

Determination of the Inactivating Alterations in Two Mutant Alleles of the *Neurospora crassa* Cross-Pathway Control Gene *cpc-1*

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ABSTRACT

cpc-1 is the locus specifying what is believed to be the major *trans*-activating transcription factor that regulates expression of amino acid biosynthetic genes subject to cross-pathway control in *Neurospora crassa*. Mutants altered at this locus are incapable of the global increase in gene expression normally seen in response to amino acid starvation. Using polymerase chain reaction methodology we have cloned and sequenced the inactive mutant allele, *cpc-1* (CD15). The *cpc-1* (CD15) mutation was found to be a single base pair deletion in codon 93 of the *cpc-1* structural gene. A second, presumed lethal, allele, *cpc-1* (j-5), also was investigated. Northern analyses with strains carrying the *cpc-1* (j-5) allele revealed that no *cpc-1* mRNA is produced. Southern and genetic analyses established that the *cpc-1* (j-5) mutation involved a chromosomal rearrangement in which a break occurred within the *cpc-1* locus, normally resident on linkage group VI; a small fragment from the left arm of linkage group VI, containing the *cpc-1* promoter region and *ylo-1*, was translocated to the right arm of linkage group I. Other studies indicate that the *cpc-1* locus itself is not essential for viability. Lethality previously attributed to the *cpc-1* (j-5) mutation is due instead to the production of progeny that are deficient for essential genes in an adjoining segment of linkage group VI. Molecular characterization of *cpc-1* (j-5) × *ylo-1 pan-2* duplication progeny indicated that *cpc-1* is normally transcribed towards the linkage group VI centromere.

STARVATION for individual amino acids in *Saccharomyces cerevisiae*, *Aspergillus nidulans* and *Neurospora crassa* results in increased expression of genes encoding several, if not most, of the amino acid biosynthetic pathway enzymes (CARSIOTIS, JONES and WESSELING 1974; CARSIOTIS and JONES 1974; PIOTROWSKA 1980; GOC and WEGLENSKI 1988; HINNEBUSCH 1988). This global response is due to increased rates of transcription of the affected genes. Extensive genetic analyses of this regulatory mechanism in yeast, termed general control of amino acid biosynthesis, have identified a hierarchy of fifteen genes involved in this complex regulatory process. The product of one of these genes, GCN4, has been studied extensively; GCN4 is a DNA-binding protein and the transcriptional activator of amino acid biosynthetic genes (HOPE and STRUHL 1985, 1987; ARNDT and FINK 1986; HINNEBUSCH 1988).

Only a few genetic loci have been implicated so far in the similar regulatory response in *Neurospora*, referred to as cross-pathway control (DAVIS 1979; BARTHELMESS 1982, 1984; D. KRÜGER, J. KOCH and I. B. BARTHELMESS, manuscript in preparation). The

majority of mutants that are defective in cross-pathway control have alterations at one locus, *cpc-1*. Mutations in this gene, like those in *GCN4* in yeast, abolish the increase in expression of amino acid biosynthetic genes normally seen in amino acid auxotrophs grown on limiting amounts of the required amino acids. *cpc-1* has been cloned and sequenced (PALUH *et al.* 1988). The deduced amino acid sequence of its polypeptide product, CPC1, shows homology to yeast GCN4 in regions known to be critical for transcription activation, and for DNA-binding (PALUH *et al.* 1988).

No alterations mapped to the *cpc-1* locus have previously been characterized at the molecular level. In this paper we identify the alterations in two *cpc-1* mutant alleles: *cpc-1* (CD15) and *cpc-1* (j-5). Strains carrying the *cpc-1* (CD15) allele do not increase transcription of amino acid biosynthetic genes under conditions of amino acid limitation. However, transcription of *cpc-1* (CD15) itself is increased under these conditions (PALUH *et al.* 1988). We have found that the *cpc-1* (CD15) allele has a single base pair deletion in codon 93 of the *cpc-1* structural gene. The second allele analyzed, *cpc-1* (j-5), was originally characterized as being lethal in the absence of an unlinked rescuing mutation, *slo*, that results in a "slow growth" phenotype (BARTHELMESS 1984). Recently, however,

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a deletion derivative of *cpc-1*, constructed by transforming *Neurospora* with disrupted *cpc-1* DNA, was shown to be viable (M. PLAMANN unpublished results). The biochemical and genetic analyses described in this paper indicate that the *cpc-1* (j-5) alteration involved a chromosomal rearrangement and that *cpc-1* is not essential for viability.

MATERIALS AND METHODS

Strains: *Neurospora crassa* strains used as sources of DNA for polymerase chain reaction (PCR) analyses, southern and northern analyses were obtained from the Fungal Genetics Stock Center (University of Kansas Medical Center): *cpc-1* (CD15) (FGSC #4264), *cpc-1* (j-5) (FGSC #4433 and #4434), and 74-OR23-1A (FGSC #8015). Strains used for genetic analyses, described in the text, were from the collection of David Perkins.

