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Characterization of *rco-1* of *Neurospora crassa*, a Pleiotropic Gene Affecting Growth and Development That Encodes a Homolog of Tup1 of *Saccharomyces cerevisiae*

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The filamentous fungus *Neurospora crassa* undergoes a well-defined developmental program, conidiation, that culminates in the production of numerous asexual spores, conidia. Several cloned genes, including *con-10*, are expressed during conidiation but not during mycelial growth. Using a previously described selection strategy, we isolated mutants that express *con-10* during mycelial growth. Selection was based on expression of an integrated DNA fragment containing the *con-10* promoter-regulatory region followed by the initial segment of the *con-10* open reading frame fused in frame with the bacterial hygromycin B phosphotransferase structural gene (*con10′-′hph*). Resistance to hygromycin results from mutational alterations that allow mycelial expression of the *con-10′-′hph* gene fusion. A set of drug-resistant mutants were isolated; several of these had abnormal conidiation phenotypes and were *trans*-acting, i.e., they allowed mycelial expression of the endogenous *con-10* gene. Four of these had alterations at a single locus, designated *rco-1* (regulation of conidiation). Strains with the *rco-1* mutant alleles were aconidial, female sterile, had reduced growth rates, and formed hyphae that coiled in a counterclockwise direction, opposite that of the wild type. The four *rco-1* mutants had distinct conidiation morphologies, suggesting that conidiation was blocked at different stages. Wild-type *rco-1* was cloned by a novel procedure employing heterokaryon-assisted transformation and ligation-mediated PCR. The predicted RCO1 polypeptide is a homolog of Tup1 of *Saccharomyces cerevisiae*, a multidomain protein that mediates transcriptional repression of genes concerned with a variety of processes. Like *tup1* mutants, null mutants of *rco-1* are viable and pleiotropic. A promoter element was identified that could be responsible for RCO1-mediated vegetative repression of *con-10* and other conidiation genes.

Neurospora crassa, a filamentous fungus, has three distinct sporulation pathways that lead to the production of either of two types of asexual spores, macroconidia and microconidia, or sexual spores, ascospores. Formation of macroconidia, known as macroconidiation or conidiation (as it will be referred to throughout this paper), has been extensively studied at the morphological level (32, 40, 53, 59, 64). Upon receipt of certain environmental cues, e.g., carbon starvation, vegetative tissue, the mycelium, initiates conidiation by producing aerial hyphae that grow away from the nutrient surface. These hyphae undergo apical budding, generating what have been termed minor constriction chains. Subsequent growth by continued apical budding leads to hyphal segments with more pronounced, major constrictions. These proconidial chains continue to differentiate by completing septation at the sites of major constrictions. Just prior to disarticulation of mature conidia, a fragile structure, termed the connective (59), is evident. The connective joins adjacent proconidia and is ultimately cleaved. The entire process of conidiation takes 12 to 24 h. Microconidia are generated by a process that is distinct from conidiation; these asexual spores are formed by "eruption" through the walls of microconidiophores (59). The sexual stage of the life cycle proceeds after maturation of mycelial hyphae into the female sex organ, the protoperithecium. Upon fertilization by cells of the opposite mating type, protoperithecia mature into perithecia which contain many asci, each derived from a single zygote and each containing eight haploid ascospores.

Early molecular studies on conidiation led to the isolation of *con* genes, genes cloned on the basis of their preferential expression during conidiation (5). These genes were analyzed in developmental studies, and it was observed that their temporal expression varied. *con-8* is expressed very early during conidiation; its expression is followed by that of *con-6* and then by that of *con-10* (46, 58). Another conidiation gene, *eas*, is expressed in a conidiation-specific manner resembling that of *con-8. eas* encodes the conidial rodlet protein, a hydrophobin that covers the surface of the macroconidium (3, 24). None of the *con* genes studied to date, nor *eas*, appears to be essential for conidiation (3, 24, 60, 68, 72). The fact that these genes are developmentally regulated and nonessential makes them ideal targets in approaches designed to identify the *cis*-acting sites and *trans*-acting factors that mediate conidiation-specific expression.

In a previous study, we described a procedure that could be used to isolate mutants that express *con-10* aberrantly (30). In the present study, we analyzed four such *trans*-acting mutants. We found that these mutants are altered at a single locus, *rco-1*. Each mutant allele was shown to be responsible for abnormal expression of *con-10* in mycelia, and each prevented completion of conidiation. Our findings suggest that *rco-1* is a regulatory gene that mediates mycelial repression of conidia-

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tion gene expression. The *rco-1* mutants were characterized by examining growth rate, morphological phenotype, and *con* gene expression; they were also analyzed in genetic and complementation studies. The wild-type *rco-1* gene was cloned by a novel transformation procedure and cosmid identification process. The polypeptide sequence predicted from the nucleotide sequence of *rco-1* suggests that RCO1 is homologous to *Saccharomyces cerevisiae* Tup1, a protein known to be a component of the Ssn6-Tup1 complex (21) that mediates repression of genes concerned with several different processes (16, 38, 52, 73).

