche Forschungsgemeinschaft. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) X91867.

¶ Recipient of a National Research Council Research Associateship. To whom correspondence should be addressed: Laboratory of Eukaryotic Gene Regulation, NICHD, National Institutes of Health, Bldg. 6A, Rm. B1A-05, Bethesda, MD 20892-2716. Tel.: 301-435-6023; Fax: 301-496-8576; E-mail: esattlegger@aghmac1.nichd.nih.gov.

[1] The abbreviations used are: 3AT, 3-amino-1,2,4-triazole; eIF, eukaryotic translational initiation factor; HRI, heme-regulated inhibitor; PKR, double-stranded RNA-dependent kinase; GCN, general amino acid control non-derepressible; CPC, cross-pathway control; PCR polymerase chain reaction; uORF, upstream open reading frame; bp, base pair(s); kb, kilobase pair(s); HisRS, histidyl-tRNA synthetase(s); RT, reverse transcriptase; DIG, digoxigenin.

spectively, which distinguishes them from other serine/threonine 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 eIF2α kinase catalytic domain, GCN2 contains a domain that resembles the histidyl-tRNA synthetases (HisRS), which was postulated to monitor amino acid availability (8).

Early work by various *N. crassa* and yeast researchers (23) indicated that uncharged aminoacyl-tRNAs that accumulate in amino acid-deprived cells are the relevant signal in the mechanism 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 binding 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 moiety (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-deficient 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* disruption allele and the phenotypic consequences of the loss of function 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*, analogous 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-2,A, arg-12^s,a arg-12$^-$,a* were obtained from the Fungal Genetics Stock Center (FGSC, University of Kansas Medical Center); the *cpc-1(j-5)* and *cpc-2(U142)* mutant strains were from the Barthelmess lab.

N. crassa cultivation on Vogel's standard medium and crossing techniques 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 $\times$ 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 concentrations 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—The ordered *N. crassa* genomic cosmid library of Vollmer and Yanofsky (33) was used to screen for *cpc-3* sequences. Plasmids used in this study were pCPC1-2 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 pBluescript SK. 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—Spheroblasts obtained from germinating 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 Irelan *et al.* (41) and Chow and Kaefer (42) were combined as follows: *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 1:1 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 $\times$ *g*). The liquid phase was again extracted with 0.3 ml of phenol:chloroform. The DNA was precipitated with 1 ml 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 membranes (Amersham Pharmacia Biotech). Probes were labeled with DIG-11-dUTP (DIG-DNA random primed labeling kit, Boehringer Mannheim). Labeling of DNA shorter than 700 bp was performed by PCR reaction (see below, except that ⅓ of dTTP was replaced by DIG-dUTP), and the labeling reaction was used directly for hybridization. Hybridization and detection of probes and stripping of probes from membranes followed the manufacturer's protocol (DIG luminescent detection kit, Boehringer Mannheim).

PCR reaction mixtures consisted of 1$\times$ PCR buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 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/cosmid 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 min in the first cycle), 1 min at the annealing temperature (5 °C lower than T_m, 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 Technologies, 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*-specific 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).

RNA 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 (N+, Amersham Pharmacia Biotech). Probing was done according to Sambrook *et al.* (43). DNA probes were radiolabeled with [α-^{32}P]dCTP (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 sequencing

TABLE I
Oligonucleotide sequences used for PCR reactions

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).

Name	Position	Sequence (from 5′ to 3′)	Restriction enzyme
cpc-3, degenerated and sequence specific oligonucleotides			
2.1 s	2414	atcgtcgaCTITTYATHCAGATGGAGTAITGYGA 42 °C	*Sal*I
2.3 a	2653	acaggatccGCIAGICCGAARTCICCHATYTT 42 °C	*Bam*HI
4.1 s	1888	cgtgcatgCTIGGRCAGGGIGGITTYGGICARGT 42 °C	*Sph*I
4.2 a	2523	GAATAAGCGCCAGATCTCA	*Bgl*II
5.1 s	2586	gccgtcgaCCACATTCACAGCCTCAACATC	*Sal*I
5.2 a	3407	gcccttaagCYCGITAICCYATICAITCYAGYAT 50 °C	*Eco*RI
6.1 s	3301	cgaggtaCCATCTTCCGGCGACAC	*Kpn*I
6.2 a	4155	atactgcAGRGTGTCGTAICGRCCRCCIGC 65 °C	*Pst*I
11.1 s	511	GAGATGATAGACCAGATTGTCGAAG	
14.1 s	−220	attagatCTTCGTTTCCACCCAA	*Bgl*II
14.2 a	612	tctgaaTTCGCTCTGCGCTAACCTT	*Eco*RI
Bml cassette			
BmlU s		tcaagatCTTCCACCCTTCCAAAAGTTTGAC	*Bgl*II
BmlL a		tgcagATCTAAACAGACATTATCATCATCATGCA	*Bgl*II
hph cassette			
11.2 a		TTCAATATCATCTTCTGTCGACCTC	

kit, Promega). A 1.6-kb *Sma*I-*Eco*RI fragment of *cpc-3* was commercially sequenced (LARK). DNA sequences were analyzed with programs DNASIS and PROSIS (Hitachi). Predicted amino acid sequences were compared with available protein sequences using the basic local alignment search tool (BLAST, see Ref. 45). Multi-alignments were performed using the GCG program (46).

Enzyme Assays—All assays were performed with crude extracts from freeze-dried mycelia. The specific activities of L-ornithine carbamoyltransferase (EC 2.1.3.3) and citrate-synthase (EC 4.1.3.7) were assayed according to Davis (47) or Flavell and Fincham (48), respectively.

Protein Isolation and Immunoblotting—Crude cell extracts of *N. crassa* were isolated by grinding fresh mycelium in liquid nitrogen, addition of equal volumes of breaking buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.2% Triton, 1 tablet of protein inhibitor mixture (complete, Boehringer Mannheim) per 50 ml of buffer, 10 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM dithiothreitol) and of glass beads (0.3–0.4 mm diameter), and subsequent vortexing 6 times for 30 s at 4 °C. After removal of cell debris (14,000 rpm, 10 min, 4 °C), Western blots were conducted by using precast gels and nitrocellulose membranes (NOVEX) according to the manufacturer's protocol. Detection of antigen-antibody complexes was performed by using horseradish peroxidase-conjugated anti-rabbit antibodies and the enhanced chemiluminescent detection system (Amersham Pharmacia Biotech).