Plasmids: pCPC1-2, which contains *cpc-1* on a 5.5 kb genomic fragment in pUC12, was described previously (PALUH *et al.* 1988). pUC118, used in DNA sequence analyses, was provided by J. VEIRA, Waksman Institute, Piscataway, N.J. pJPT7C2, used for *in vitro* transcription/translation experiments, contains a *RsaI* to *EcoRI* 1.2-kb cDNA fragment from pM031 (PALUH *et al.* 1988) cloned into pBluescript (Stratagene) at the *HincII* and *EcoRI* sites, then recloned into pT7-7 (TABOR and RICHARDSON 1985) at the *XbaI* and *SalI* sites. The DNA sequence between positions 1176 and 1180 (AGGGC) was modified by site-directed mutagenesis (BAUER *et al.* 1985; KUNKEL 1985) to resemble an *E. coli* ribosome binding site. A *RsaI*-*BamHI* fragment from pJPCD1541 (described in the text), containing the *cpc-1* (CD15) mutational change, was incorporated into pJPT7C2 in place of native *cpc-1* sequences. The resulting plasmid was designated pJPT7CD15. The genomic clone of the *cpc-1* structural gene, pMO61, has been described (PALUH *et al.* 1988).

PCR analysis and DNA sequencing: Genomic DNAs for PCR (MULLIS and FALOONA 1987) and southern analyses were isolated as described (METZENBERG and BAISCH 1981) with suggested modifications (STEVENS and METZENBERG 1982). Samples containing 3 to 5 μ g undigested genomic DNA from 74A (wild type), *cpc-1* (CD15), *cpc-1* (j-5) or 0.1 μ g of *SalI* linearized, CsCl-purified pCPC1-2 control DNA, were incubated with 0.2 to 5.0 μ g each of two nonpurified oligonucleotide primers and amplified for a total of 40 cycles using an Ericomp temperature cyler. Cycle conditions were as follows: [94° 1 min, 37° 2 min, 74° 2 min] for 15 cycles, [74° 1 min] for 5 cycles; repeat. The reaction buffer and taq polymerase conditions were according to the recommendations of Perkin-Elmer/Cetus. PCR products were examined by electrophoresis on 0.8% agarose gels run in 0.5 \times TBE buffer. TBE is: 90 mM Tris-HCl (pH 8.3), 90 mM boric acid and 2.5 mM EDTA. DNA sequencing was performed by the dideoxy chain termination method (SANGER, NICKLEN and COULSON 1977), using Sequenase (United States Biochemical, Cleveland, Ohio).

***In vitro* transcription/translation:** *In vitro* transcription was performed as recommended (United States Biochemical). Translation was carried out using 0.5 to 3.0 μ l of a 50 μ l *in vitro* transcription mixture and 30 μ Ci [α^{35} S]-L-methionine (>800 Ci/mmol; Amersham) in a 25 μ l reaction volume. Rabbit reticulocyte lysates were used as described (Promega Biotec, Madison, Wisconsin).

Northern analysis: Total cellular RNA was prepared as described (KINNAIRD *et al.* 1982). RNA species were sepa-

rated by electrophoresis on 1.4% agarose/6% formaldehyde gels (LEHRACH *et al.* 1977), and transferred to nitrocellulose (MANIATIS, FRITSCH and SAMBROOK 1982). For hybridization, DNA probes were prepared by incorporating [α^{32} P] dCTP using the "Random Primed DNA Labeling Kit" (Boehringer) to a specific activity of at least $0.5\text{--}2 \times 10^8$ cpm/ μ g DNA using 0.1–0.5 μ g DNA in a total volume of 20 μ l.

Southern analyses: DNAs were fractionated on 1.0% agarose/Tris-borate-EDTA gels, transferred to genescreen (NEN Research Products, Boston, Massachusetts) and probed with [α^{32} P]dCTP random-hexamer-labeled *cpc-1* fragments (FEINBERG and VOGELSTEIN 1983) as described (MANIATIS, FRITSCH and SAMBROOK 1982).

Crosses with *cpc-1* (j-5): Standard procedures were employed (DAVIS and DE SERRES 1970).

RESULTS

PCR analyses of *cpc-1* (CD15) and *cpc-1* (j-5) alleles. Pairs of oligonucleotides were chosen for PCR analyses that would prime the synthesis of fragments of *cpc-1* 0.2–1.8 kb in length. Undigested genomic DNA isolated from 74A (wild type) or linearized pCPC1-2 DNA containing the entire *cpc-1* transcription unit were used as control DNAs for adjusting annealing temperatures and extension times (MATERIALS AND METHODS).

PCR fragments were generated using genomic DNA isolated from strains carrying the *cpc-1* (CD15) or *cpc-1* (j-5) alleles. A series of overlapping DNA fragments which encompassed the entire *cpc-1* transcriptional unit, were produced. One tenth of each PCR reaction mixture was electrophoresed on agarose gels, transferred to Genescreen, and probed with *cpc-1* DNA fragments to identify specific *cpc-1* PCR DNA products.