MATERIALS AND METHODS

Strains and growth conditions. Strain CH10 is a derivative of Fungal Genetics Stock Center (FGSC) strain 462 (*his-3 A*). It was prepared by homologous integration of plasmid pCH10 at the *his-3* locus (30). Plasmid pCH10 contains a translational fusion of the first 40 codons of *con-10* to the bacterial hygromycin B phosphotransferase gene, *hph* (12). pCH10 also contains the 520-bp segment of the *con-10* promoter that precedes the *con-10* transcription start site (30). Integration of pCH10 at the *his-3* locus generates a tandem duplication of *his-3* sequences flanking the *con-10′-′hph* fusion. This strain was used to isolate the hygromycin-resistant mutants described in this paper. Mutant strains CH10-101, CH10-119, CH10-121, CH10-125, and CH10-129a were derived from strain CH10 after UV mutagenesis and selection for hygromycin resistance. All backcrosses were performed with either ORS *a* (FGSC 2490) or 74-OR23-1 *A* (FGSC 987). A *nic-3 arg-10 a* strain (from D. Perkins) was used for the introduction of auxotrophic markers into mutant strains. An a^{m1} *ad-3B cyh-1* (FGSC 4564) strain was used as a helper strain for genetic crosses with heterokaryons formed with female sterile strains (43). Mapping was performed with an *acr-2 a* strain (FGSC 4044). An *mt a* strain containing a deletion allele of *mtr* (SR33) and a tightly linked *col-4* mutation (70007c) was provided by David Stadler. This strain was backcrossed to 74-OR23-1 *A*, and 730 progeny were screened for *mtr* single mutants. Two were obtained, of which one, 179-51 *a*, was crossed to *rco-1* mutant strain CH10-101 *A*, and the progeny were screened for the genotype *mtr rco-1 his-3* (approximately half of the progeny should be histidine auxotrophs because of efficient inactivation of *his-3* by repeat-induced point mutation (RIP) (55) of the tandem duplication of *his-3* in CH10-101). The *cot-1* [C102(t)] *wc-1* (ER45) *A* strain (strain 152-48) was used to prepare a heterokaryon with *mtr rco-1 his-3 A*, for use in heterokaryon-assisted transformation of the latter strain. Conidia from the heterokaryon were used in transformation experiments designed to detect a complementing clone.

Strains were grown in Vogel's minimal salts (13), supplemented as necessary. Colonial growth on solid agar was promoted by addition of Tergitol to the growth medium (57). Growth rates were determined by a modification of the race tube method (13). Tubes were marked once or twice daily, and the average migration per hour was determined. Crosses were performed on synthetic crossing medium (13). Random ascospores were picked and germinated as described previously (44). All nutritional supplements were obtained from Sigma.

Spiral growth was observed either on slants or plates containing Vogel's minimal salts with 1.5% sucrose and 1.5% agar. A single ascospore was transferred to each slant and induced to germinate by heating at 60°C for 1 h. For plates, an approximately 1-mm^3 agar piece with mycelia from a 2- to 4-day-old slant culture was transferred to the center of the plate. For plates and slants, cultures were incubated at 34°C and scored for directionality of spiral growth after 16 to 48 h. Hyphal growth was photographed with a Wild dissecting microscope with 6× objective and 10× ocular lenses. Kodak Technical Pan 2415 film (ASA 125) was used.

Heterokaryon-assisted transformation. For heterokaryon-assisted transformation, we constructed the triple mutant *rco-1 mtr his-3* by crossing the *con-10′-′hph rco-1* strain (CH10-101A) with a strain bearing *mtr* (179-51a). A heterokaryon was then prepared from the *rco-1 mtr his-3 A* and *cot-1 wc-1 A* strains by mixing mycelia of the two strains on a minimal agar slant and incubating the slant at 34°C. Under these conditions, only the heterokaryon could grow. All mutant alleles are recessive. *cot-1* homokaryons grow as tiny compact colonies at 34°C but normally at 25°C (45). Homokaryons carrying *mtr* are resistant to the toxic amino acid analog *p*-fluorophenylalanine (FPA [15 mg/liter]), whereas heterokaryons for *mtr* or strains containing normal *mtr* are sensitive to this analog (61). Conidia from the heterokaryon were generated by being cultured in 250-ml Erlenmeyer flasks containing 50 ml of minimal agar medium with 1.5% sucrose grown at 34°C in the dark for 1 to 2 days, followed by growth in constant light at 34°C for 4 to 6 days. Collection, germination, spheroplasting, and transformation of conidia were performed as described previously (67). Each transformation was performed with approximately 5×10^7 competent conidia and 5 μg of cosmid DNA. Each cosmid contained the gene conferring hygromycin B (*hph*) resistance. The transformation mixture was resuspended in 20 ml of top regeneration agar containing 0.6 M sucrose and 15 mg of FPA per liter and was plated onto 150-mm-diameter petri dishes containing minimal agar supplemented with 0.005% Tergitol, 1.5% sucrose, 50 mg of histidine per liter, 15 mg of FPA per liter, and 175 U of hygromycin B per ml. There were 50 pools of cosmid DNA, each CsCl-purified, representing each of the Orbach-Sachs genomic cosmid library pools of ~96 clones (41). The transformation plates were incubated at 34°C with a 12-h light-dark cycle. After 5 days of incubation, plates were screened for colonies exhibiting extensive aerial growth with evidence of conidiation (orange growth).