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 302-bp PCR product, called 2.1–2.3, was obtained using 2.1 and 2.3 as primers (Fig. 1*B*) 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 cosmid 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. 1*B*) 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. 1*C*), a restriction map was derived (Fig. 1*A*). Sequencing of PCR fragments and subcloned restriction fragments or direct sequencing of cosmid 17:5D 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. 1*B*) 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*

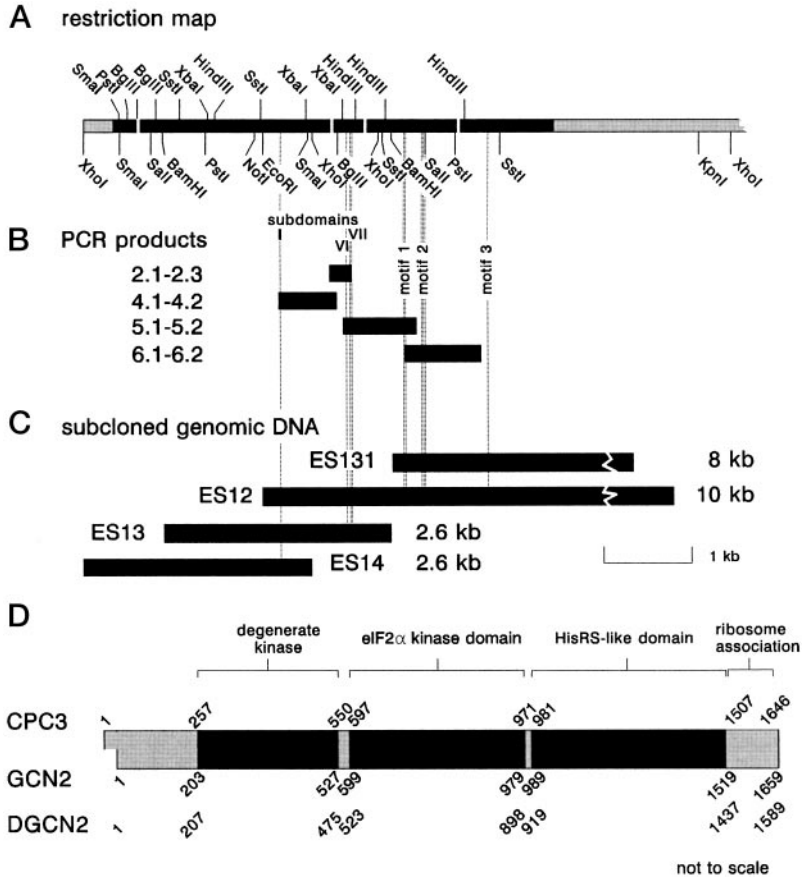

FIG. 1. **Molecular organization of the *N. crassa cpc-3* gene, subcloned fragments, and domain structure of the encoded polypeptide.** The positions of eIF2α kinase subdomains I, VI, and VII and the positions of motifs 1–3 in the HisRS-like domain are indicated. *A,* restriction map indicating the exon/intron structure; *cpc-3* is indicated by a *black bar* and introns are shown in *white*. *B,* PCR amplification products obtained from genomic DNA (2.1–2.3, 4.1–4.2) or cosmid clone 17:5D (5.1–5.2, 6.1–6.2). *C,* subcloned restriction fragments from cosmid clone 17:5D. *D,* domain structure of CPC3 (accession number X91867), GCN2 (U51030), and DGCN2 (U80223) in relation to amino acid positions (8, 18).

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).

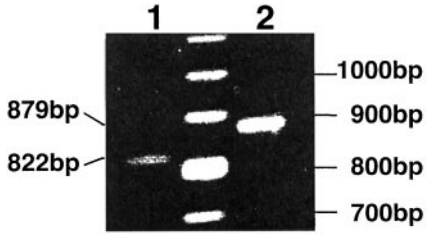

FIG. 2. **Confirmation of the putative *N. crassa cpc-3* translational start site as part of the *cpc-3* transcript using RT-PCR analysis.** Reverse transcription on *N. crassa* total RNA with primer 14.2 (Table I), located 3′ from intron 1, was followed by PCR amplification using the primer pair 14.1/14.2 (flanking intron 1, with 14.1 representing the most 5′ available sequence, *i.e.* located 220 bp upstream of the putative translational start site) yielding a fragment of 822 bp (*lane 1*). As control, the same primer pair was used in a PCR reaction on genomic DNA as template, resulting in a 879-bp fragment (*lane 2*).

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. 1*D*):

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 *versus* DGCN2) identity and 64/61/60% similarity. This domain in CPC3 contained 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

