The *clp1* Gene of the Mushroom *Coprinus cinereus* Is Essential for *A*-Regulated Sexual Development

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ABSTRACT

Sexual development in the mushroom *Coprinus cinereus* is under the control of the *A* and *B* mating-type loci, both of which must be different for a compatible, dikaryotic mycelium to form between two parents. The *A* genes, encoding proteins with homeodomain motifs, regulate conjugate division of the two nuclei from each mating partner and promote the formation of clamp connections. The latter are hyphal configurations required for the maintenance of the nuclear status in the dikaryotic phase of basidiomycetes. The *B* genes encode pheromones and pheromone receptors. They regulate the cellular fusions that complete clamp connections during growth, as well as the nuclear migration required for dikaryosis. The *AmutBmut* strain (326) of *C. cinereus*, in which both *A*- and *B*-regulated pathways are constitutively activated by mutations, produces, without mating, dikaryon-like, fertile hyphae with clamp connections. In this study we isolated and characterized *clampless1-1* (*clp1-1*), a mutation that blocks clamp formation, an essential step in *A*-regulated sexual development, in the *AmutBmut* background. A genomic DNA fragment that rescues the *clp1-1* mutation was identified by transformations. Sequencing of the genomic DNA, together with RACE experiments, identified an ORF interrupted by one intron, encoding a novel protein of 365 amino acids. The *clp1-1* mutant allele carries a deletion of four nucleotides, which is predicted to cause elimination of codon 128 and frameshifts thereafter. The *clp1* transcript was normally detected only in the presence of the A protein heterodimer formed when homokaryons with compatible *A* genes were mated. Forced expression of *clp1* by promoter replacements induced clamp development without the need for a compatible *A* gene combination. These results indicate that expression of *clp1* is necessary and sufficient for induction of the *A*-regulated pathway that leads to clamp development.

SEXUAL development in the mushroom *Coprinus cinereus* is under the control of the *A* and *B* mating-type genes (Kimura 1952). Basidiospores (sexual spores), which are produced on the mushroom, germinate to give haploid homokaryons. Homokaryons are normally sterile. When two homokaryons with compatible alleles at both *A* and *B* genes are mated, both *A*- and *B*-regulated pathways operate to give the fertile dikaryon with the characteristic clamp structure (see Raper 1966; Casselton and Olesnicky 1998). Compatible *B* genes promote exchange and reciprocal migration of the nuclei between the two mating partners for dikaryosis. The dikaryon is a prolonged mycelial stage in which the two nuclei from each mating partner remain paired without fusing in each cell. Compatible *A* genes promote conjugate mitotic division of the two nuclei with an associated clamp connection. One nucleus divides in the clamp and the other in the hypha just beneath the clamp, which results in the formation of a binucleate tip cell and uninucleate clamp and subterminal cells. *B* gene compatibility is again required for fusion of the clamp with the subterminal cell to complete the clamp connection, allowing the clamp cell nucleus to enter the subterminal cell. Thus the dikaryotic mycelium carrying the two different nuclei in each hyphal compartment is established. However, the *AmutBmut* homokaryotic strain of *C. cinereus*, in which both *A*- and *B*-regulated pathways are constitutively activated by mutations, produces, without mating, dikaryon-like fertile hyphae with clamp connections (Swamy *et al.* 1984). In this strain, two genetically identical nuclei pair in each cell (Swamy *et al.* 1984).

Molecular analysis in *C. cinereus* revealed that the *A* genes encode proteins with homeodomain motifs while the *B* genes encode pheromones and pheromone receptors (see Casselton and Olesnicky 1998). The *A* mating-type proteins fall into two classes, designated HD1 and HD2, on the basis of conserved but distinct homeodomain motifs (Kües *et al.* 1992, 1994). A compatible mating is recognized by the ability of allelic forms of HD1 and HD2 proteins to heterodimerize. The heterodimerization brings together potential DNA-binding and activation domains of a putative dikaryon-specific transcription factor (see Casselton and Olesnicky 1998). Similar homeodomain proteins are encoded by the *A* mating-type genes of another homobasidiomycete, *Schizophyllum commune* (Stankis *et al.* 1992; Specht

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TABLE 1

***Coprinus cinereus* strains used in this study**

Strain	Genotype/description	Source
5302	*A2B2*	This laboratory
5401	*A1B2*	This laboratory
KF_2#1	*A91B92*	This laboratory
326	*AmutBmut pab1-1*	P. J. Pukkila
292	*A3B1 trp1-1,1-6*	P. J. Pukkila
CLP1	*AmutBmut pab1-1 clp1-1*	This study
$CLP1F_1$#44	*AmutBmut pab1-1 trp1-1,1-6 clp1-1*/a progeny of CLP1 × 292	This study
5337#1	*A8B7 pcc1-1*	Murata *et al.* (1998)
$A_{ON}1$	*A43/A42 B43 ade8-1 trp3-1/trp3*$^+$	L. A. Casselton
$B_{ON}1$	*A43 B43/B42 ade8-1 trp3-1/trp3*$^+$	L. A. Casselton

$A_{ON}1$ and $B_{ON}1$ were constructed by transforming AT8 (*A43B43 ade8-1 trp3-1*) with the *A42* and *B42* loci, respectively (L. A. Casselton, unpublished results).

et al. 1992), and the *b* mating-type genes of the heterobasidiomycetes *Ustilago maydis* (Gillissen *et al.* 1992) and *U. hordei* (Bakkeren and Kronstad 1993). The HD1 and HD2 proteins resemble the a1 and α2 homeodomain mating-type proteins of *Saccharomyces cerevisiae* (see Casselton and Olesnicky 1998). Recently, we identified a *C. cinereus* gene, *pcc1*, in which mutation leads to clamp development in the absence of a compatible *A* gene complement and thus the heterodimeric A protein transcription factor (Murata *et al.* 1998). The *pcc1* gene encodes a high mobility group domain transcription factor (Murata *et al.* 1998) that is suggested to act as a suppressor in a pathway leading to clamp development without mating (Y. Murata and T. Kamada, unpublished results). How the A protein heterodimer activates the pathway leading to clamp development still remains to be elucidated, however.