Cloning genomic *rco-1* and its cDNA. Sequences corresponding to the ends of the inserts of the complementing cosmid clones were isolated and amplified by ligation-mediated PCR (LM-PCR [9, 37]). The linker sequences are the standard set (linker long strand and linker primer, 5′-GCGGTGACCCGGGA GATCTGAATTC-3′; linker short strand, 5′-GAATTCAGATC-3′). Unique oligonucleotide primers (primer T3-side, 5′-TAACCCTCACTAAAGGGATCC -3′; primer T7-side, 5′-TACGACTCACTATAGGGATCC-3′) corresponding to the pMOcosX vector (41) sequences flanking the cloning site (elongation from those primers extends into the insert sequence) were used for cloning the ends of the inserts. Genomic DNA was isolated from mycelia of the complemented transformants grown in 50 ml of Vogel's minimal medium containing 1.5% sucrose, 50 mg of histidine per ml, and 175 U of hygromycin B per ml in a 125-ml Erlenmeyer flask at 34°C grown with agitation for 24 h. The mycelia were lyophilized, mechanically pulverized, extracted with phenol-chloroform, and RNase treated (27). One microgram of genomic DNA was digested to completion as described above with restriction enzymes leaving blunt ends, such as *Rsa*I, *Alu*I, *Pvu*II, and *Eco*RV (usually two to four different digests for each clone). Ligation to the linker was performed for 1 h at room temperature. The PCR mixtures contained 5 μl of ligation reaction mixture, 20 pmol of vector-specific primer (T3 or T7), and 10 pmol of linker primer, in a total volume of 50 μl. Cycling conditions were 94°C for 1 min, 65°C for 2 min, and 72°C for 2 min for 35 cycles. Products were visualized by running 5 to 10 μl on a 1.5% agarose gel. To prepare probes from these fragments, the LM-PCR was carried out in the presence of digoxigenin-labeled dUTP (Boehringer Mannheim) as a nonradioactive probe. The specific complementing cosmid was identified by colony hybridization screening of 96 clones representing the corresponding pool of clones which complemented *rco-1*. The procedure of Engler-Blum et al. (17) was used for probe hybridization and chemiluminescence detection.

The cDNA clone was synthesized by PCR with a random cDNA library (6) linearized by *Xho*I. The PCR mixture contained 100 ng of linearized plasmid pool, 200 nM (each) primer (primer 1, 5′-GGACAACCGCCCCAACTACG-3′; primer 2, 5′-CGCATTGCAACTCCGCTGGTG-3′), 200 μM (each) deoxynucleoside triphosphates, 5 U of *Taq* polymerase (Boehringer Mannheim), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$ in a total volume of 100 μl covered by 100 μl of mineral oil. Cycling conditions were denaturation for 2 min at 95°C; 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min; and final extension at 72°C for 10 min. The PCR product was ligated to pCRII vector (Invitrogen) and transformed into *Escherichia coli* XLI-Blue (Stratagene) with selection for ampicillin and kanamycin resistance.

Double-stranded DNA sequencing was performed by the chain-termination method (51) with ^{35}S-labeled α-dATP or ^{33}P-labeled γ-ATP. DNA and amino acid sequence comparison analyses were performed with the sequence analysis software package (version 5) of the University of Wisconsin Genetics Computer Group (14) or as described in reference 28 or 62.

RFLP mapping of *rco-1*. Cosmids X1:B1 and X14:D7 were used as probes for restriction fragment length polymorphism (RFLP) mapping of *rco-1*. The small RFLP strain set was used for mapping (35). *Eco*RI-digested cosmid DNA was used to make probes by random hexamer priming in the presence of digoxigenin-labeled dUTP as described by the manufacturer (Boehringer Mannheim).

Nucleic acid techniques. All nucleic acid manipulation techniques were performed according to standard procedures (50). RNA was isolated as described previously (49), with the exception that the extraction buffer contained EDTA (extraction buffer was 100 mM Tris-HCl [pH 7.9], 100 mM LiCl, 1% sodium dodecyl sulfate, 1 mM dithiothreitol, 10 mM EDTA). Flasks containing 50 ml of Vogel's N medium (13) plus 1.5% sucrose were inoculated with approximately 5×10^5 conidia, and the cultures were grown in constant light with shaking at 200 rpm at 34°C for 20 h. Approximately 50 mg (wet weight) of mycelia collected by vacuum filtration or mature conidia collected by centrifugation was flash frozen in liquid nitrogen and immediately added to a 2.0-ml screw-cap tube containing 0.45 ml of phenol, 0.45 ml of chloroform, 0.55 ml of extraction buffer, and 1.5 g of zirconium oxide beads (0.5 mm in diameter). The tube was shaken in a mini-Beadbeater (Biospec Products) for two 60-s periods and centrifuged. After two phenol extractions, the RNA was precipitated with ethanol. RNA was dissolved in diethylpyrocarbonate-treated water, and the concentration was determined with a spectrophotometer. Ten micrograms of total RNA was separated in 1.5% agarose–formaldehyde gels for Northern (RNA) blot analysis, as described previously (49). The radiolabeled RNA probes used for *con-6*, *con-8*, *con-10*, and *eas* transcript detection were synthesized in vitro with either T3 or T7 RNA polymerase from plasmids pCON6-6, pRB2, pBW100, and pEAS, respectively (30). For the β-tubulin probe, the *Sal*I fragment from pBT3 (39) containing the β-tubulin gene was isolated and labeled by random priming (50).

Other techniques. Mutagenesis and selection conditions were as described by Madi et al. (30). Samples for scanning electron microscopy were prepared as described by Springer (56). A JEOL2010 scanning electron microscope was used.

Nucleotide sequence accession number. The *rco-1* sequence accession number is U57061.