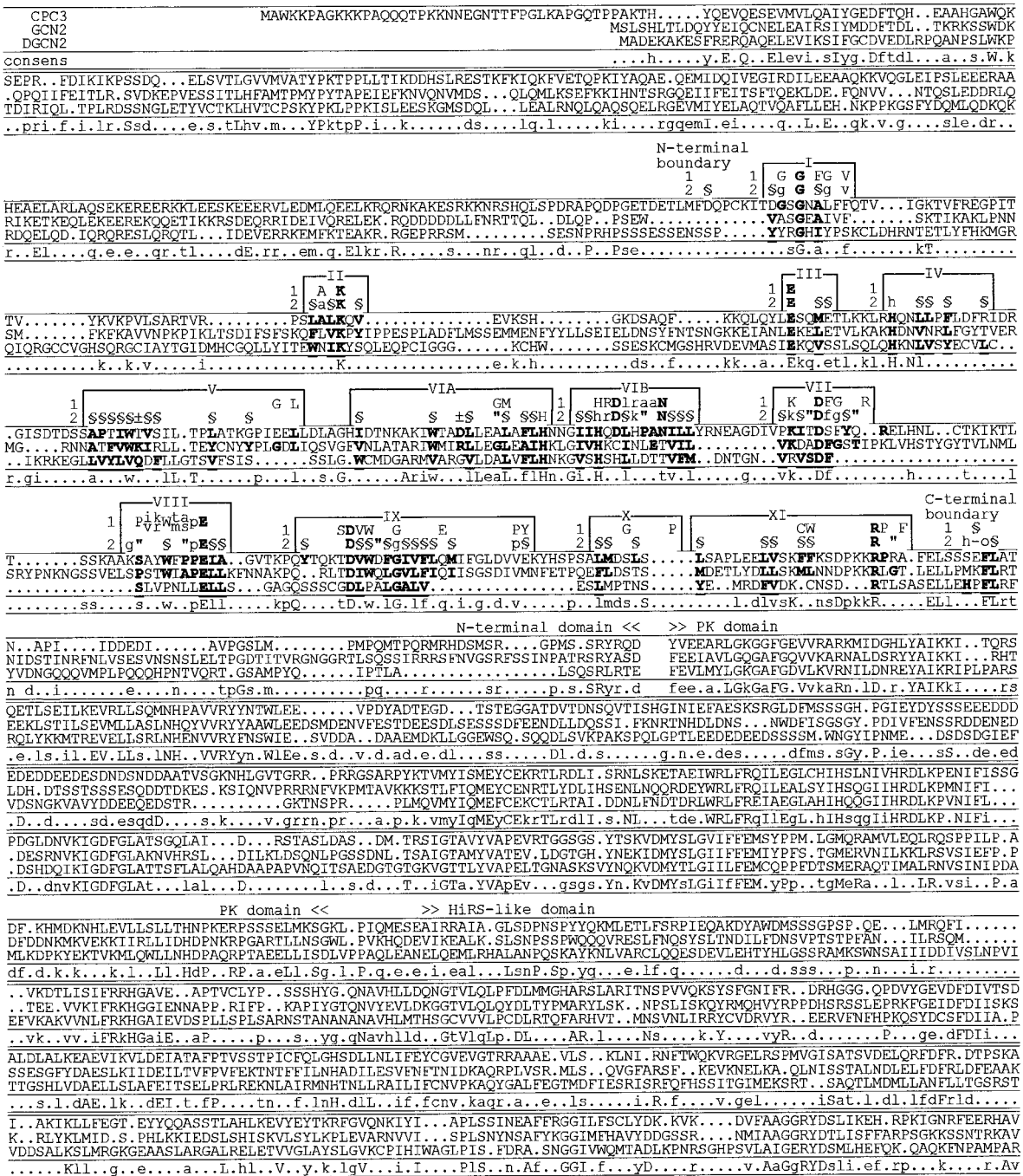

FIG. 3. **Multiple sequence alignment of *N. crassa* CPC3 (accession number X91867), yeast GCN2 (Ref. 6, U51030), and *Drosophila* DGCN2 (Ref. 15, U80223) using GCG program PileUp (gap creation penalty 6, gap extension penalty 2), with some manual adjustment in areas of low similarity.** The N- (≫) and C-terminal (≪) ends of the CPC3 N-terminal domain, eIF2α 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 (*1*) and serine/threonine kinase-specific sequences (*2*) are indicated *above* the alignment of the degenerate kinase region (12, 56, 80). Positions conserving nonpolar residues (§, FYWIMVLA), polar residues (±, HRKDENQ), small residues with near neutral polarity (“, 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.

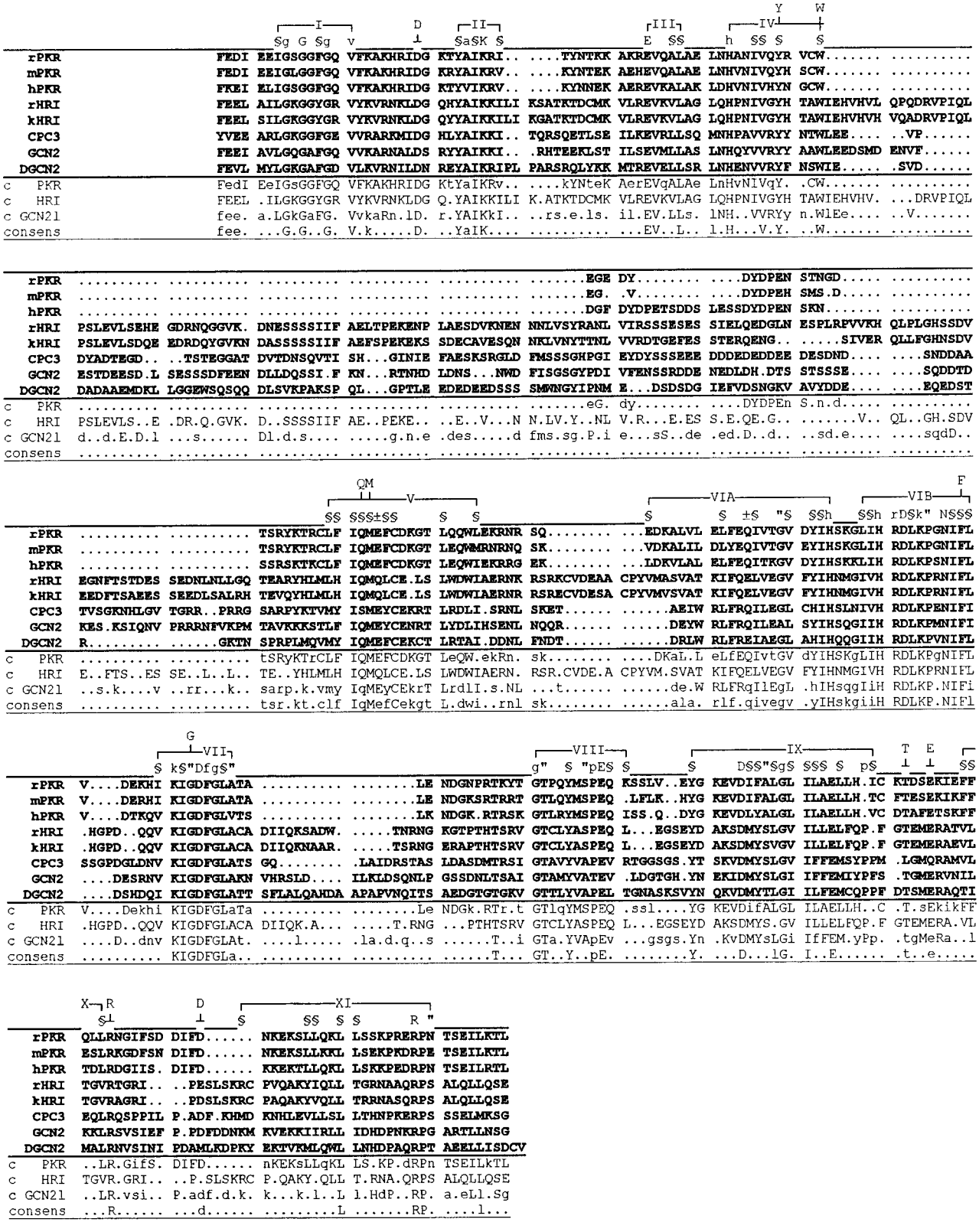

FIG. 4. **Multiple sequence alignment of all known eIF2α kinase domains (Program GCG, gap creation penalty 10, gap extension penalty 2: PKR from rat (*rPKR*, accession number L29281), mouse (*mPKR*, accession number Q03963), and human (*hPKR*, accession number P19525); HRI from rat (*rHRI*, accession 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*. Kinase subdomains (*roman numerals*) and their characteristic amino acids (12) are indicated *above* the sequences. Positions conserving nonpolar residues (§), polar residues (±), and small residues with near neutral polarity (") are indicated. *Capital letters* represent positions with high conservation and *lowercase letters* modest conservation.