In this article, we describe isolation and molecular characterization of the *clampless1-1* (*clp1-1*) mutation that blocks clamp development in the *AmutBmut* strain (326) of *C. cinereus* in which both *A*- and *B*-regulated pathways are constitutively activated by mutations in both mating-type genes. The *clp1* gene encodes a novel protein of 365 amino acid residues. Normal expression of *clp1* is dependent on the presence of the A protein heterodimer. Forced expression of *clp1* by promoter replacements induced clamp development in the absence of the A protein heterodimer. *clp1* is not transcribed in the *pcc1-1* mutant, despite the fact that the mutant exhibits clamps. On the basis of these results, we suggest a possible role for Clp1 in the *A*-regulated pathway.

MATERIALS AND METHODS

Strains and culture conditions: Strains of *C. cinereus* listed in Table 1 were used. Malt extract/yeast extract/glucose (MY) medium (Rao and Niederpruem 1969) solidified with 2% (w/v) agar in 9-cm petri dishes was used for routine mycelial cultures. Slants of MY agar medium in test tubes were used for fruiting, the same medium without agar in 9-cm petri dishes for mycelial cultures for extraction of DNA and RNA. MY medium was supplemented with 100 mg/liter of tryptophan for culture of tryptophan requiring strains. The minimal medium was that of Shahriari and Casselton (1974) modified by Binninger *et al.* (1987). Cultures were maintained at 28° under a 12-hr light/12-hr dark regime unless otherwise stated.

Microscopy: Presence or absence of clamps in mycelial colonies on agar plates was examined under a Nikon bright-field microscope. For micrographs, hyphae were grown on thin MY agar film on glass slides and observed under a Zeiss microscope equipped with phase-contrast optics.

Measurement of oidia number: A small piece of agar medium with mycelium (1 × 1 × 1 mm) was inoculated in the center of an MY agar plate and incubated for 10 days under a 12-hr light/12-hr dark regime or continuous darkness. Oidia produced were harvested in water by adding 5 ml of water to the surface of the culture and then gently scratching the culture surface with a glass rod. The number of oidia in the suspension was measured with a hemocytometer.

Mutagenesis: Strain 326 (*AmutBmut pab1-1*) was mutagenized by restriction enzyme-mediated integration (REMI) using plasmid pPHT1 carrying the hygromycin B resistance gene as a positive selectable marker in *C. cinereus* (Cummings *et al.* 1999). The plasmid (4 μg) was digested with 60 units of *Hin*dIII in 20–27.2 μl of reaction mixture at 37° overnight. Protoplasts from oidia of strain 326 were transformed with the reaction mixture containing linearized pPHT1 and *Hin*dIII according to the procedure described by Binninger *et al.* (1987) with minor modifications. They were then spread on regeneration agar plates (Binninger *et al.* 1987) supplemented with 10^{-4} M *p*-aminobenzoic acid (PAB). After incubation for 1 day, the plate was overlaid with 5 ml of regeneration medium supplemented with 10^{-4} M PAB and 600 μg/ml hygromycin B and incubated for 2 weeks. Hygromycin B-resistant transformants appeared and were transferred onto minimal medium supplemented with 10^{-4} M PAB and 150 μg/ml hygromycin B to purify the transformed mycelium.

Genetic techniques: Crosses were made by laying two inocula (1 × 1 mm) 1 mm apart on MY agar plates. Basidiospore germlings were isolated at random using a chisel-shaped needle under a dissecting microscope (Miles *et al.* 1966).

DNA manipulations: Cosmid and plasmid DNA were isolated with the FlexiPrep kit (Pharmacia Biotech, Piscataway, NJ). Genomic DNA from *C. cinereus* was prepared as described by Zolan and Pukkila (1986).

TABLE 2

PCR primers used

Primer	Sequence
GSP1	GAGATTCAAGCACCTGGCGAATG
GSP2	AGTTTGCACGACGCTTCACGACG
NGSP1	AGGGAGATAGGAATTGTGAGCGG
NGSP2	AACAGGCAAAGCGACGGGATG
CLS1	tgtaaaacgacggccagtTGGATTGCTTCGT TTCAAGG
CLA1	caggaaacagctatgaccGGTTGAAGTT GATGGAGCAG
CLS2	tgtaaaacgacggccagtCAAGCAACTTGTC GAACAGG
CLA2	caggaaacagctatgaccCGACCTGCACATC CTTCAAG
CLS3	tgtaaaacgacggccagtAATCGATATAG GATGCTCG
CLA3	caggaaacagctatgaccGTGTTGCGAAC GAGTCGACG
CLS4	tgtaaaacgacggccagtCCGCCTACCTT TACCACAAG
CLA4	caggaaacagctatgaccATAAGGAGGTT GAGCAGGCG
CLS5	tgtaaaacgacggccagtGAACCATCCATTC CATCTCG
CLA5	caggaaacagctatgaccTAACGGGCAAGT GGAGGAAG
TB-SphI	ACATGCATGCTTCATTTAA ACGGCTTC
CL1-TB	CTGTCTCAGAACTGGCATGCTGG GAACGCGAGGTCA
TB-CL1	TGACCTCGCGTTCCCAGCATGCCAG TTCTGAGACAG
CL1-SalI	ACGCGTCGACGGTCGCGC CATGCTT
act1	CATGCCATGGCAGTTCTGAGACAG CACGTT
act2	CATGCCATGGTGACTCGGTCACA CAAAGAG