No discrepancies in predicted fragment lengths were observed between PCR DNA fragments generated from *cpc-1* (CD15) DNA or from control DNAs. PCR DNA fragments generated from *cpc-1* (j-5) DNA were as predicted for the *cpc-1* structural gene and for part of the *cpc-1* mRNA leader region, but no PCR DNA fragments were obtainable that included the entire region encoding the 5' segment of the *cpc-1* transcript (data not shown).

Sequence analysis and cloning of *cpc-1* (CD15). PCR-generated DNA fragments, that contained overlapping segments of the *cpc-1* (CD15) structural gene were isolated from agarose gels and sequenced directly and/or cloned into pUC118, and then sequenced. Sequencing of several *cpc-1* (CD15) PCR DNA fragments identified a single base pair deletion removing a G of codon 93 of the *cpc-1* structural gene (Figure 1). No other changes were detected. This was confirmed by sequencing several isolates of *cpc-1* (CD15) PCR products cloned into pUC118.

For *in vitro* synthesis experiments, the intact *cpc-1* (CD15) structural gene was cloned from genomic DNA using PCR methods (PCR analyses and MATE-

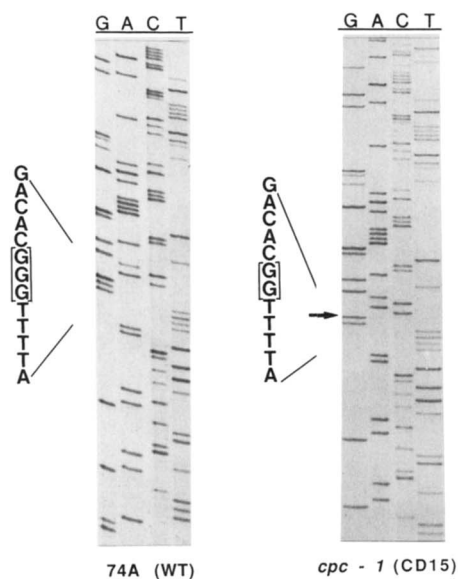


FIGURE 1.—DNA sequence comparison of a segment of *cpc-1* isolated from 74A(wild type) and a single representative isolate of *cpc-1* (CD15) cloned into pUC118. An arrow indicates the deletion of a single G residue in *cpc-1* (CD15). The sequence immediately surrounding the site of alteration is shown alongside the reaction lanes. Brackets indicate the actual site of the deletion.

RIALS AND METHODS). A 1.15-kb DNA fragment was generated which contained the entire *cpc-1* (CD15) structural gene, including 130 bp of upstream and downstream sequences. DNA fragments from several PCR reactions were isolated and cloned into the *Hinc*II site of pBluescript and sequenced to confirm the single base pair change. One of these isolates was designated pJPCD1541.

Analysis of *cpc-1* (CD15)-directed *in vitro* translation product: pJPT7C2, pMO61 and pJPT7CD15 contain the cDNA copy of the *cpc-1* structural gene, the genomic copy of *cpc-1*, or *cpc-1* of the mutant *cpc-1* (CD15), respectively, inserted downstream from a T7 promoter located within the vector (MATERIALS AND METHODS). mRNAs synthesized from these constructs using T7 RNA polymerase were translated *in vitro* using rabbit reticulocyte lysate.

cpc-1 encodes a polypeptide 268 amino acids in length, with a predicted molecular mass of 30 kD. The CPC1 polypeptide has an aberrant mobility (42 kD) which presumably is due to the highly charged carboxyl end of the protein (PALUH *et al.* 1988). *In vitro* translation of mRNA derived from the genomic copy of the *cpc-1* structural gene, which contains an in-frame intron that encodes 17 additional amino acids, yields a polypeptide 285 amino acids in length. This polypeptide migrates on SDS-polyacrylamide gels with a mobility of 45 kD (PALUH *et al.* 1988).

The position of the frameshift mutation in *cpc-1* (CD15) leads to the prediction that its specified polypeptide should be 220 amino acids in length, and should have a molecular mass of 25 kD. Since the last

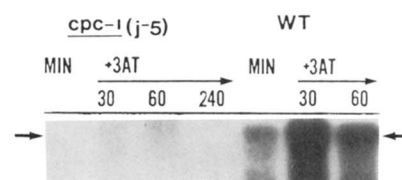


FIGURE 2.—RNA northern analyses with electrophoretically fractionated total cellular RNA from 74A(wild type) and a strain carrying the *cpc-1* (j-5) allele. RNA samples were taken from cultures grown in minimal medium (MIN) and 30 min., 60 min. or 240 min. following addition of 3AT to 6 mM. *cpc-1* mRNA was detected with a labeled probe consisting of plasmid DNA containing the *cpc-1* gene. Arrows indicate the position of *cpc-1* mRNA.

127 amino acids of this polypeptide would be translated from a shifted reading frame, the aberrant mobility observed with native CPC1 would not be expected.

The expected translation products were detected when pJPT7C2 and pMO61 mRNAs were used as templates but no discrete products were observed with *cpc-1* (CD15) mRNA prepared using the pJPT7CD15 template (data not shown).