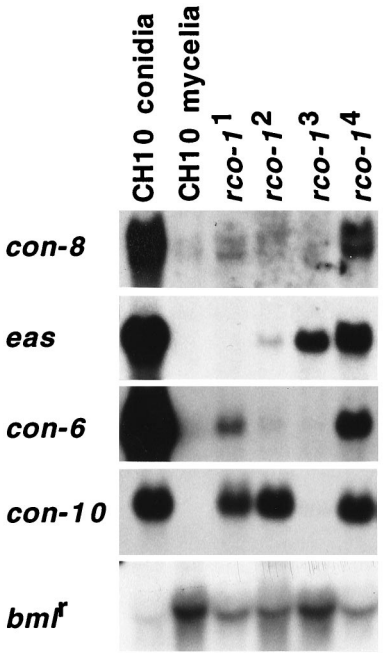

FIG. 1. Gene expression in *rco-1* mutants. *con* gene expression was assessed with mRNAs isolated from conidia and mycelia of the parental strain, CH10, and from mycelia of the *rco-1* mutants. *con-10* mRNA was elevated in all *rco-1* mutants (although only slightly so for *rco-1*^{3} in the blot displayed). The pattern of *con* gene expression was unique for each mutant. The strain bearing the *rco-1*^{3} allele displays the least severe loss of *con* gene regulation; only *eas* mRNA is markedly elevated. In *rco-1*^{4}, all of the *con* genes are expressed at high levels. β-Tubulin mRNA (*bml*^{r}) was measured as a control to establish the relative loading of mRNA in each lane.

RESULTS

Isolation of hygromycin B-resistant mutants of strain CH10. Strain CH10 carries a single copy of a *con-10′-′hph* translational fusion integrated at the *his-3* locus (30). When grown vegetatively, this strain is resistant to moderate levels of hygromycin B (70 U/ml), but is inhibited by higher levels (580 U/ml) (30). To obtain hygromycin-resistant mutants, CH10 conidia were irradiated with UV light to 84% survival, and the mutagenized conidia were embedded in an agar medium containing 580 U of hygromycin B per ml. Resistant colonies appeared in 5 days. Thirty of the fastest growing colonies were selected for analysis. The colony morphology of some of the resistant mutants suggested that they might be defective in conidiation. These 30 mutants were purified, and homokaryotic strains were obtained by three sequential platings of conidia or hyphal fragments from individual colonies on hygromycin B-containing agar.

After purification, 5 of the 30 mutants had readily distinguishable conidiation phenotypes. Of these, mutants CH10-101 and CH10-125 were aconidial, mutants CH10-119 and CH10-121 produced proconidial chains that failed to release individual conidia and thus were conidial separation defective, and CH10-129a exhibited sparse and irregular aerial growth with very few conidia. To determine if the alteration in any of these mutants was *trans*-acting, we screened the mutants for mycelial expression of the endogenous copy of *con-10*. Elevated levels of *con-10* mRNA were detected in all five mutant strains (Fig. 1; CH10-129a data not shown).

Complementation analysis with *rco* mutants. Complementation tests were performed with the various mutants with forced heterokaryons. The mutants were first crossed with a *nic-3 arg-10* strain to introduce auxotrophic markers into the mutant background. Approximately half of the progeny from these crosses contained the *con-10′-′hph* translational fusion between a tandem duplication of *his-3* generated during integration. Such tandem duplications are highly susceptible to RIP (55) during sexual crosses; *his-3* was inactivated in the *con-10′-′hph* segregants. *rco* mutant strains containing different auxotrophic mutations (either *nic-3*, *arg-10*, or *his-3*) were combined to make forced heterokaryons which grew on minimal medium. Pairwise prototrophic combinations containing nuclei of CH10-101, -119, -121, and -125 in heterokaryons remained conidiation defective; these mutants were noncomplementing. However, heterokaryons formed with CH10-129a and each of the other *rco* mutants exhibited wild-type conidiation. Therefore, there appear to be two *rco* complementation groups represented: one, *rco-1*, containing four mutant alleles and the other in strain CH10-129a. In addition, since CH10-129a complemented each of the other mutants, their alterations must be recessive with respect to the conidiation-defective phenotype.

TABLE 1. Summary of the characteristics of *rco-1* mutants

Strain	Allele	Linear growth rate (mm/h)	Conidiation[a]	Female fertility[b]	Spiral growth[c]
CH10	*rco-1*$^+$	5.0	+	+	cw
CH10-101	*rco-1*^{1}	1.5	acon	−	ccw
CH10-119	*rco-1*^{2}	1.6	csd	−	ccw
CH10-121	*rco-1*^{3}	1.2	csd	−	ccw
CH10-125	*rco-1*^{4}	1.1	acon	−	ccw

[a] +, normal conidiation; acon, aconidial; csd, conidial separation defective.
[b] +, fertile; −, sterile.
[c] cw, clockwise spiral hyphal growth; ccw, counterclockwise spiral hyphal growth.

Crosses were performed between the different mutants, and at least 100 ascospores were picked from each cross and analyzed. Germination rates were variable (ca. 33 to 70%). For all pairwise crosses between CH10-101, -119, -121, and -125, no wild-type progeny were obtained, indicating tight linkage of their mutations. For crosses between CH10-129a and the other mutants, the phenotypes of the progeny indicated that the CH10-129a alteration was unlinked to the changes in any of the other mutants (data not shown). These results are consistent with the complementation analyses. We designated the altered locus in mutants CH10-101, -119, -121, and -125 as *rco-1* and the mutant alleles as *rco-1*^{1}, *rco-1*^{2}, *rco-1*^{3}, and *rco-1*^{4}, respectively. The altered locus in CH10-129a was named *rco-2*.