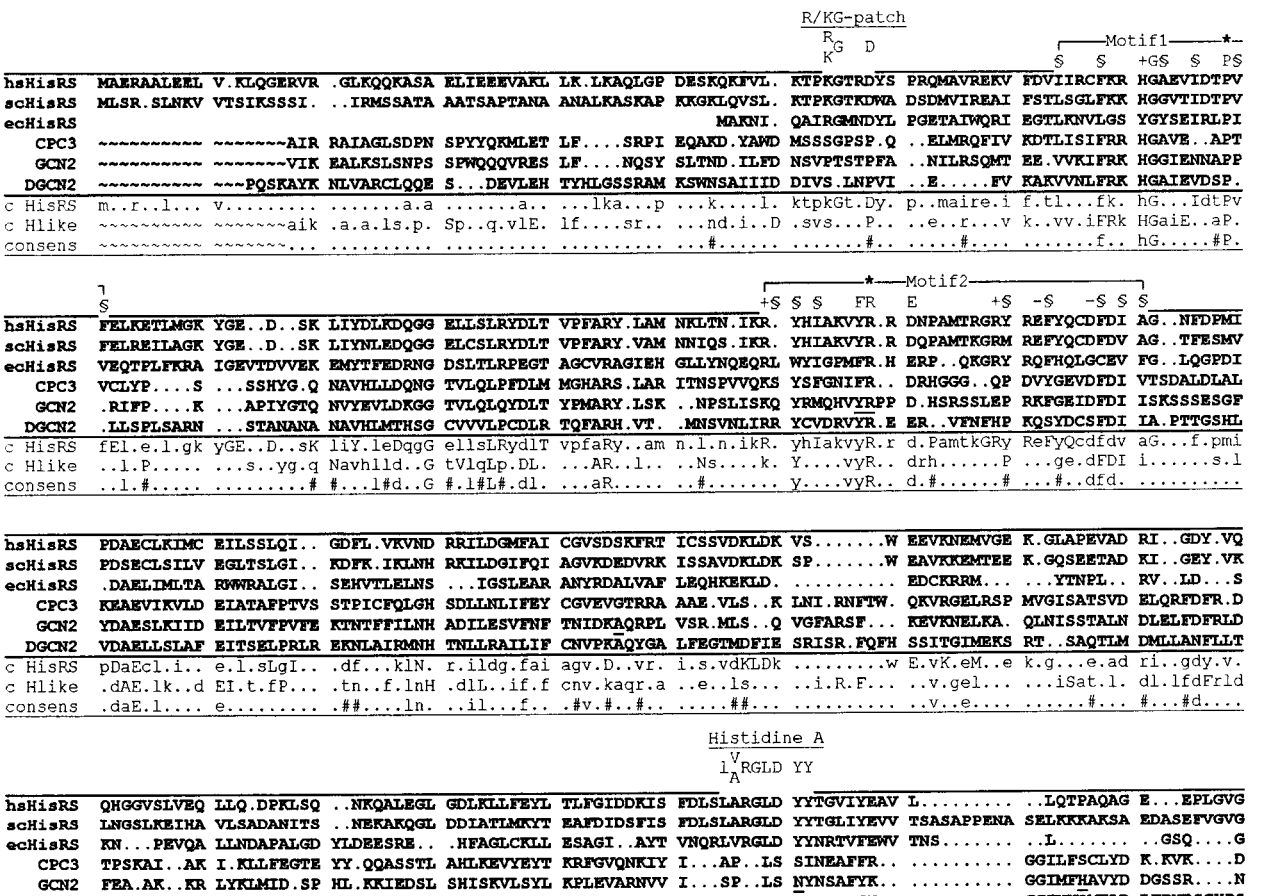

FIG. 5. **Multi-alignment of HisRS-like domains of CPC3, GCN2, and DGCN2 (explanation see Fig. 4) and genuine HisRS sequences (Program GCG, gap creation penalty 4, gap extension penalty 2).** HisRS sequences were taken from human (*hsHisRS*, accession number P12081), yeast (*scHisRS*, accession number 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), +positive (HRK), −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.