Northern analyses of *cpc-1* (j-5): 74A(wild type) and a strain carrying the *cpc-1* (j-5) allele were grown on minimal medium (DAVIS and DE SERRES 1970) or under conditions of amino acid starvation [in the presence of the inhibitor of histidine biosynthesis, 3-amino-1,2,4-triazole (3AT)]. Total cellular RNA was isolated from 150 mg lyophilized mycelial samples and the RNA species were separated electrophoretically (MATERIALS AND METHODS). To verify equal loading and transfer of RNA samples, filters were first probed with α^{32} P-labeled pBT6 plasmid DNA containing the β -tubulin structural gene (ORBACH, PORRO and YANOFESKY 1986). The filters were then stripped and reprobated with a labeled DNA fragment from pCPC1-2 (Figure 2).

The *cpc-1* mRNA level was low when total RNA was isolated from wild-type cultures grown in minimal medium, but was clearly induced 30 min after 3AT treatment. By contrast, *cpc-1* (j-5) total RNA did not contain detectable levels of *cpc-1* mRNA even following treatment with 3AT for up to 240 min.

Southern analysis of *cpc-1* (CD15) and *cpc-1* (j-5). Approximately 10 μ g of genomic DNAs isolated from 74A(wild type) and from strains carrying the *cpc-1* (CD15) or *cpc-1* (j-5) allele were digested with restriction endonucleases and the fragments separated electrophoretically. The DNA fragments used as hybridization probes either contained the region corresponding to the 5' end of the *cpc-1* transcript, or flanked this region.

Restriction patterns generated with digested *cpc-1* (CD15) and 74A(wild type) genomic DNAs were found to be identical (data not shown). The probe used was a 1.4 kb *Hind*III-*Sph*I fragment which contains the 5' noncoding region of the *cpc-1* transcript