Phenotypic characterization of *rco-1* mutants. The morphological phenotypes of the four *rco-1* mutants are summarized in Table 1. CH10, the parent, is indistinguishable from typical wild-type strains with respect to morphology, fertility, and growth rate. Scanning electron microscopy was performed with each of the *rco-1* mutants to define its morphological block in conidiation. After 48 h of growth on solid medium, a wild-type strain displays abundant proconidial chains and individual, released, conidia (Fig. 2A, arrow b). Mature conidia are released from proconidial chains by severance of the specialized attachment structure, the connective (59) (Fig. 2A, arrow a). The aconidial mutant *rco-1*^{1} produced aerial hyphae, but there was no indication of the change in hyphal morphology that is normally associated with minor or major constriction chain formation (Fig. 2B). *rco-1*^{3} produced major constriction chains but no free conidia; double cross-walls were evident, indicating that development had proceeded to a late stage in conidiation and then was arrested (Fig. 2C, arrow c). *rco-1*^{4} also was ac-

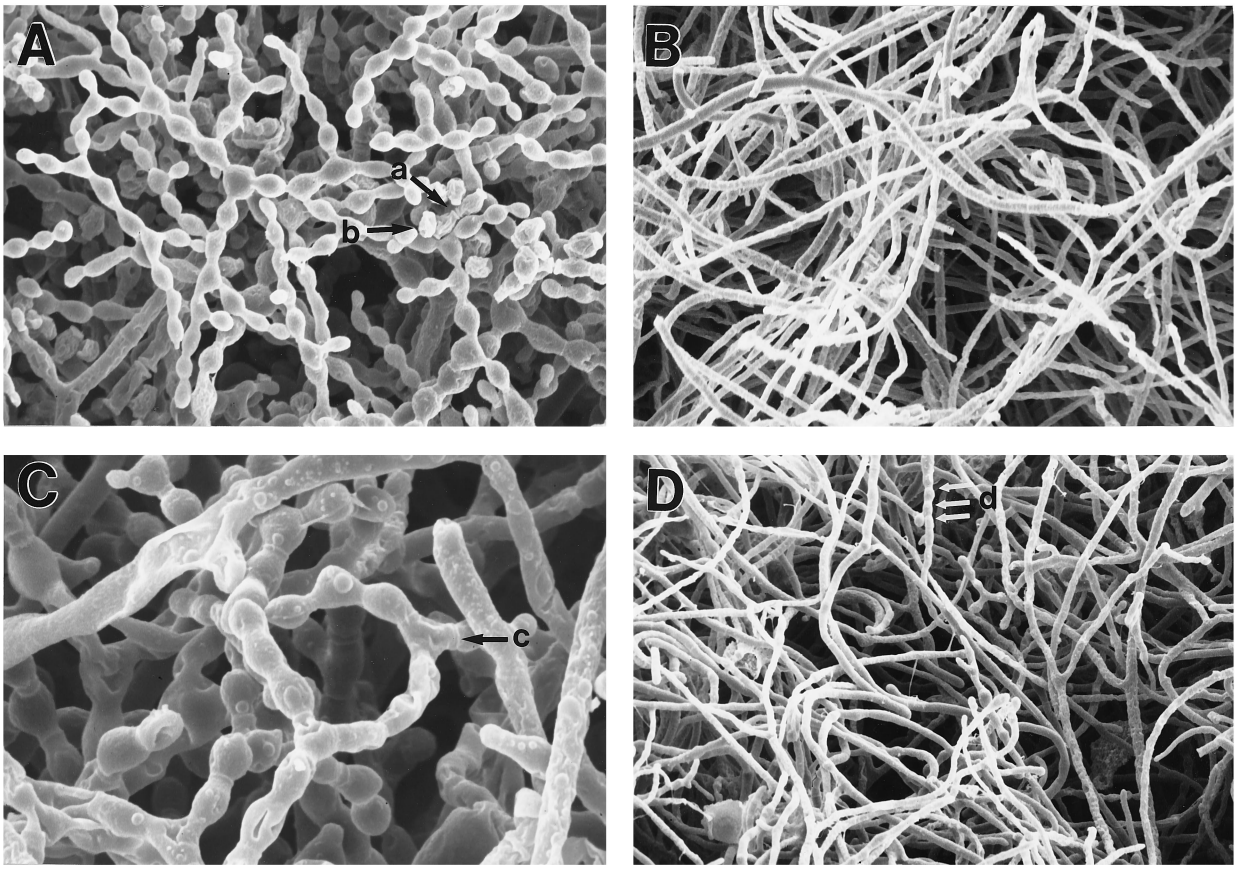

FIG. 2. Mutant morphologies. Scanning electron micrographs displaying wild-type (A) and mutant (B to D) conidiation phenotypes. (A) In the wild type, conidiation involves formation of major constriction chains that mature to proconidia that are held together by thin connective structures (arrow a). These connective structures are ultimately disrupted to yield free conidia (arrow b). (B) The strain with *rco-1*^{1} is aconidial and does not exhibit major constriction chain formation. (C) The strain with *rco-1*^{3} has a conidial separation phenotype and produces major constriction chains that do not mature and release free conidia (arrow c). (D) The strain with *rco-1*^{4} also is aconidial but occasionally shows minor constriction chain budding (arrows d). Magnification: A, ×700; B, ×350; C, 1,400; D, ×350. Photographs were taken at approximately the same time after a shift to conidiation-inducing conditions.

onidial and occasionally showed minor constriction chain budding (Fig. 2D, arrow d). The phenotypes of *rco-1*^{2} and RIP *rco-1* mutants (see below) were similar to that of *rco-1*^{3} (data not shown).

All four mutant alleles of *rco-1* failed to produce protoperithecia, the female reproductive organs, and therefore are female sterile. Female fertility was restored by combining nuclei of each *rco-1* mutant allele with nuclei of the *a*^{m1} helper strain in a heterokaryon (43). These heterokaryons make normal protoperithecia in which only the *rco-1* nucleus can participate in karyogamy and meiosis. *rco-1* mutants functioned normally as male parents in crosses.