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

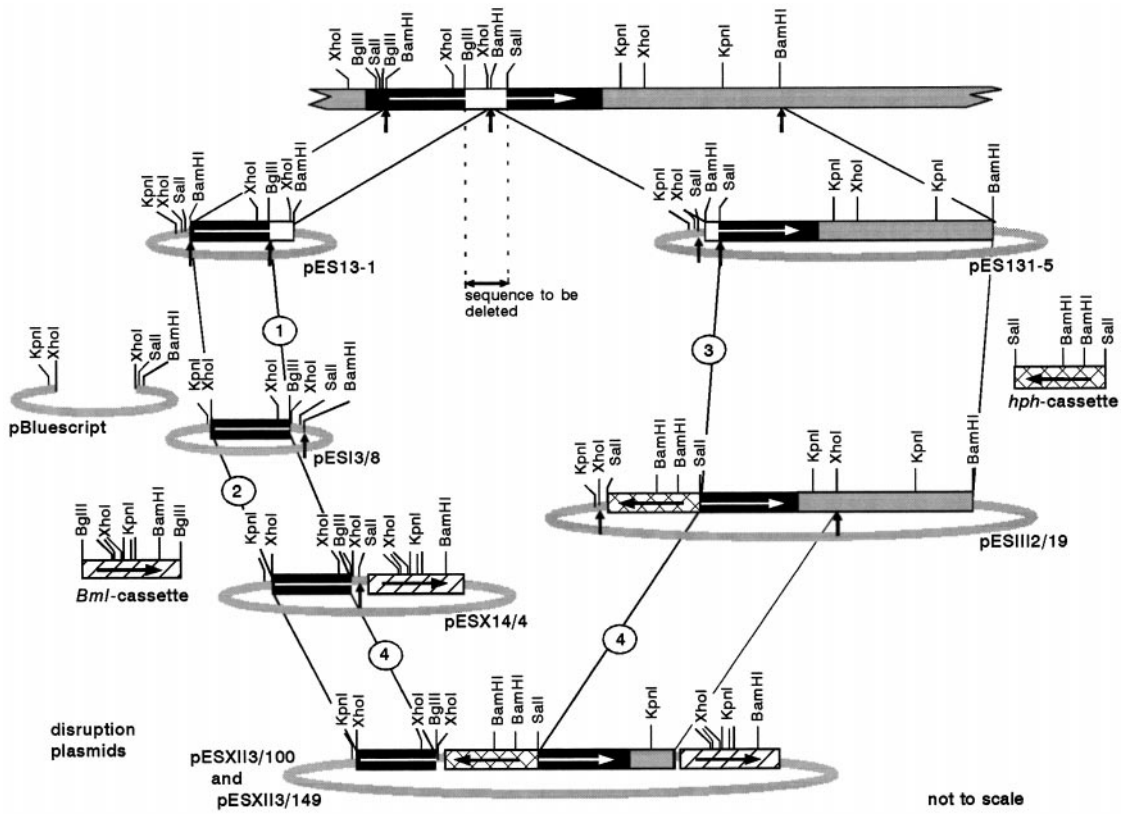

FIG. 6. **Construction of the *cpc-3::hph* disruption plasmid.** The deletion construct of *cpc-3* was engineered with the subcloned contiguous *Bam*HI restriction fragments ES13 (in plasmid pES13-1) and ES131 (in plasmid pES131-5) (Fig. 1) as follows. The 2-kb *Bam*HI-*Bgl*II *cpc-3* fragment (*black bar*) of pES13-1 was cloned blunt-ended into *Xho*I-digested pBluescript resulting in plasmid pESI3/8. *Bgl*II digestion resulted in the loss of a 0.6-kb inner *cpc-3* sequence (*white bar*). The *Bml* cassette (*hatched bar*) was PCR-amplified from pBT6 (35) using primers *Bml*U/*Bml*L provided 5′ with *Bgl*II sites. The *Bgl*II-restricted PCR fragment was cloned into the compatible *Bam*HI site of pESI3/8 resulting in plasmid pESX14/4. (Function of Bml^R was confirmed by transformation of *N. crassa* with pES14/4 and detection of benomyl-resistant colonies.) *Sal*I fragment (*cross-hatched bar*) from plasmid pCSN43 containing the *hph* cassette was ligated into similarly digested pES131-5 upstream of the *N. crassa cpc-3* sequences (flanking the disruption at the 3′ side), resulting in plasmid pESIII2/19. *Sal*I digestion of pES131-5 deleted a 0.4-kb fragment of inner *cpc-3* sequence, which together with the 0.6-kb deletion resulting from step 1 amounted to a 1.0-kb deletion of the *cpc-3* sequence (deleting parts of the eIF2α kinase and HisRS-like domains). The *Xho*I fragment of pESIII2/19 carrying the *hph* cassette and a 3.5-kb *N. crassa* 3′ sequence was inserted into the compatible *Sal*I site of pESX14/4 in between the 5′ *cpc-3* sequence and the *Bml* cassette, resulting in disruption plasmids pESXII3/100 and pESXII3/149.

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 LVRGLDYY (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 $tRNA^{His}$. However, *GCN2* mutations in the HisRS-like domain are known that lead to either inactivation (*gcn2* alleles) or constitutive activation ($GCN2^c$ 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 (9). Both residues are conserved in CPC3 and DGCN2. In contrast, some of the $GCN2^c$ mutations affect amino acids that are not conserved in CPC3 or DGCN2 (*e.g.* $GCN2^c$*-N1295D* and $GCN2^c$*-H1308Y*, see Ref. 25) (Fig. 5). Quite the opposite, the Ala residue altered by the $GCN2^c$*-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 data bases 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. 1*D*). 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;

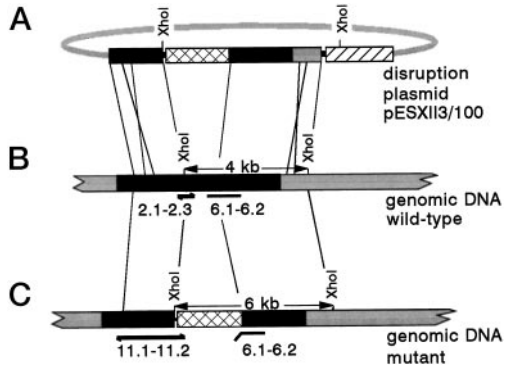

FIG. 7. **Strategy to disrupt and partially delete the *N. crassa cpc-3* gene.** Integration of the plasmid-borne *cpc-3* disruption construct (*A*) via a homologous double cross-over event on either side of the *hph* cassette should replace the *cpc-3* wild-type allele (*B*) by the mutant allele *cpc-3::hph* (*C*). Homologous integration should be indicated by 1) stable hygromycin resistance but 2) benomyl sensitivity and 3) amplification of a 2.1-kb PCR fragment with primer pair 11.1/11.2 (but no amplification with primers 2.1/2.3 in homokaryotic *cpc-3::hph* strains). 4) In a Southern analysis of *Xho*I-digested genomic DNA of the mutant, a 6-kb fragment (as opposed to a 4-kb fragment in the wild-type) should be detected using the 6.1–6.2 fragment (Fig. 1*B*) as probe. *black bar cpc-3* gene, *cross-hatched bar hph* cassette, *hatched bar Bml* cassette.

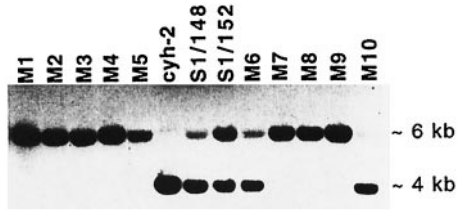

FIG. 8. **Test for homologous integration of the disruption construct *cpc-3::hph* at the *cpc-3* locus by Southern analysis.** 10 μg of *Xho*I-digested DNA of the recipient strain *cyh-2*, the primary transformant S1/152, and 10 of its microconidial subcultures (*M1–10*), and the primary transformant S1/148 were electrophoretically separated and probed with 6.1–6.2 DNA (Fig. 1*B*). DNA of microconidial strain S1/152M10 (*M10*) showed a signal at 6 kb after longer exposure. S1/152, S1/148, and S1/152-derived microconidial strains contained a 6-kb signal, expected after gene replacement; however, the primary transformants and S1/152-derived subcultures M6 and M10 additionally possessed the native 4-kb fragment present in the *cyh-2*-recipient strain, indicative of heterokaryosis. By using all sequences involved in the disruption plasmid as probe, no further signals were obtained indicating that no additional ectopic integrations had occurred (not shown).

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. 7) 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 pESXII3/100 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 both 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 (coded 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 uninduced 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*^{s} background. The bradytrophic *arg-12*^{s} allele encodes for an enzyme with drastically reduced OCT activity (47). An *arg-12*^{s}*;cpc-3* double mutant was found to grow almost at the wild-type rate without arginine supplementation (like *arg-12*^{s}*;cpc-2*, see Ref. 30), whereas an *arg-12*^{s}*;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*^{s} 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.