Lowercase letters indicate −21M13 or M13 reverse sequence added to the designed primers. The underlined sequences are *Sph*I, *Sal*I, and *Nco*I sites. The double-underlined sequences are a part of the promoter of the β1-tubulin gene of *C. cinereus* and its antisense sequence.

Southern and Northern analysis: DNA or RNA was transferred to Hybond-N^+ (Amersham, Arlington Heights, IL) according to Sambrook *et al.* (1989). The enhanced chemiluminescence (ECL) direct system (Amersham) and the Gene Images system (Amersham) were used for probe labeling and detection for Southern and Northern analysis, respectively.

CHEF electrophoresis of *C. cinereus* chromosomal DNA: Plugs for clamped homogeneous electric fields (CHEF) electrophoresis were prepared as described by Muraguchi and Kamada (1998). To separate larger chromosomes of *C. cinereus*, CHEF electrophoresis was run using 120 ml of 0.8% chromosomal grade agarose (Bio-Rad) gels at 1.8 V/cm for 144 hr with a pulse time of 1200 sec in 0.5 × TBE (45 mm Tris base, 45 mm boric acid, 1 mm EDTA) at 18°, exchanging the buffer every day.

Chromosome IV-specific cosmid library screening, *clp1* gene cloning, and sequencing: A cosmid library of chromosome IV from the wild-type strain (5302) was constructed as described by Zolan *et al.* (1992). The vector (LLC5200) contains the *C. cinereus trp1* gene as a selectable marker (Pukkila and Casselton 1991). The library was composed of 1248 clones (96 clones × 13 plates). Groups of 12 clones were cultured on a plate of Luria broth/ampicillin solid medium, mixed, and subjected to miniprep with the FlexiPrep kit (Pharmacia Biotech). The pooled DNAs (1.0 μg) were used to transform protoplasts from strain CLP1F_1#44 (*AmutBmut pab1-1 trp1-1, 1-6 clp1-1*) as described by Binninger *et al.* (1987). Fifty to sixty trp^+ transformants were isolated with each pooled DNA. They were cultured on minimum medium supplemented with 10^{-4} m PAB for 3 days to purify the transformed mycelium and then transferred to MY plates to examine for the presence or absence of clamps. Five of the 52 trp^+ transformants from a single pool of DNA, 3A, produced clamps. Subsequent sib selection revealed that a single cosmid clone, 3A3, has the rescuing activity.

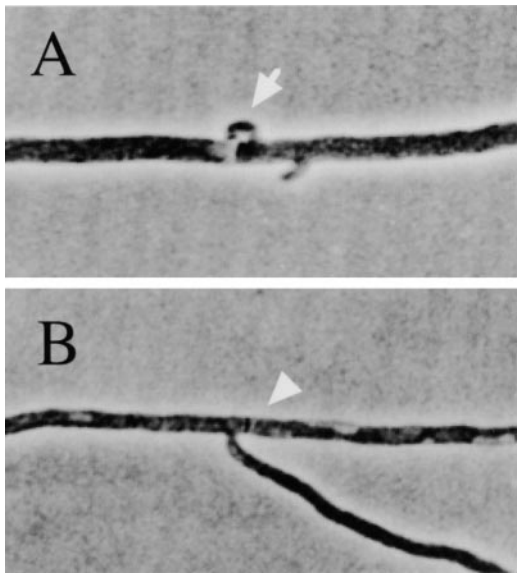

Figure 1.—Phase contrast micrographs showing parts of hyphae of the *AmutBmut* strain 326 (A) and the *clp1-1* mutant (B). The arrow indicates a clamp and the arrowhead a septum without a clamp.

Clone 3A3 was digested with *Apa*I, *Hin*dIII, *Pst*I, *Sac*I, or *Xba*I, and each digest was used to transform strain CLP1F_1#44. None of the restriction enzymes destroyed the rescuing activity. *Apa*I digestion produced six fragments (25, 6, 4.5, 3, 2.5, and 1.5 kb). The 25-kb fragment, which contains the whole sequence of LLC5200, was subcloned by self-ligation and its *clp1* activity was examined by transformation. The other fragments were ligated into the *Apa*I site of pGEM-7zf + (Promega, Madison, WI) and examined for the *clp1* activity by cotransformation with pCc1003 carrying the intact *trp1* gene (Skrzynia *et al.* 1989). The 4.5-kb *Apa*I fragment had the rescuing activity. This 4.5-kb fragment was then deleted from each side with the nested deletion kit (Pharmacia Biotech) and sequenced with a model 373S DNA sequencer (Perkin-Elmer, Norwalk, CT) using a PRISM dye primer cycle sequencing kit (Perkin-Elmer). The deletion products were also examined for the *clp1* activity. The genomic DNA sequencing of *clp1* and the deduced amino acid sequence data reported in this article will appear in the DDBJ/EMBL/GenBank databases under accession no. AB034196.