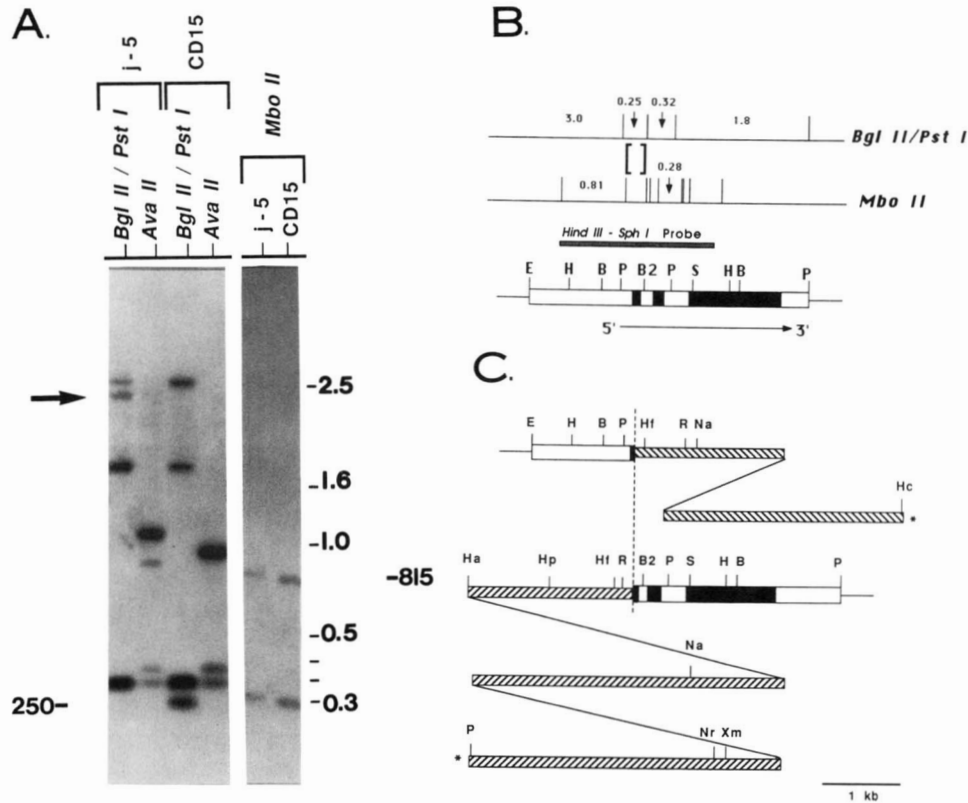


FIGURE 3.—Southern analysis of *cpc-1* (*j-5*) genomic DNA. (A) Genomic DNA isolated from 74A(wild type), *cpc-1* (*j-5*) and *cpc-1* (CD15) was digested with *Pst*I and *Bgl*II, *Ava*II or *Mbo*II and separated electrophoretically. The filters shown were probed with the 1.4-kb *Hind*III–*Sph*I 5' *cpc-1* fragment (defined in B). The arrow indicates the fragment mentioned in the text. (B) Diagram of the *cpc-1* region and restriction maps obtained with *Pst*I plus *Bgl*II, and *Mbo*II digests. The three solid blocks in the *cpc-1* diagram indicate the location of the two short upstream open reading frames, present within the *cpc-1* mRNA leader region, and the *cpc-1* structural gene. The arrow below the solid blocks indicates the segment corresponding to the *cpc-1* transcript. The two horizontal lines with tick marks (top of diagram) represent restriction maps expected for digests with either *Pst*I plus *Bgl*II, or *Mbo*II. Brackets indicate the 170-bp segment containing the breakpoint. Predicted fragment sizes in kb are inserted between tick marks. The 1.4-kb *Hind*III–*Sph*I probe is indicated. (C) Summary of southern analysis of *cpc-1* (*j-5*). Large bars indicate portions of the *cpc-1* genetic region as shown in B. Small bars indicate foreign DNA attached to *cpc-1* DNA in the region of discontinuity indicated by the dashed line. Asterisks located at the end of these small bars indicate an undefined end. The scale for B and C is indicated. Abbreviations for B and C are as follows: E (*Eco*RI), H (*Hind*III), B (*Bam*HI), P (*Pst*I), Hf (*Hinf*I), R (*Rsa*I), Na (*Nae*I), B2 (*Bgl*II), Hc (*Hinc*II), NR (*Nar*I), Ha (*Hae*III), Hp (*Hpa*II), S (*Sph*I).

and upstream sequences (Figure 3B). However, a 250-bp *Pst*I–*Bgl*II fragment present in digested 74A(wild type) DNA was absent from *cpc-1* (*j-5*) DNA digests; a new, 2.0-kb band, was present in the *cpc-1* (*j-5*) digests (Figure 3A). This result indicates that there is a chromosomal break within the 250-bp *Pst*I–*Bgl*II region of *cpc-1* (*j-5*) (Figure 3B). An *Mbo*II digest of *cpc-1* (CD15) and *cpc-1* (*j-5*) DNAs, probed with the 1.4-kb *Hind*III–*Sph*I DNA fragment, contains an 815-bp *Mbo*II fragment which partially overlaps the 250-bp *Pst*I–*Bgl*II fragment (Figure 3, A and B). The smaller *Mbo*II fragment, located in the region of the breakpoint, was too small to be detected by southern analyses. The extent of overlap between these two fragments resolves the breakpoint of *cpc-1* (*j-5*) to within a 170-bp *Mbo*II–*Bgl*II region (Figure 3, A and B, brackets).

Using DNA probes that flank this *Mbo*II–*Bgl*II 170 bp segment the breakpoint in the *cpc-1* (*j-5*) allele was further localized to within a 100 bp segment, starting

40 bp downstream from the *cpc-1* transcription start site. At least 5.0 kb of foreign DNA was determined to be attached to the *cpc-1* locus 5' to the break point, and at least 10 kb of foreign DNA was attached to the *cpc-1* locus 3' to the break point. A partial restriction map of these foreign DNA segments is shown in Figure 3C.

Crosses with the *cpc-1* (*j-5*) mutant: Initial genetic analysis of *cpc-1* (*j-5*) suggested that it represented a lethal allele of *cpc-1* that could only be identified in a strain carrying an unlinked modifier, *slo*, that causes a “slow growth” phenotype (BARTHELMESS 1984). A cross of *cpc-1* (*j-5*) with wild type produces three classes of progeny which were classified as *slo*⁺ *cpc-1*⁺, *slo* *cpc-1*⁺ (barren in subsequent crosses) and *slo* *cpc-1*. The lack of *slo*⁺ *cpc-1* progeny was interpreted to indicate the lethal nature of the *cpc-1* (*j-5*) allele in the absence of the *slo* mutation.

During the course of our work the observation was made that a cross of *cpc-1* (*j-5*) by wild type produced

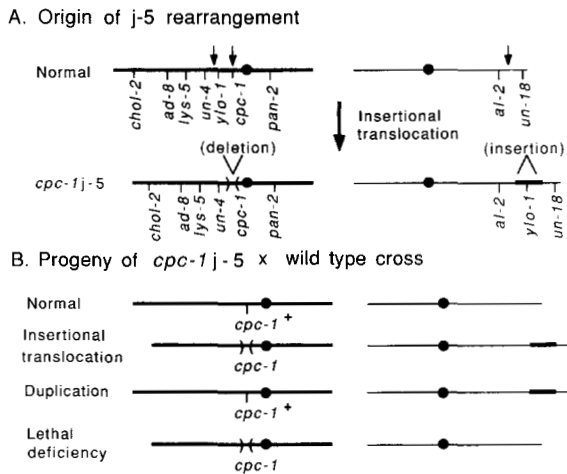


FIGURE 4.—Model of *cpc-1* (j-5) chromosome rearrangement formation (A) and predicted meiotic products of a *cpc-1* (j-5) × wild type cross (B). Thick lines represent donor chromosome segments, linkage group VI, thin lines represent recipient chromosome segments, linkage group I, and filled circles represent respective centromeres. The vertical arrows above the two lines (A) represent approximate chromosome breakpoints. The precise location of the linkage group I breakpoint with respect to the *al-2* and *un-18* markers has not been determined. The genetic markers used in the analysis of *cpc-1* (j-5) are indicated below the illustrated chromosomes. The indicated gene order is correct but the distances shown are not to scale with respect to known values (PERKINS *et al.* 1982).