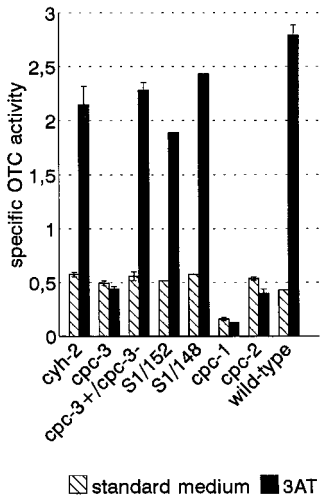

FIG. 9. **Effect of the *cpc-3* disruption on the derepression of L-ornithine carbamoyltransferase (*OTC*) in histidine-deprived mycelia.** Specific activity was measured in the *cyh-2* recipient strain, homokaryotic *cpc-3::hph* mutants (cpc-3, average of strains S1/152M1, -2, -3, -4, -5, -7, -8, -9), heterokaryotic *cpc-3*$^+$*/cpc-3::hph* transformants (cpc-3+/cpc-3−, average of strains S1/152M6 and M10), and heterokaryotic primary transformants S1/148 and S1/152 after exponential growth on standard medium or 4 h after supplementation with 6 mM (final) 3AT, respectively. For comparison strains with mutations at the *cpc-1(j-5)* and *cpc-2(U142)* loci and the wild-type were investigated under the same conditions.

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. 10*A*) 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. 10*A*), with the mRNA increased to 58% of the wild-type level (Fig. 10*B*). 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-2*;*cpc-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

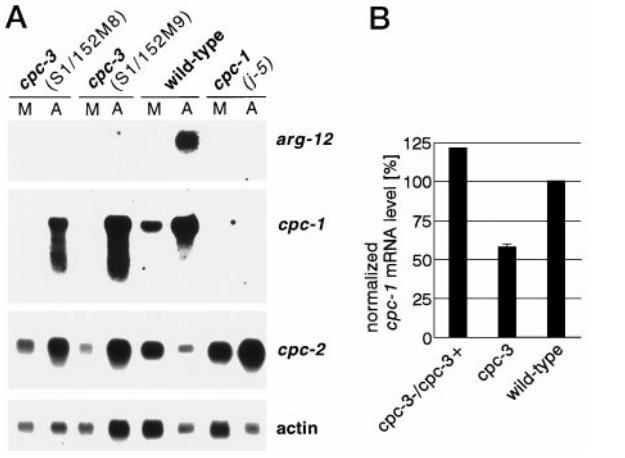

FIG. 10. **Investigation of gene expression in *cpc-3* mutants.** Northern analysis was performed with total cellular RNA of *N. crassa* grown exponentially on standard medium (*M*) or 4 h after supplementation with 6 mM (final) 3AT (*A*). *A,* the same membrane containing RNA from the homokaryotic *cpc-3* mutants S1/152M8 and M9, the wild-type, and a *cpc-1(j-5)* strain was probed with [α-^{32}P]dCTP-labeled plasmids carrying the *arg-12*, *cpc-1*, *cpc-2*, or actin genes, respectively, the latter an internal control for equal loading. *B,* quantitative estimation of *cpc-1* mRNA levels of the heterokaryotic strain S1/152M6 (cpc-3−/cpc-3+), *cpc-3* mutants (average of S1/152M8 and M9), and wild-type after supplementation of 3AT. The signal intensities of the hybridizing probes *cpc-1* and actin were calculated using NIH Image, and the amount of *cpc-1* mRNA was normalized to the actin mRNA level of each sample, respectively. The *cpc-1* mRNA level of wild-type was set to 100%. The *cpc-1* mRNA levels of the *cpc-3* mutants did not vary significantly (S1/152M8, 58%; M9, 60%), and therefore the average level is shown including the standard variation.

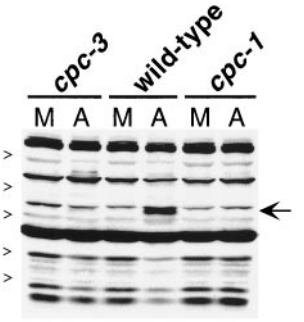

FIG. 11. **Effect of *cpc-3* mutation on cellular CPC1 protein level.** CPC1 was detected by immunoblot analysis of crude cell extracts derived from wild-type, and *cpc-1(j-5)* and *cpc-3* (S1/152M9 once backcrossed with wild-type) mutants, after exponential growth in minimal medium (*M*) or 4 h after supplementation of 6 mM (final) 3AT (*A*). Aliquots of each sample containing 50 μg 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.

leads to an increase in *cpc-1* mRNA level but not to derepression of *arg-12* transcription, a target gene of CPC1. To obtain additional evidence that *cpc-3* functions at a posttranscriptional step to increase *cpc-1* expression in amino acid-starved cells, we studied CPC1 protein levels (Fig. 11). Consistent with previous observations (49) CPC1 is undetectable under nonstarvation conditions but readily detectable under amino acid deprivation. In contrast, the *cpc-3* mutant did not show any detectable CPC1 under starvation conditions. Because CPC1 is undetectable in starved *cpc-3::hph* mycelium, it is impossible to calculate the reduction in CPC1 expression conferred by the *cpc-3* mutation. However, we estimate that the CPC1 level is at least 10-fold greater in the wild-type *versus cpc-3* mutant, a much greater difference than the 1.7-fold higher amount of *cpc-1* mRNA in wild type. These findings support the idea that *cpc-3* is a translational activator of *cpc-1* expression.

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 CPC3 kinase domain contains many of the conserved features observed previously for the eIF2α kinases GCN2, HRI, and PKR (57). The only continuous amino acid stretches uniquely conserved in the GCN2-like proteins are "WRLFRXIXEXL" in subdomain VIA and "VVRY" 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 $tRNA^{Asp}$ (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 $tRNA^{His}$ 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 eIF2α 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 minihelices 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 $tRNA^{His}$ species, the extra nucleotide G_{-1}, 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* mutations in an *arg-12*^{s} background (26, 27). We found that *arg-12*^{s}*;cpc-3* double mutants grew in unsupplemented medium.

The extensive structural similarity to GCN2 suggested that CPC3 has a function in general amino acid control equivalent to that of GCN2, namely translational activation of *cpc-1*, the *GCN4* homologue of *N. crassa*. Consistent with this conclusion, *cpc-1* mRNA becomes associated with larger polysomes after transfer of *N. crassa* to histidine starvation medium (71), and there are two uORFs in the *cpc-1* mRNA leader (see Ref. 3, accession number J03262). The 5′ leader of *GCN4* mRNA contains four uORFs necessary for gene-specific translational activation of *GCN4* expression by GCN2, but the first and fourth

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·Met-tRNA$_i^{Met}$ ternary complex formation by phosphorylation of eIF2α.

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 eIF2α in amino acid-starved *N. crassa* cells. By using isoelectric focusing gels, increased phosphorylation of eIF2α under amino acid deprivation was shown in yeast (83). Because antibodies against *N. crassa* eIF2α are not available, and the yeast eIF2α antibodies do not appear to cross-react with the *N. crassa* protein (data not shown), we could not test this prediction. The sequences surrounding Ser-51 in yeast eIF2α, the phosphorylation site recognized by GCN2, PKR, and HRI (84), are highly conserved between yeast, mammals, and *Drosophila* (85); however, the sequence of *N. crassa* eIF2α is not known. For the eIF2α 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 *N. crassa cpc-3* does not point out distinct differences in the mechanism of general control between *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 eIF2α kinase linked to a HisRS-like domain. This might indicate a general metabolic requirement for eIF2α kinases activable by uncharged tRNA, providing the means to down-regulate general translation and induce a starvation response protein like CPC1 or GCN4. *N. crassa cpc-3* or yeast *gcn2*Δ 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.