Determination of the *clp1-1* mutation site: To sequence the *clp1-1* mutant allele, we carried out PCR on the genomic DNA

TABLE 3

Effect of the *clp1-1* mutation on production of oidia

Strain	No. of oidia ($\times 10^7$/plate)	
	12-hr light/12-hr dark	Continuous darkness
5302 (*A2B2* + +)	151.0	61.8
326 (*AmutBmut pab1-1* +)	4.1	0.4
CLP1 (*AmutBmut pab1-1 clp1-1*)	11.6	2.7

Values are means of three measurements.

of the CLP1 mutant using the following five pairs of sense and antisense primers that cover the entire length of the open reading frame (ORF) of the *clp1-1* gene: CLS1, CLA1, CLS2, CLA2, CLS3, CLA3, CLS4, CLA4, CLS5, CLA5 (Table 2). To sequence the PCR products directly, we added a −21M13 primer sequence (18 bp) to the end of each sense primer sequence and a M13 reverse primer sequence (18 bp) to that of each antisense primer sequence.

Northern analysis: For total RNA, mycelia grown in MY liquid medium in 9-cm petri dishes for 7–8 days were harvested on a nylon filter, washed thoroughly with sterile water, and then frozen in liquid nitrogen. The frozen mycelia were ground into fine powder with a mortar and pestle. RNA was extracted from the powder as described by Yeager Stassen *et al.* (1997). About 15 μg of total RNA was fractionated by electrophoresis in 1.3% agarose formaldehyde gel. The 0.9-kb *Eco*RI-*Sal*I fragment from the *clp1* gene (see Figure 3) was used as the probe.

5′-RACE and 3′-RACE experiments: *clp1* cDNA was amplified using a cDNA library synthesized with the Marathon cDNA amplification kit (CLONTECH, Palo Alto, CA) by Muraguchi and Kamada (1998) as a template and a nest of gene-specific primers (GSP1, GSP2, NGSP1, NGSP2) (Table 2). PCR was performed with AmpliTaq Gold DNA polymerase (Perkin-Elmer) and the PCR products were cloned into pGEM-T Easy vector (Promega), according to the manufacturer's instructions, and sequenced as described above.

Replacements of the promoter of *clp1* with those of *C. cinereus* β1-tubulin and actin genes: The *clp1* ORF fused to the promoter of the β1-tubulin gene was constructed by sequential PCR amplifications using Pfu DNA polymerase (Stratagene, La Jolla, CA). In the first step, 393 bp upstream of the β1-tubulin coding sequence with a 3′ extension of the 5′ terminal 18 bp of the *clp1* coding sequence and 839 bp from the *clp1* start codon to the unique *Sal*I site in *clp1* (see Figure 3) with a 5′ extension homologous to 18 bp upstream of the β1-tubulin coding sequence were amplified: the former was amplified using pBl204KS carrying the β1-tubulin gene (Matsuo *et al.* 1999) as the template and primers TB-SphI and CL1-TB. TB-SphI (27-mer) is the 5′ terminal 17 bp of the β1-tubulin gene promoter with a 10-bp 5′ extension having a *Sph*I site, which is located 377–382 bp upstream of the *clp1* start codon. CL1-TB (36-mer) is the antisense sequence of 18 bp upstream of the β1-tubulin start codon with a 5′ extension homologous to the 5′ terminal 18 bp of the *clp1* coding sequence. The latter was amplified using the 4.5-kb *Apa*I fragment as the template and primers TB-CL1 and CL1-SalI. TB-CL1 is a 5′ extension that is homologous to 18 bp upstream of the β1-tubulin start codon with a 3′ extension of the 5′ terminal 18 bp of the *clp1* coding sequence, and CL1-SalI is the antisense sequence of a unique *Sal*I site in *clp1* and its upstream 15 bp. Because primers CL1-TB and TB-CL1 contain complementary 5′ extensions, two PCR products containing a region of overlapping homology are generated. In the second step, PCR was performed on the mixture of the two PCR products with primers TB-SphI and CL1-SalI to produce *clp1* fused to the β1-tubulin promoter. The ~1.2-kb region extending from the *Sph*I site to the *Sal*I site in the 3.2-kb fragment in pGEM-7zf+, which is a deletion product from the 4.5-kb *Apa*I fragment in pGEM-7zf+ (see Figure 3), was replaced with the second PCR product digested by *Sal*I and *Sph*I. Sequencing analysis showed that the construct has the intact *clp1* fused to the intact β1-tubulin promoter. Tryptophan-requiring homokaryotic strain 292 was cotransformed with the construct and pCc1003 carrying the intact *trp1* gene. As a control, strain 292 was cotransformed with the intact 3.2-kb fragment in pGM-7zf+ and pCc1003.

To replace the promoter of *clp1* with that of a *C. cinereus* actin gene, the sequence from the *clp1* start codon to the end of the *clp1* terminator with the modification of the sequence around the start codon from CAATGC to the *Nco*I site, CCATGG, and with a 3′ extension with an *Nco*I site was amplified using the 4.5-kb *Apa*I fragment as the template and primers act1 and act2 (Table 2). The amplified product was digested with *Nco*I and ligated to the *Nco*I site of plasmid pLJ2, which carries the promoter of the actin gene and the intact *trp1* gene, so that the 5′ terminal of the *clp1* coding sequence is connected to the actin gene promoter (L. A. Casselton and W. J. Cummings, unpublished data). Strain 292 was transformed with the construction.