The linear growth rates of the *rco-1* mutant and wild-type strains were compared on minimal agar in linear race tubes over a period of several days (Table 1). The linear growth rate of each mutant strain was fairly uniform; however, it was only one-fifth to one-half that of the parent, CH10.

Young hyphae of filamentous fungi growing on an agar surface often exhibit spiral growth (29, 63). *N. crassa* hyphae normally spiral in a clockwise direction when viewed from above (Fig. 3A). The young hyphae of the *rco-1* mutants spiralled in a counterclockwise direction (Fig. 3B). Spiral growth was observed with wild-type and *rco-1* mutants on fresh agar regardless of the type of inoculum (i.e., ascospores or mycelia or conidia for the wild type). With wild-type and *rco-1* strains, spiral growth could no longer be detected after 12 to 24 h of incubation at 34°C.

Regulation of *con* genes in *rco-1* mutants. Expression of *con-6*, *con-8*, *con-10*, and *eas* and of a control gene encoding β-tubulin was determined by Northern analyses with RNA isolated from several sets of mycelial cultures grown in liquid medium (Fig. 1). Since *con-10* expression is known to be influenced by light, circadian rhythm, and nutritional status (11), we grew our cultures under uniform conditions of temperature, light, time of growth and media, and inoculum size. Despite this, we observed some variation in the abundance of mRNA for these genes in different RNA preparations. Northern blots from one set of RNA preparations are shown in Fig. 1. Four mRNAs were measured. *con-8* mRNA is normally present in postexponential mycelial cultures and during the very early stages of conidiation. *eas* mRNA appears about 4 h after induction of conidiation, when minor constriction chains are first formed. *con-6* mRNA is detected approximately 6 h after induction of conidiation. *con-10* mRNA accumulates about 8 to 10 h after induction of conidiation, during the period of major constriction chain formation. As shown in Fig. 1, mRNA for each of these genes accumulated in conidia of the parental wild-type strain, CH10, but was absent from extracts of wild-type mycelial cultures. Occasionally a faint *con-8* mRNA band was observed in wild-type mycelia. In mycelial extracts of the

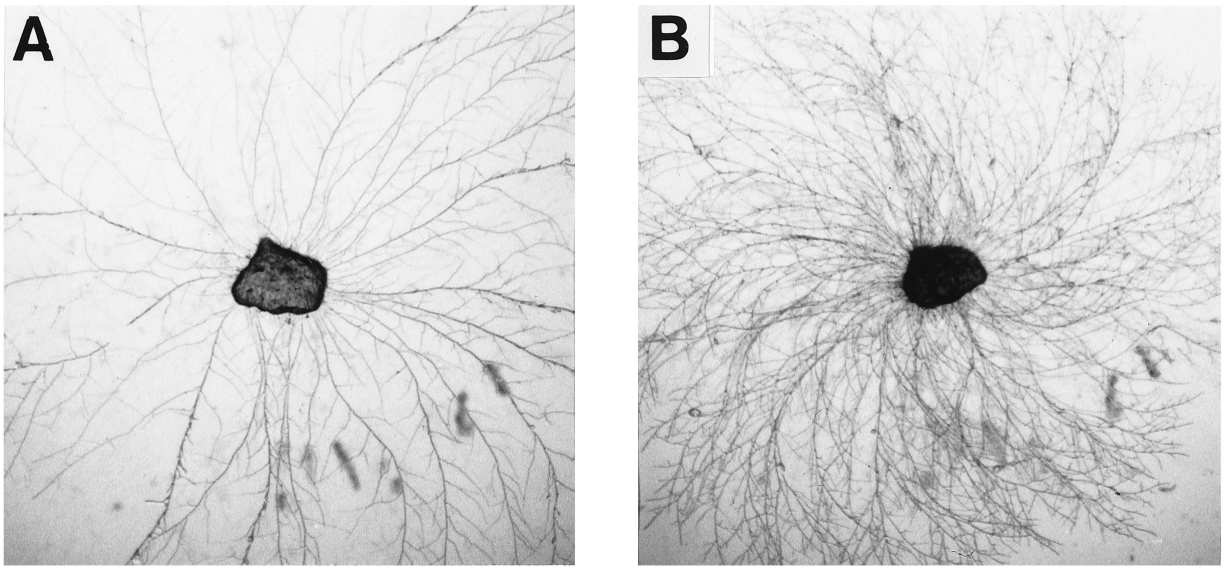

FIG. 3. Spiral hyphal growth in *rco-1* mutant strain CH10-125. Directionality of spiral growth of hyphae as seen on agar after 16 to 30 h of incubation at 34°C for the wild type (A) and strain CH10-125 *rco-1*^{4} (B). Cultures were viewed from above. The observed hyphae were at the surface-air interface. In the other *rco-1* mutants, spiral growth was identical to that of the strain with *rco-1*^{4} (not shown).

mutants, very low levels of *con-6* mRNA were generally present, except for samples from mutant *rco-1*^{4}, for which a strong signal was observed. In the Northern blot shown in Fig. 1, *con-6* mRNA was also observed in *rco-1*^{1}. However, in other RNA preparations, *con-6* mRNA was not detected in *rco-1*^{1}. *con-8* mRNA was detected in *rco-1*^{1} and *rco-1*^{4}, and *con-10* mRNA, as mentioned, was detected in all of the mutants. However, in the RNA preparation used for the blot shown in Fig. 1, *con-10* mRNA was barely detectable in *rco-1*^{3}. *eas* mRNA was generally present at a very low level in *rco-1*^{1} mycelia and at appreciable levels in mycelia from *rco-1*^{2}, *rco-1*^{3}, and *rco-1*^{4}. Mutant *rco-1*^{4} generally had the highest levels of conidiation gene mRNAs.