Acknowledgments—We thank Robert Metzenberg and Ronald Wek for helpful comments; Michael Plamann for the *N. crassa* actin gene; and Charles Yanofsky for the anti-CPC1 antiserum.

REFERENCES

1. Carsiotis, M., and Jones, R. F. (1974) *J. Bacteriol.* **119,** 889–892
2. Carsiotis, M., Jones, R. F., and Wesseling, A. C. (1974) *J. Bacteriol.* **119,** 893–898
3. Paluh, J. L., Orbach, M. J., Legerton, T. L., and Yanowsky, C. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 3728–3732
4. Hinnebusch, A. G. (1997) *J. Biol. Chem.* **272,** 21661–21664
5. Wanke, C., Eckert, S., Albrecht, G., van Hartingsveldt, W., Punt, P. J., van den Hondel, C. A., and Braus, G. H. (1997) *Mol. Microbiol.* **23,** 23–33
6. Wang, P., Larson, T. G., Chen, C. H., Pawlyk, D. M., Clark, J. A., and Nuss, D. L. (1998) *Fungal Genet. Biol.* **23,** 81–94
7. Roussou, I., Thireos, G., and Hauge, B. M. (1988) *Mol. Cell. Biol.* **8,** 2132–2139
8. Wek, R. C., Jackson, B. M., and Hinnebush, A. G. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 4579–4583
9. Wek, S. A., Zhu, S., and Wek, R. C. (1995) *Mol. Cell. Biol.* **15,** 4497–4506
10. Samuel, C. E. (1993) *J. Biol. Chem.* **268,** 7603–7606
11. Pavitt, G. D., Ramaiah, K. V., Kimball, S. R., and Hinnebusch, A. G. (1998) *Genes Dev.* **12,** 514–526
12. Hanks, S. K., and Hunter, T. (1995) *FASEB J.* **9,** 576–596
13. Chen, J.-J., Throop, M. S., Gehrke, L., Kuo, I., Pal, J. K., Brodsky, M., and London, I. M. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 7729–7733
14. Mellor, H., Flowers, K. M., Kimball, S. R., and Jefferson, L. S. (1994) *J. Biol. Chem.* **269,** 10201–10204
15. Meurs, E., Chong, K., Galabru, J., Thomas, N. S. B., Kerr, I. M., Williams, B. R. G., and Hovanessian, A. G. (1990) *Cell* **62,** 379–390
16. Icely, P. L., Gros, P., Bergeron, J. J. M., Devault, A., Afar, D. E. H., and Bell, J. C. (1991) *J. Biol. Chem.* **266,** 16073–16077
17. Mellor, H., Flowers, K. M., Kimball, S. R., and Jefferson, L. S. (1994b) *Biochim. Biophys. Acta* **1219,** 693–696
18. Santoyo, J., Alcalde, J., Mendez, R., Pulido, D., and de Haro, C. (1997) *J. Biol. Chem.* **272,** 12544–12550
19. Proud, C. G. (1995) *Trends Biochem. Sci.* **20,** 241–246
20. Robertson, H. D., and Mathews, M. B. (1996) *Biochimie* (*Paris*) **78,** 909–914
21. Chen, J.-J., and London, I. M. (1995) *Trends Biochem. Sci.* **20,** 105–108
22. de Haro, C., Mendez, R., and Santoyo, J. (1997) *FASEB J.* **10,** 1378–1387
23. Sachs, M. S. (1996) in *The Mycota: Biochemistry and Molecular Biology* (Brambl, R., and Marzluf, G. A., eds) Vol. 4, pp. 315–345, Springer-Verlag, Berlin
24. Wek, R. C. (1994) *Trends Biochem. Sci.* **19,** 491–496
25. Ramirez, M., Wek, R., and Hinnebusch, A. G. (1991) *Mol. Cell. Biol.* **11,** 3027–3036
26. Davis, R. H. (1979) *Genetics* **93,** 557–575
27. Barthelmess, I. B. (1982) *Genet. Res.* **39,** 169–185
28. Catcheside, D. E. A. (1991) *Fungal Genet. Newsl.* **38,** 71
29. Krüger, D. (1996) *Expressionsstudien an Potentiellen Trans-aktiven Regulationselementen und Ihren Zielgenen: Ein Beitrag zur Aufklärung des Molekularen Mechanismus der Allgemeinen Kontrolle der Aminosäurebiosynthesen von Neurospora crassa.* Ph.D. thesis, University of Hannover, Germany
30. Krüger, D., Koch, J., and Barthelmess, I. B. (1990) *Curr. Genet.* **18,** 211–215
31. Müller, F., Krüger, D., Sattlegger, E., Hoffmann, B., Ballario, P., Kanaan, M., and Barthelmess, I. B. (1995) *Mol. Gen. Genet.* **248,** 162–173
32. Davis, R. H., and deSerres, F. J. (1979) *Methods Enzymol.* **57,** 79–143
33. Vollmer, S. J., and Yanofsky, C. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 4867–4873
34. Flint, H. J., and Wilkening, J. (1986) *Mol. Gen. Genet.* **203,** 110–116
35. Orbach, M. J., Porro, E. B., and Yanofsky, C. (1986) *Mol. Cell. Biol.* **6,** 2452–2461
36. Staben, C., Jensen, B., Singer, M., Pollock, J., Schechtman, M., Kinsey, J., and Selker, E. (1989) *Fungal Genet. Newsl.* **36,** 79–81
37. Jacobs, M., Wnendt, S., and Stahl, U. (1990) *Nucleic Acids Res.* **18,** 1653
38. Zabarovsky, E. R., and Winberg, G. (1990) *Nucleic Acids Res.* **18,** 5912
39. Ebbole, D. J., and Sachs, M. S. (1990) *Fungal Genet. Newsl.* **37,** 17–18
40. Lee, S. B., Milgroom, M. G., and Taylor, J. W. (1988) *Neurospora Newsl.* **35,** 23–24
41. Irelan, J., Miao, V., and Selker, E. U. (1993) *Fungal Genet. Newsl.* **40,** 24
42. Chow, T. Y.-K., and Kaefer, E. (1993) *Fungal Genet. Newsl.* **40,** 25–27
43. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., pp. E34–35, 9.31–9.62, 7.52, and 1.90–1.104, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
44. Sokolowsky, V., Kaldenhoff, R., Ricci, M., and Russo, V. E. A. (1990) *Fungal Genet. Newsl.* **37,** 41–43
45. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol.* **215,** 403–410
46. Devereux, J., Haeberli, P., and Smithies, O. (1984) *Nucleic Acids Res.* **12,** 387–395
47. Davis, R. H. (1962) *Arch. Biochem. Biophys.* **97,** 185–191
48. Flavell, R. B., and Fincham, J. R. S. (1968) *J. Bacteriol.* **95,** 1063–1068
49. Ebbole, D. J., Paluh, J. L., Plamann, M., Sachs, M. S., and Yanofsky, C. (1991) *Mol. Cell. Biol.* **11,** 928–934
50. Sattlegger, E. (1996) *Identifikation, Molekulare und Funktionelle Charakterisierung des cpc-3 Gens: Ein Beitrag zur Aufklärung der Allgemeinen Kontrolle der Aminosäurebiosynthesen von Neurospora crassa.* Ph.D. thesis, University of Hannover, Germany
51. Metzenberg, R. L., Stevens, J. N., Selker, E. U., and Morzycka-Wroblewska, E. (1984) *Neurospora Newsl.* **31,** 35–39
52. Metzenberg, R. L., and Grotelueschen, J. (1995) *Fungal Genet. Newsl.* **42,** 82–90
53. Gurr, S. J., Unkles, S. E., and Kinghorn, J. R. (1981) in *Gene Structure in Eukaryotic Microbes* (Kinghorn, J. R., ed) pages 93–139, IRL Press at Oxford University Press, Oxford
54. Bruchez, J. J. P., Eberle, J., and Russo, V. E. A. (1993) *Fungal Genet. Newsl.* **40,** 85–87