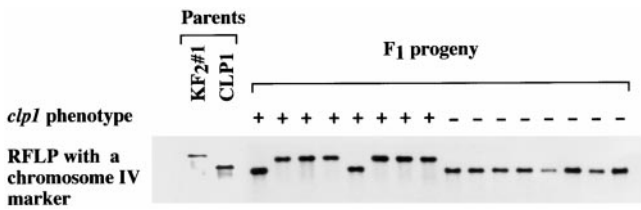

Figure 2.—RFLP analysis of the progeny from the cross CLP1 (*AmutBmut pab1-1 clp1-1*) × KF_2#1 (*A91B92* + +). The progeny were scored for the wild-type forming clamps (+) and the *clp1-1* mutant not forming clamps (−) in the *Amut* background. A selection of eight wild type and eight mutant progeny were then scored for RFLP using enzyme *Bam*HI and the chromosome IV marker, Hiroshima II#11.

RESULTS

Isolation and genetic analysis of the *clp1-1* mutation: The *clp1-1* mutant that fails to form clamps was found among 3225 hygromycin-resistant transformants of strain 326 *(AmutBmut pab1-1*) after REMI mutagenesis (Figure 1). The mutant did not exhibit any visible sign

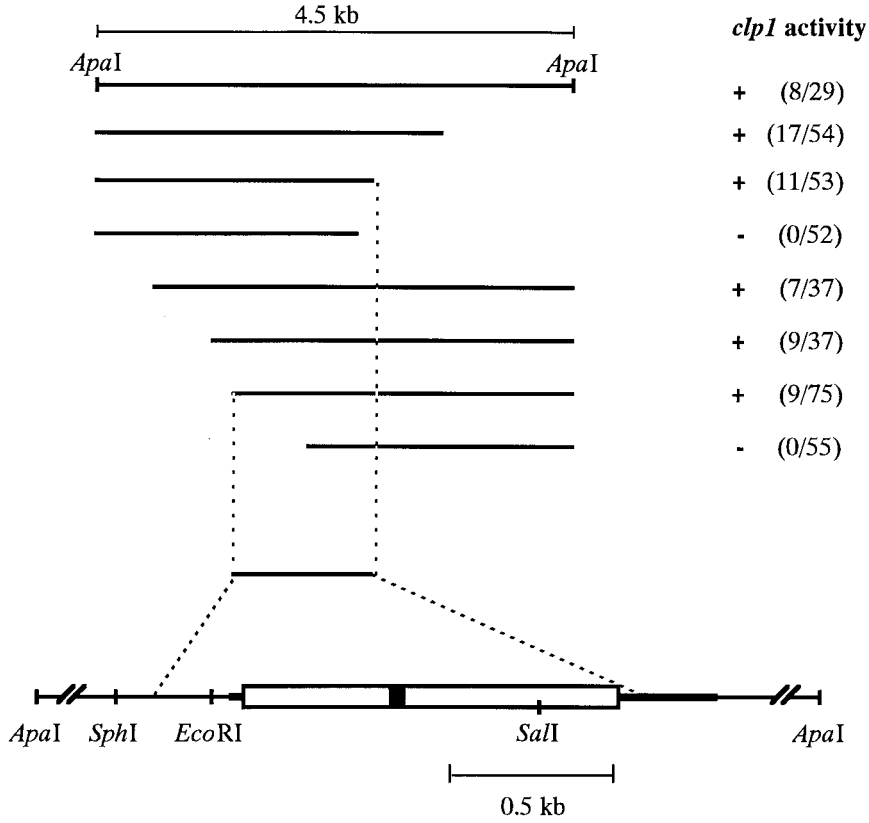

FIGURE 3.—Deletion products of the 4.5-kb *Apa*I fragment and the activity that rescues the *clp1-1* mutation. The values in parentheses are clamp-forming trp$^+$ transformants/total trp$^+$ transformants examined. The thick line, open box, and solid box indicate the region transcribed, a deduced ORF, and the intron, respectively.

of fruiting although the parental strain formed fertile fruit bodies constitutively. Activation of the *A*-regulated pathway in sexual development has been correlated with repression of asexual sporulation (TYMON *et al.* 1992) and the *AmutBmut* strain from which the *clp1* mutant was derived produces few asexual oidia. The *clp1* mutation led to a marked increase in the numbers of oidia produced (Table 3).

When CLP1 (*AmutBmut pab1-1 clp1-1*) was mated to the wild-type homokaryon, KF_2#1, dikaryotic hyphae with clamp connections emerged from the margin of the colony of KF_2#1, indicating that the mutation is recessive. We isolated 111 progeny from the dikaryon. Of the 111 progeny, 62 were PAB requiring and 49 were prototrophic. Most of the PAB-requiring progeny are considered to carry *Amut* because the *A* mating-type locus is closely linked to *pab1* at 0.5 map units (TAKEMARU 1982). We examined the PAB-requiring progeny for the presence or absence of clamps. Of 62, 29 exhibited clamps in their hyphae whereas 33 did not. This result shows that the clampless phenotype in the *Amut* background is due to a mutation in a single chromosomal gene, which we designated *clp1*. We then examined the 62 progeny for hygromycin resistance and found that hygromycin resistance and the clampless phenotype segregated independently in the progeny (date not shown). The *clp1-1* mutation is not, therefore, a direct result of plasmid insertion. We carried out genetic linkage analyses using chromosome markers and found that *clp1* was linked to a restriction fragment length polymorphism (RFLP) marker, Hiroshima II #11 (Figure 2), which is located on chromosome IV, as separated by a CHEF gel (PUKKILA 1993).