only 75% black (viable) spores. The presence of 25% defective white spores is characteristic of an insertional translocation that generates viable duplications and nonviable deficiencies (PERKINS 1974). Examination of unordered asci from the above cross showed: 14.6% 8 black:0 white (8B:0W) spores, 64.7% 6B:2W, 19.3% 4B:4W, 0.5% 2B:6W, 0.5% 0B:8W ($N = 218$). The high frequency of 6B:2W asci with approximately equal numbers of 8B:0W and 4B:4W asci is consistent with the hypothesis that the *cpc-1* (j-5) strain contains a chromosomal rearrangement with one breakpoint near a centromere (at *cpc-1*) and the other breakpoint far from the centromere of another chromosome. Therefore, the progeny from the above cross should consist of four classes: normal wild type, insertional translocation, viable duplication and lethal deficiency (Figure 4). It would be predicted that the duplication class would be barren (this is typical for Neurospora duplications) and the *cpc-1* progeny lacking the chromosome with the translocated part of linkage group VI would be present as white, inviable spores (Figure 4B). The original hypothesis required the progeny containing the modifier *slo* to be barren when segregated with *cpc-1*⁺ but fertile when segregated with *cpc-1* (j-5).

If the insertional translocation hypothesis is correct then recessive markers that enter the cross with the normal sequence parent will be expressed in the duplication progeny if they map outside the translocated segment, but will be heterozygous and not expressed if they map within the translocated segment. There-

fore, linkage group VI recessive markers covered by the duplication should be expressed at a 1⁻:2⁺ ratio among the progeny and linkage group VI recessive markers not covered should be expressed at a 2⁻:1⁺ ratio (see Figure 4B).

Crosses of *cpc-1* (j-5) with *cho1-2 ad-8 lys-5 un-4 pan-2* and *ylo-1 pan-2* strains indicated that *ylo-1*, but not the other linkage group VI markers, was likely included in the duplication (Table 1A and 1B). Thus, there was a low *ylo-1/ylo-1*⁺ progeny ratio (29:41) relative to the *pan-2/pan-2*⁺ progeny ratio (42:28) (Table 1B). Additional analysis of the progeny from the *cpc-1* (j-5) × *ylo-1 pan-2* cross showed that all of the *ylo-1 cpc-1*⁺ progeny were fertile and all the *ylo-1*⁺ *cpc-1*⁺ progeny were barren as predicted by the insertional translocation hypothesis. (The original hypothesis of an unlinked modifier would have predicted that half of each class would be barren.) These observations provide strong evidence that the *cpc-1* (j-5) strain contains a chromosomal rearrangement which resulted from a break within the *cpc-1* genetic region, and that a segment of linkage group VI including *ylo-1* but not *un-4*, has been inserted into another chromosome, far from its centromere.

The recipient chromosome was tentatively identified by the cross *cpc-1* (j-5) × *alcoy; csp-2* (method of PERKINS *et al.* 1969). Analysis of the progeny of this cross showed a 1:2 ratio of *al-1/al-1*⁺ which indicated that the other breakpoint was likely to reside in either the right arm of linkage group I or linkage group II. In addition, 90 of 91 *al-1*⁺ progeny were *ylo-1*⁺. This close linkage is consistent with the concept that *ylo-1* is within the segment of linkage group VI that was inserted into another chromosome. A follow up cross of *cpc-1* (j-5) × *al-1; fl* (linkage group I and II markers, respectively) produced a 1:2 ratio of *al-1/al-1*⁺ progeny but a 1:1 ratio of *fl/fl*⁺ progeny indicating that the right arm of linkage group I is likely to be the site where the segment of linkage group VI was inserted. An additional cross of *cpc-1* (j-5) × *al-2 un-18* (two markers within the right arm of linkage group I) also produced 1:2 ratios of *al-2/al-2*⁺ and *un-18/un-18*⁺ progeny. In addition, no *al-2 cpc-1* or *un-18 cpc-1* progeny were observed. These results confirm that linkage group I is the recipient chromosome, but the precise position of the insertion site with respect to the linkage group I markers could not be inferred from the data (data not shown).