Cloning and mapping of *rco-1*. Efficient transformation of *N. crassa* requires the use of conidial spheroplasts as recipient cells. Since the *rco-1* mutants are aconidial, we could not use the standard transformation procedure and complementation to identify the wild-type gene. This obstacle was circumvented by developing a heterokaryon-assisted approach that allowed us to recover transformants derived from conidial spheroplasts containing only nuclei of the aconidial mutant. A schematic diagram of the procedure we used is shown in Fig. 4. The *rco-1*^{1} allele was used, since it has the most severe phenotype with respect to lack of aerial growth and conidiation. The *rco-1*^{1} strain was crossed with a strain containing *mtr*, a locus that confers resistance to the toxic amino acid analog FPA. From the progeny of this cross, we isolated the triple mutant *rco-1*^{1} *mtr his-3*. *his-3* inactivation occurred during the cross by the RIP process (55) and, as mentioned previously, was a consequence of the presence of a tandem duplication of *his-3* at the *his-3* locus of the original *rco-1* strain. This strain and a strain containing the colonial temperature-sensitive mutation *cot-1* (restricted colonial growth and no conidia at elevated temperatures) were combined to form a heterokaryon. Since all of the mutations employed are recessive, the resulting heterokaryons grew and conidiated normally at 34°C on minimal agar. Conidia from the heterokaryon were collected, and spheroplasts were prepared for transformation. DNAs from each of 50 pools of ~96 clones of a cosmid library were used for transformation, selecting for hygromycin B resistance encoded by the cosmid vector (41, 42). Conidia typically have one to four nuclei (20); therefore, three classes of conidia will be formed by this heterokaryon. Two classes will be homokaryotic; these will have nuclei that are either *his-3 mtr* and contain the mutant *rco-1* locus or are *cot-1*. The heterokaryotic conidia will contain both types of nuclei. Suspensions of transformed spheroplasts were plated on agar containing FPA, histidine, and hygromycin B at 34°C. Conidia containing the *cot-1* nucleus, both homokaryotic and heterokaryotic, were inhibited by FPA because the *cot-1* nucleus contains the wild-type *mtr* locus. This locus allows uptake of FPA. Thus, the only hygromycin-resistant colonies which would be able to grow with FPA

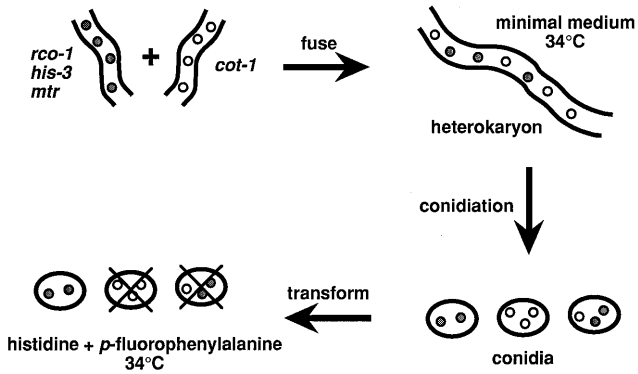

FIG. 4. Schematic of the steps in heterokaryon-assisted transformation. Heterokaryon formation was forced between the aconidial mutant (*rco-1*^{1} *his-3 mtr*) and the helper strain containing the temperature-sensitive *cot-1* mutation by selection for growth on minimal medium at the *cot-1* nonpermissive temperature, 34°C. Conidia were collected from the heterokaryon, and competent spheroplasts were prepared. The spheroplasts were transformed with cosmid DNA and plated on minimal agar supplemented with histidine, FPA, and hygromycin B and incubated at 34°C. Selection was for transformants that were resistant to hygromycin B and FPA and that could grow at 34°C. The colonies that formed were screened for complemented transformants, i.e., colonies exhibiting aerial growth and conidiation.

would be derived from homokaryotic conidia containing *his-3 mtr* and the mutant *rco-1* locus.

After 5 days of incubation, plates representing two different pools, X1 and X14, contained several complementing colonies which grew significantly faster than the other colonies and had aerial growth with limited conidiation. Sib selection (1, 67) was initially used in attempts to isolate the specific clone in each pool but was ineffective. Instead we turned to LM-PCR (37) to generate probes corresponding to the ends of the inserts of the cosmids that had complemented the *rco-1* mutant. To do this, genomic DNA was isolated from a complemented transformant from each of the X1 and X14 pool plates. Amplicons corresponding to each end of the DNA from the X1 pool-complemented transformant and to one end of the DNA from the X14 pool-complemented transformant were generated by LM-PCR. Nonradioactive probes were produced and used to probe colony blots containing the 96 clones of the relevant cosmid library pools. For the X1 pool, both of the probes only hybridized to the B1 clone; only the D7 clone hybridized with the X14 pool probe (data not shown). Restriction digests prepared with the two clones indicated that they had overlapping, but nonidentical inserts (data not shown). The common sequences in complementing cosmids X1:B1 and X14:D7 were assumed to contain *rco-1*.