Cloning of *clp1*: To clone the *clp1* gene, we constructed a chromosome IV-specific library from a wild-type strain, 5302, and screened it for a DNA fragment that complements the *clp1-1* mutation. We identified a single cosmid clone, 3A3, that rescues the *clp1-1* mutation. Sixty-three percent (12/19) of trp$^+$ transformants with clone 3A3 exhibited clamps. We scored the 16 progeny used for the experiment shown in Figure 2 for RFLP using the the clone 3A3 as a probe, which showed that the 3A3 region is closely linked to the *clp1* locus (recombinants: 0/16). This strongly suggests that the fragment does not contain an extragenic suppressor of the *clp1-1* mutation, but *clp1* itself. Digestion of clone 3A3 with *Apa*I followed by tests of the *clp1* rescuing activity revealed that a 4.5-kb *Apa*I fragment from 3A3 contained *clp1*. We further localized *clp1* to 1479 bp in the 4.5-kb fragment by deletions (Figures 3 and 4). The 1479 bp contain 404 bp upstream of the start codon and 49 bp downstream of the stop codon (see below).

Identification of the *clp1* ORF: Genomic DNA sequencing, together with 5′-RACE and 3′-RACE experiments, identified an ORF interrupted by one intron of 53 nucleotides (nt), which is predicted to encode a

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1       TTGGTTCCCAGGAATTACCCTTTCTGGAAGAAAACTGCCGGCGAAGGCAAAAGTGGATTGGGATGCAAACAAATGCACACATAGTCTCGC
91      TACGCGATTTCTGCCTTTTTGGGTAAAAATGCTGGATTGCTTCGTTTCAAGGATTGGGTCGATTGGTTTTGCGAATTCGCCTATAAAACC
181     AGGTGCGGGGCCGTGTTGGTTCGGTCACTGTTCCCCCAGTTCAAGCGTTCCTCGTCAACAAGCAACTTCCCCTCAAGTCTCCCGCCCTCT
271     TCTGCCACAATGCCAGTTCTGAGACAGCACGTTCAAGCACGACGGGCCGACGCGGAGAATATGCCTCCCGCCCCGGCCGTCAAAGGCGTT
                M  P  V  L  R  Q  H  V  Q  A  R  R  A  D  A  E  N  M  P  P  A  P  A  V  K  G  V    27

361     GCCGCCAACCGCCTCCGAAGCAGTTTGCACGACGCTTCACGACGACGACGAGGACGCGCAACCAACACACCCCGCAACTCCAAGCAACTT
         A  A  N  R  L  R  S  S  L  H  D  A  S  R  R  R  R  G  R  A  T  N  T  P  R  N  S  K  Q  L    57

451     GTCGAACAGGCAAAGCGACGGGATGTCATGAAACCCAAATCGACTTCTACTGGCTCGCTCAAATTCACTATTACTTCGCCTCCATCGACT
         V  E  Q  A  K  R  R  D  V  M  K  P  K  S  T  S  T  G  S  L  K  F  T  I  T  S  P  P  S  T    87

531     GATGTTCAAATGAGCGACGCTGCTCCATCAACTTCAACCCCGCTCACAATTCCTATCTCCCTCCCCAAGGAACTTGCTCGACCACAGTTC
         D  V  Q  M  S  D  A  A  P  S  T  S  T  P  L  T  I  P  I  S  L  P  K  E  L  A  R  P  Q  F   117

631     AAGGAAGTTCCTCGCGAGGCTCTCATGGCTGTGAACCCAGAGCTTGCCGATACCGATCCTGAATACATTCGCCAGGTGCTTGAATCTCAC
         K  E  V  P  R  E  A  L  M  A  V  N  P  E  L  A  D  T  D  P  E  Y  I  R  Q  V  L  E  S  H   147

721     GGGCCTCAgtaagtgactccttttttctttgataaccgagcatttgctaataatcgatatagGATGCTCGCCTCTCTCTCAAATGTCAAAG
         G  P  Q                 i  n  t  r  o  n                    M  L  A  S  L  S  N  V  K   159

811     CCACCCTCACCACCAATGCTCTCCCCTCCGAGATCGAAGTCAGCTTGAAGGATGTGCAGGTCGCACCATCGCACATGGTCGCCGTTTTTG
         A  T  L  T  T  N  A  L  P  S  E  I  E  V  S  L  K  D  V  Q  V  A  P  S  H  M  V  A  V  F   189

901     GCCCTGCCCCCGCGAACTGCTGTACCCAAACCCCCGCAAAGTCACCCTCTATCCCGTCCACAGCTTCGTGCTCGCTGCCCACTGCTCGAACC
         G  P  A  P  R  T  A  V  P  K  P  R  K  V  T  L  Y  P  V  H  S  F  V  L  A  A  H  C  S  N   219

991     TCCCACCATTCCCACCTGCAGCAACCATCCCTTCGGCACCCGCATCGAGCGACGACAAAGTTCGCCTGCCCGTGCGCTCTCTCTGCCTCC
         L  P  P  F  P  P  A  A  T  I  P  S  A  P  A  S  S  D  D  K  V  R  L  P  V  R  S  L  C  L   249