Southern analysis of a duplication progeny from the *cpc-1* (j-5) × *ylo-1 pan-2* cross. Southern analysis of *cpc-1* (j-5) established that the chromosome break which disrupted *cpc-1* DNA occurred in a 100 bp segment, starting 40 bp downstream from the *cpc-1* transcription start site (Figure 3). Restriction endonuclease generated fragments that overlap the breakpoint are not observed by southern analysis of *cpc-1*

TABLE 1
Linkage group VI marker duplication analysis

A. Cross: <i>cpc-1</i> (j-5) A × <i>chol-2 ad-8 lys-5 un-4 pan-2</i> a				
Genetic marker	Number of progeny ^a			
	Prototrophs	Auxotrophs		
<i>chol-2</i>	39	49		
<i>ad-8</i>	31	57		
<i>lys-5</i>	29	59		
<i>un-4</i> ^b	28	60		
<i>pan-2</i>	27	61		

B. Cross: <i>cpc-1</i> (j-5) A × <i>ylo-1 pan-2</i> a					
Expected crossover type	Progeny phenotype			Number of progeny ^c	
	Ylo	Cpc	Pan	Fertile ^e	Barren ^e
Parental	+	-	+	24	0
	-	+	-	26	0
Single crossover <i>ylo-cpc</i>	+	+	-	0	14
	-	-	+	0	0
Single crossover <i>cpc-pan</i>	+	-	-	2	0
	-	+	+	3	0
Double crossover	+	+	+	0	1
	-	-	-	0	0
				55	15

ylo-1-pan-2 recombination: 6/70 = 9%.

Expected number of duplications: $1/3 \times 70 = 23$.

Duplications are expected to be barren and to be Cpc⁺ Ylo⁺ if *ylo-1* is included in the translocated segment (see Figure 4). All apparent recombinants in the column headed "Fertile" are bona fide crossovers, but apparent *ylo-cpc* crossovers in the column headed "Barren" are due instead to the presence of *ylo*⁺ in the transposed duplicated segment.

All Cpc⁻ progeny produce ~75% black ascospores in test crosses × *fl*. All fertile Cpc⁺ progeny produce 95–98% black ascospores in test crosses × *fl*.

^a 88 of 100 black ascospores germinated.

^b *un-4* strains grow at 25°C but not at 34°C.

^c *cpc-1* function was determined by the ability to grow on solid minimal media supplemented with 3AT (0.1 mg/ml).

^d Seventy of 100 black ascospores germinated.

^e Fertility was determined by follow up crosses with *fl*^p tester strains. The 15 Ylo⁺ Cpc⁺ apparent recombinants are in fact not crossovers between *ylo-1* and *cpc-1* but are *ylo*⁺/*ylo*⁻ duplications.

(j-5) DNA; instead, two new fragments are observed. From the genetic analysis of *cpc-1* (j-5), it follows that one new fragment will extend from *cpc-1* DNA into linkage group VI sequences and the other new fragment will extend from *cpc-1* DNA into linkage group I sequences. Southern analysis of a *ylo-1*⁺ *pan-2* (barren) duplication strain, obtained from the *cpc-1* (j-5) × *ylo-1 pan-2* cross (Table 1B, Figure 4B), would be expected to identify the new fragment that extends from *cpc-1* DNA into linkage group I sequences. The other hybrid fragment would not be observed because the duplication strain contains a complete copy of *cpc-1* on linkage group VI.

Genomic DNAs from 74A(wild type), *cpc-1* (j-5) and a *ylo-1*⁺ *pan-2* duplication strain were digested with *Hind*III restriction endonuclease and analyzed

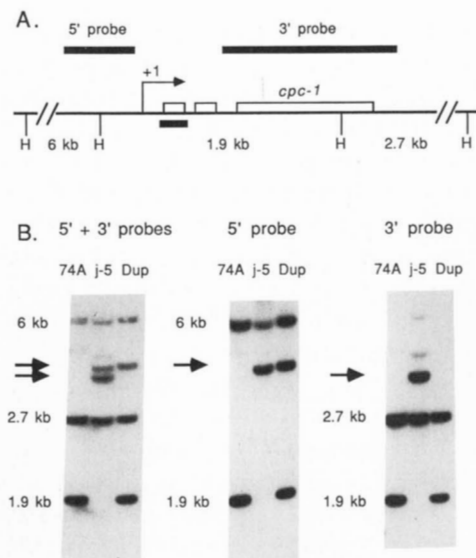


FIGURE 5.—Southern analysis of a duplication progeny from the *cpc-1* (j-5) × *ylo-1 pan-2* cross. (A) Physical map of the *cpc-1* transcription unit. The long horizontal line represents *cpc-1* DNA and flanking regions, pairs of diagonal lines through this line represent DNA not shown, *Hind*III sites (H) are indicated below the line along with the distances between the respective sites, the bar below the line represents the approximate location of the breakpoint that occurred in *cpc-1* (j-5). Above the line the transcription start site and direction of transcription is indicated (+1), the two short and one long rectangular boxes represent the two short open reading frames in the *cpc-1* leader region and the *cpc-1* structural gene, respectively, and the two bars above the line represent the DNA segments used as 5' and 3' probes. (B) Genomic DNAs isolated from 74A(wild type), *cpc-1* (j-5) and a *ylo-1*⁺ *pan-2* duplication strain were digested with *Hind*III, separated electrophoretically and hybridized with the indicated probes. The two arrows indicate the two hybrid *Hind*III fragments (4.1 and 3.7 kb) found in *cpc-1* (j-5) (see text). Note that only the larger fragment (4.1 kb) is found in the duplication strain and it is only observed with the 5' probe.

by southern analysis using two probes that recognize *cpc-1* DNA 5' or 3' to the *cpc-1* (j-5) breakpoint (Figure 5A). As shown in Figure 5B, the 1910 bp *Hind*III fragment that overlaps the breakpoint is present using strain 74A(wild type) DNA, but is absent using *cpc-1* (j-5) DNA. As expected, the use of the 5' and 3' probes together identified two new *Hind*III fragments (4.1 and 3.7 kb) in *cpc-1* (j-5). The larger of these two fragments (4.1 kb) is also present in the duplication strain. Additional analysis showed that this hybrid fragment is observed in the duplication strain when the 5' probe is used but not when the 3' probe is used (Figure 5B). This result indicates that *cpc-1* DNA 5' to the breakpoint, plus additional linkage group VI DNA, was inserted into linkage group I.