55. Edelmann, S. E., and Staben, C. (1994) *Exp. Mycol.* **18,** 70–81
56. Hanks, S. K., Quinn, A. M., and Hunter, T. (1988) *Science* **241,** 40–52
57. Ramirez, M., Wek, R., Vazquez de Aldana, C. R., Jackson, B. M., Freeman, B., and Hinnebusch, A. G. (1992) *Mol. Cell. Biol.* **12,** 5801–5815
58. Carter, C. W. (1993) *Annu. Rev. Biochem.* **62,** 715–48
59. Arnez, J. G., Augustine, J. G., Moras, D., and Franklyn, C. S. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 7144–7149
60. Arnez, J. G., Harris, D. C., Mitschler, A., Rees, B., Franklyn, C. S., and Moras, D. (1995) *EMBO J.* **14,** 4143–4155
61. Aberg, A., Yaremchuk, A., Tukalo, M., Rasmussen, B., and Cusack, S. (1997) *Biochemistry* **36,** 3084–3094
62. Rühlmann, A., Cramer, F., and Englisch, U. (1997) *Biochem. Biophys. Res. Commun.* **237,** 192–201
63. Ruff, M., Krishnaswamy, S., Boeglin, M., Poterszman, A., Mitschler, A., Podjarny, A., Rees, B., Thierry, J. C., and Moras, D. (1991) *Science* **252,** 1682–1689
64. Cusack, S., Hartlein, M., and Leberman, R. (1991) *Nucleic Acids Res.* **19,** 3489–3498
65. Raben, N., Boriello, F., Amin, J., Horwitz, R., Fraser, D., and Plotz, P. (1992) *Nucleic Acids Res.* **20,** 1075–1081
66. Zhu, S., Sobolev, A. Y., and Wek, R. C. (1996) *J. Biol. Chem.* **271,** 24989–24994
67. Aronson, B. D., Lindgren, K. M., Dunlap, J. C., and Loros, J. J. (1994) *Mol. Gen. Genet.* **242,** 490–494
68. Hilton, J. L., Kearney, P. C., and Ames, B. N. (1965) *Arch. Biochem. Biophys.* **112,** 544–547
69. Kolanus, J., Michalczyk, J., Flint, H. J., and Barthelmess, I. B. (1990) *Mol. Gen. Genet.* **223,** 443–448
70. Yan, W., Augustine, J., and Francklyn, C. (1996) *Biochemistry* **35,** 6559–6568
71. Luo, Z., Freitag, M., and Sachs, M. S. (1995) *Mol. Cell. Biol.* **15,** 5235–5245
72. Grant, C. M., and Hinnebusch, A. G. (1994) *Mol. Cell. Biol.* **14,** 606–618
73. Himeno, H., Hasegawa, T., Ueda, T., Watanabe, K., Miura, K., and Shimizu, M. (1989) *Nucleic Acids Res.* **19,** 7855–7863
74. Francklyn, C., and Schimmel, P. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 8655–8659
75. Francklyn, C., Shi, J.-P., and Schimmel, P. (1992) *Science* **255,** 1121–1125
76. Rudinger, J., Felden, B., Florentz, C., and Giege, R. (1997) *Bioorg. Med. Chem.* **5,** 1001–1009
77. Sprinzl, M., Hartmann, T., Weber, J., Blank, J., and Zeidler, R. (1989) *Nucleic Acids Res.* **17,** (suppl.) R1–R172
78. Hinnebusch, A. G. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 6442–6446
79. Hinnebusch, A. G. (1985) *Mol. Cell. Biol.* **5,** 2349–2360
80. Hanks, S. K., and Quinn, A. M. (1991) *Methods Enzymol.* **200,** 38–81
81. Eriani, G., Delarue, M., Poch, O., Gangloff, J., and Moras, D. (1990) *Nature* **347,** 203–206
82. Doolittle, R. F. (1986) *Of URFs and ORFs: A Primer on How to Analyze Derived Amino Acid Sequences*, pp. 15–18, University Science Books, Mill Valley, CA
83. Dever, T. E., Feng, L., Wek, R. C., Cigan, A. M., Donahue, T. F., and Hinnebusch, A. G. (1992) *Cell* **68,** 585–596
84. Dever, T. E., Chen, J. J., Barber, G. N., Cigan, A. M., Feng, L., Donahue, T. F., London, I. M., Katze, M. G., and Hinnebusch, A. G. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 4616–4620
85. Qu, S., and Cavener, D. R. (1994) *Gene* (*Amst.*) **140,** 239–242
86. Romano, P. R., Garcia-Barrio, M. T., Zhang, X., Wang, Q., Taylor, D. R., Zhang, F., Herring, C., Mathews, M. B., Qin, J., and Hinnebusch, A. G. (1998) *Mol. Cell. Biol.* **18,** 2282–2297
87. Hope, I. A., and Struhl, K. (1985) *Cell* **43,** 177–188
88. Mandel, M., and Higa, A. (1970) *J. Mol. Biol.* **53,** 159–162
89. Qiu, H., Garcia-Barrio, M. T., and Hinnebusch, A. G. (1998) *Mol. Cell. Biol.* **18,** 2697–2711