1081    CATCCCCAGAAAACTACGCGCCTCTCGCCGCCTACCTTTACCACAAGCAAACGACCACCCTCCTCAACGCCATGCTCCCCAGCCCCGTTC
         P  S  P  E  N  Y  A  P  L  A  A  Y  L  Y  H  K  Q  T  T  T  L  L  N  A  M  L  P  S  P  V   279

1171    CTCACAACTTTGAAATCGAACAAAAGCATGGCGCGACCGTCGACTCGTTCGCAACACGGCTAGGGCGCACCTACACCTTTGAAAAACTTC
         P  H  N  F  E  I  E  Q  K  H  G  A  T  V  D  S  F  A  T  R  L  G  R  T  Y  T  F  E  K  L   309

1261    TTTTGAACACCATGATGATCCACGGCCTCTGGCAGAACGCCTGCGCACTGGGCATCCACGACCAGCTGCTCTGGGACACGATCGATTTGG
         L  L  N  T  M  M  I  H  G  L  W  Q  N  A  C  A  L  G  I  H  D  Q  L  L  W  D  T  I  D  L   339

1351    CATGGCAGGTCATGCTCACTGCTCTTGCAGTCAGCACAGGCAAGCCATCGCTCATGATCTCTTCCGTTTCTACCGACTAAGCCACCAATC
         A  W  Q  V  M  L  T  A  L  A  V  S  T  G  K  P  S  L  M  I  S  S  V  S  T  D  *           365