DISCUSSION

In yeast the response to starvation for single amino acids has been shown to involve complex interactions between positive and negative-acting factors (HINNEBUSCH 1988). The end result, increased transcription of the genes of amino acid biosynthesis, requires bind-

ing of the central *trans*-activating regulatory protein, GCN4, to one or more copies of its target sequence, ATGACTCAT, associated with each responding gene (HOPE and STRUHL 1985; ARNDT and FINK 1986). The cloning of the gene for GCN4 and extensive analysis of its polypeptide product, and the recent cloning and analysis of genes and proteins that modulate GCN4 expression, have provided a wealth of information on the mechanism of general control in *Saccharomyces cerevisiae*. A similar general coregulated response to amino acid starvation is seen in other fungi, although until recently, no genes encoding proteins similar in function to GCN4 had been cloned. Genetic and biochemical studies with *Neurospora* had identified one gene, *cpc-1*, as the principal regulatory gene responsible for cross-pathway control (BARTHELMESS 1982; PALUH *et al.* 1988). Mutations in this gene prevent the transcriptional response characteristic of this regulatory mechanism (FLINT and WILKENING 1986). In agreement with these observations, the predicted amino acid sequence of CPC1 indicates that this polypeptide has residue identities with GCN4 of yeast in two functionally important regions, GCN4's transactivation and DNA binding domains. Recent *in vitro* analyses have also established that CPC1 is a dimeric DNA binding protein (J. L. PALUH unpublished results) that recognizes the same target sequence as GCN4 (D. EBBOLE, J. L. PALUH, M. PLAMANN, M. S. SACHS and C. YANOFSKY, manuscript in preparation). In the studies described in this paper we determined the genetic changes in two inactive alleles of *cpc-1*: *cpc-1* (CD15) and *cpc-1* (j-5). The *cpc-1* (CD15) allele was cloned from genomic DNA using PCR technology. Sequence analyses showed that the *cpc-1* (CD15) mutation involved a single base pair deletion that introduces a frameshift in codon 93 of the *cpc-1* coding region. This frameshift should result in the production of a polypeptide 220 amino acids in length, in which the last 127 amino acids are specified by a shifted reading frame. The mutant polypeptide is predicted to lack those segments that are believed to be responsible for *trans*-activation, DNA binding, and dimerization. Attempts to produce a discrete *cpc-1* (CD15) polypeptide *in vitro*, using rabbit reticulocyte lysates, were unsuccessful.

The second mutant, *cpc-1* (j-5) was initially described as a lethal allele whose lethality was suppressed by an unlinked second mutation, *slo*, resulting in a "slow growth" phenotype. Our biochemical and genetic analyses of *cpc-1* (j-5) requires reinterpretation of the original results. We have established that the *cpc-1* (j-5) mutation involved a chromosomal break that disrupted the *cpc-1* functional unit. Southern analysis showed that the break was within a 100 bp segment, starting 40 bp downstream from the transcription start of *cpc-1*, and that foreign DNA now

resides on either side of the break. A partial restriction map of the foreign DNAs was obtained. Genetic studies have shown that a small segment of the left arm of linkage group VI, including part of the *cpc-1* functional unit and containing *ylo-1*, was translocated to the right arm of linkage group I. Additional southern analysis of a duplication progeny from a *cpc-1* (j-5) × *ylo-1 pan-2* cross provided physical evidence that it was the *cpc-1* promoter region, through *ylo-1* of linkage group VI, that was inserted in the right arm of linkage group I. The fact that the gene order on linkage group VI is known to be *ylo-1-cpc-1*-centromere (DAVIS 1979) allows one to conclude that *cpc-1* is normally transcribed towards the linkage group VI centromere. Consistent with the genetic disruption found in *cpc-1* (j-5), strains with this allele were incapable of synthesizing *cpc-1* mRNA. These observations indicate that the lethality associated with the *cpc-1* (j-5) mutation was not due to *cpc-1* inactivation, but rather was due to a chromosomal segment deficiency in one-fourth of the progeny produced in a cross with a normal allele. The observation that a specific deletion of the entire *cpc-1* gene is viable supports this interpretation (M. PLAMANN unpublished results). Thus, in *Neurospora crassa* as in *Saccharomyces cerevisiae*, the gene that specifies the key transcriptional activator regulating expression of amino acid biosynthetic genes is a dispensable gene.

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