1441    ACTCATATCATCCTATGGCTTCAAGCTCTTATGCATTAT
```

FIGURE 4.—Nucleotide and predicted amino acid sequences of the *clp1* gene of *C. cinereus*. The number of the first nucleotide of each line is indicated on the left margin, and the last amino acid of each line is indicated on the right margin of the figure. The intron sequence is shown in lowercase letters. The four nucleotides deleted in the *clp1-1* mutant allele are double underlined. The 18 nucleotides that show similarity to the conserved *hsg* in *S. cerevisiae* (GOUTTE and JOHNSON 1988) and a TATA box in the promoter region are underlined.

protein of 365 amino acids (Figure 4). The 5′ splice site, GTAAGT, agrees with the consensus sequence GTRNGT found for filamentous fungi and the 3′ splice site, TAG, with the consensus sequence YAG (GURR *et al.* 1987). The predicted protein contains no obvious structural motifs nor shows extensive similarity to any sequences represented in the database. The *clp1* mRNA is predicted to have a 63-nt 5′- and a 315-nt 3′-untranslated region. The promoter region of *clp1* contains a TATA box 45–39 bp upstream of the predicted translational start site. A total of 110 bp upstream of the TATA box there was a sequence (GATG, 11-nt spacing, ACA) similar to the conserved *hsg* motif (GATG, 9-nt spacing, ACA) that exists in the promoter regions of the haploid-specific genes in *S. cerevisiae* (GOUTTE and JOHNSON 1988).

***clp1-1* mutant allele:** We performed PCR amplifications on the genomic DNA of the *clp1-1* mutant using five pairs of primers that were designed on the basis of the wild-type *clp1* sequence and sequenced the PCR products directly. Comparison of the sequence of the *clp1-1* mutant allele with that of the wild-type gene revealed that the four bases at positions 661–664 (GTGA) were deleted in the *clp1-1* mutant allele, which is predicted to cause elimination of codon 128 and frameshifts thereafter (Figure 4).

Developmental regulation of *clp1* transcription: Using a 1.0-kb *Eco*RI-*Sal*I fragment from the *clp1* gene (see Figure 3) as a probe, we examined total RNAs from various strains (Figure 5). As expected from the *clp1* cDNA analysis, we identified a transcript at ~1.5 kb, in the parental *AmutBmut* strain, the *clp1-1* mutant, the dikaryon, and an *A*-on homokaryon. Transcription of *clp1* was clearly increased in the *clp1-1* mutant as compared with the parental *AmutBmut* strain. The *clp1* transcript was not detected in the wild-type homokaryon or in a *B*-on homokaryon. We also found that *clp1* was not transcribed above the level of detection in the *pcc1-1* mutant, which exhibits clamps constitutively (MURATA *et al.* 1998).

Forced expression of *clp1*: The above results suggest a pathway in which the A protein heterodimer promotes the expression of *clp1*, which in turn induces clamp development. To examine whether the expression of *clp1* induces clamp development in the absence of the A protein heterodimer, we produced a construct in which the promoter region of *clp1* was replaced with that of the *C. cinereus* β1-tubulin gene and introduced it into a tryptophan-requiring homokaryon 292 by cotransformation with pCc1003 carrying the intact *trp1*. We found that 26% (12/46) of trp^+ transformants exhibited clamps. The clamps formed, however, were un-

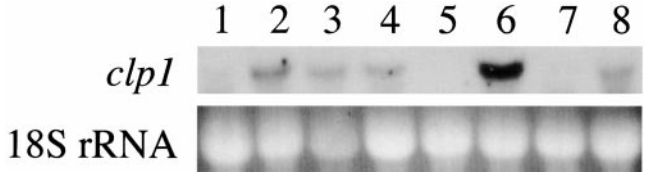

FIGURE 5.—Expression of the *clp1* gene. For each lane, 15 μg of total RNAs from the following were electrophoresed: wild-type homokaryon 5302 (lane 1), wild-type dikaryon 5302 × 5401 (lane 2), the *AmutBmut* strain 326 (lane 3), the $A_{ON}1$ strain (lane 4), the $B_{ON}1$ strain (lane 5), the CLP1 mutant (lane 6), the *pcc1-1* mutant 5337#1 (lane 7), and homokaryon 292, in which *clp1* fused to the β1-tubulin gene promoter was introduced (lane 8). After the ribosomal RNA was visualized under UV, the gel was blotted and hybridized. The probe was the 1.0-kb *Eco*RI-*Sal*I fragment (see Figure 3). After hybridization, the blot was exposed to X-ray film.

fused ones because the *B*-regulated pathway is not activated in the transformants. Northern blot analysis showed that *clp1* was transcribed in a transformant exhibiting clamps (Figure 5, lane 8). A similar result was obtained with *clp1* connected with the promoter of a *C. cinereus* actin gene: 35% (24/68) of trp^+ transformants exhibited clamps. As a control, we introduced the intact *clp1* gene into the homokaryon and found that no trp^+ transformants exhibited clamps (0/31).

DISCUSSION

C. cinereus is a heterothallic species in which mating is under the control of the *A* and *B* mating-type loci. The *A*- and *B*-regulated pathways are normally activated to produce the fertile dikaryon when two homokaryons with different *A* and *B* alleles are mated (see CASSELTON and OLESNICKY 1998). In the present study we took advantage of the fact that the *AmutBmut* homokaryotic strain carrying mutations in both mating-type loci produces dikaryon-like, fertile hyphae with clamp connections without the need for mating. We identified a mutation, *clp1-1*, that blocks clamp formation, an essential step in *A*-regulated sexual development. The *clp1* gene was cloned as a DNA fragment that rescues the *clp1-1* mutant phenotype. An ORF that is predicted to encode a novel protein of 365 amino acid residues was identified within this fragment. *clp1* transcripts were detected only in mycelia containing compatible *A* genes and thus able to generate the A protein heterodimer predicted to activate the *A* pathway in sexual development. We showed that *clp1* transcripts are present in dikaryons, *A*-on mycelia, and homokaryons carrying the *A* mutation present in strain 326, which is known to generate a chimeric protein with heterodimer activity (KÜES *et al.* 1994), but are absent in the *pcc1* mutant that activates the *A* developmental pathway in the absence of a compatible combination of *A* genes. Significantly, we found that forced expression of *clp1* by promoter replacements bypassed the requirement for compatible *A* genes. Taken together, these results indicate that *clp1* is working downstream of the *A* genes and that it is a likely target for the A protein heterodimer. *clp1* transcription is necessary and sufficient for induction of clamp formation in the *A* pathway.

The element in the promoter region responsible for activation of the *clp1* expression remains to be characterized and its ability to bind the A protein heterodimer still has to be tested. However, it seems significant that an 18-bp sequence (GATG, 11-nt spacing, ACA) was identified 218–201 bp upstream of the *clp1* start codon that is similar to the conserved *hsg* motif (GATG, 9-nt spacing, ACA) in *S. cerevisiae* (GOUTTE and JOHNSON 1988). The *hsg* motif has been shown to bind the heterodimer of MATa1 and MATα2 encoded by the a and α mating-type genes in yeast (GOUTTE and JOHNSON 1988), proteins that resemble the proteins encoded by the *A* mating-type genes in *C. cinereus* (see CASSELTON and OLESNICKY 1998). The MATa1/MATα2 heterodimer is a transcriptional repressor so it is particularly relevant that a target gene for the corresponding bE/bW protein heterodimer of *U. maydis* has been identified recently. The bE/bW heterodimer binds an *hsg*-like element (GATG, 9-nt spacing, ACA) in the promoter of this gene and acts as a transcriptional activator (ROMEIS *et al.* 2000). The interaction between two homeodomain proteins is obviously a conserved mechanism in regulating sexual development in *S. cerevisiae* and basidiomycete fungi, but whether the heterodimer acts as a repressor or as an activator depends on other functional domains within the proteins.

The *clp1-1* mutation led to increased levels of *clp1* transcription. This suggests a feedback mechanism that regulates the level of *clp1* transcription, in which Clp1 and/or some downstream factor(s) may be involved. The presumed regulating factor(s) as well as element(s) in the promoter region responsible for this regulation remain to be identified.

The Clp1 protein does not show extensive similarity to any sequences represented in the database and does not contain obvious structural motifs. At the present time it is not known what function Clp1 plays in the *A* pathway. We have previously suggested that the clamp pathway is repressed in homokaryons by the HMG-domain protein encoded by *pcc1*. This was based on the fact that loss-of-function mutations in *pcc1* lead to clamp formation without mating (MURATA *et al.* 1998; Y. MURATA and T. KAMADA, unpublished results). The A protein heterodimer may in some way release the *A* pathway from Pcc1 repression. *clp1* is not transcribed in the *pcc1-1* mutant, despite the fact that the mutant exhibits clamps, suggesting that loss of Pcc1 function bypasses a normally essential A protein-dependent step involving Clp1. If our interpretation is correct, Clp1 cannot relieve Pcc1 repression at the transcriptional level because *pcc1* transcripts are still detected in *A*-on and dikaryotic mycelia (MURATA *et al.* 1998) but this would not preclude a post-translational mechanism.

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