Delimitation of *rco-1* within the X1:B1 cosmid insert was accomplished by transforming the heterokaryon with the X1:B1 DNA digested with a variety of restriction enzymes. An unrelated cosmid, G:18:A5, was included in the transformations to allow selection for hygromycin resistance. After 4 days of incubation of transformation plates at 34°C, X1:B1 DNA digested with *Bst*EII, *Cla*I, *Kpn*I, *Not*I, *Sal*I, *Xba*I, or *Xho*I had some colonies which exhibited rapid growth, indicating complementation of *rco-1*^{1}. Transformations with DNA digested with *Bam*HI, *Eco*RI, *Hin*dIII, or *Pst*I yielded only small, slowly growing colonies, suggesting that one or more restriction sites for these enzymes lie within *rco-1*. X1:B1 DNA digested with *Sal*I or *Xba*I and ligated under conditions promoting recircularization of the vector and one end of the insert was found to complement *rco-1*^{1}. Subsequently, a 4-kb *Xba*I-*Cla*I fragment from the *Xba*I-shrunken plasmid described above still complemented *rco-1*^{1}. This *Xba*I-*Cla*I fragment was also present within the X14:D7 cosmid insert.

The *rco-1* clones X1:B1 and X14:D7 were used to map the *rco-1* locus by restriction fragment length polymorphism (RFLP) (34, 35). Both *rco-1* clones had the same RFLP pattern as LZE4 (34) (data not shown), which is located near the centromere of linkage group III. To confirm this assignment, each *rco-1* allele was crossed with *acr-2*, a locus linked to the centromere of linkage group III. Each of the four *rco-1* alleles showed linkage to *acr-2*, giving recombination frequencies of 7 to 17% (data not shown).

RNA preparations isolated from different tissues and differing in the stage of development and physiological state were analyzed for *rco-1* expression. With the *Xba*I-*Cla*I fragment as the probe, only a ~2.5-kb RNA species appears in Northern blots. Under all conditions examined, the *rco-1* mRNA level was essentially constant (reference 25 and data not shown); therefore, expression of *rco-1* appears to be independent of development and physiological state.

Nucleotide sequence of *rco-1* and the predicted sequence of its polypeptide product. Both strands of the *Eco*RI-*Xba*I fragment of the genomic *rco-1* clone, as well as 343 bp upstream of the *Eco*RI site, were sequenced (Fig. 5). An open reading frame interrupted by four introns was established by isolating and sequencing *rco-1* cDNA clones. The introns are 153, 75, 60, and 73 bp in length. Their 5′ and 3′ ends and lariat sequences are well conserved (15). The deduced amino acid sequence of RCO1 reveals that it is a polypeptide of 604 amino acid residues with a predicted molecular mass of 66 kDa (Fig. 5). The predicted amino acid sequence of RCO1 shows overall homology with that of the Tup1 protein of *S. cerevisiae* (46.3% identity) (62, 69) (Fig. 6). Tup1 and Ssn6 comprise a protein complex that is required for repression of several apparently unrelated genes, such as glucose-repressible genes and **a**-specific genes (38, 52). Tup1 and Ssn6 are physically associated proteins that are believed to interact with *trans*-acting DNA-binding factors that serve as repressors (70).

RIP alleles of *rco-1*. Presumed null alleles of *rco-1* were created by the RIP process (55). This requires the introduction of a second copy of *rco-1* into the genome, followed by a sexual cross. RIP should result in the introduction of multiple GC-to-AT changes in both copies of *rco-1* in the nucleus containing the duplication (54). For these experiments, a second copy of *rco-1* was introduced by transformation with a vector that allowed homologous targeting to the *am* locus, thereby conferring resistance to hygromycin B (7). Seven resistant transformants were generated, and all were shown by Southern blot analysis to have a single integrated copy of *rco-1* with a size of approximately 2.1 kb at the *am* locus (data not shown). Each transformant was crossed with the wild type, and the resulting ascospores were picked, germinated, and grown at 25°C (Table 2). After 24 h of incubation, progeny were scored for their spiral growth phenotype. Of the 326 spores picked from the seven crosses, 62% had extensive clockwise hyphal growth, 29% had slow counterclockwise hyphal growth, 8% had indeterminant spiral growth, and 20% failed to germinate. After 7 days at 25°C, all of the clockwise spiral progeny had profuse and normal aerial growth with conidia as observed in wild-type strains. The counterclockwise progeny had somewhat less aerial growth with numerous conidiophores which lacked free conidia, as observed in some of our UV-induced *rco-1* mutant strains. This class of progeny was common and is likely to represent null mutants due to efficient introduction of multiple point mutations by RIP (55). Selker (54) observed that strains with duplications of nonessential genes of 2.6 kb and greater, when crossed with the wild type, typically yield ~25% of progeny with a null mutant phenotype for the duplicated gene. Since seven independent *rco-1* RIP crosses yielded typical percentages of viable progeny, and in each cross about 25% of the progeny had the *rco-1* mutant phenotype—the conidiation defect and counterclockwise spiral growth—it is likely that many of these represent the null phenotype (Table 2). Single progeny from each of the seven crosses which had counterclockwise growth and a conidial separation phenotype were tested for fertility. Every putative RIP mutant isolate tested was female sterile and failed to form protoperithecia (data not shown). These aconidial, sterile, presumed null mutants most closely resemble the *rco-1*^{2} and *rco-1*^{3} alleles, the least severe of the four UV-induced *rco-1* alleles we have studied. The third, minor class (Table 2) had a variety of phenotypes ranging from normal conidiation to almost total lack of aerial growth.

DISCUSSION

A *con-10′-'hph* translational fusion (30), expressed from a 520-bp segment of the *con-10* promoter, was used to isolate mutants with altered *con-10′-'hph* expression. *con-10* is normally expressed during conidiation, but not during mycelial growth. Of 30 mutants recovered that expressed the translational fusion during mycelial growth, 5 were aconidial and had elevated levels of the transcript of the endogenous, intact *con-10* gene. These *trans*-acting mutations were presumed to

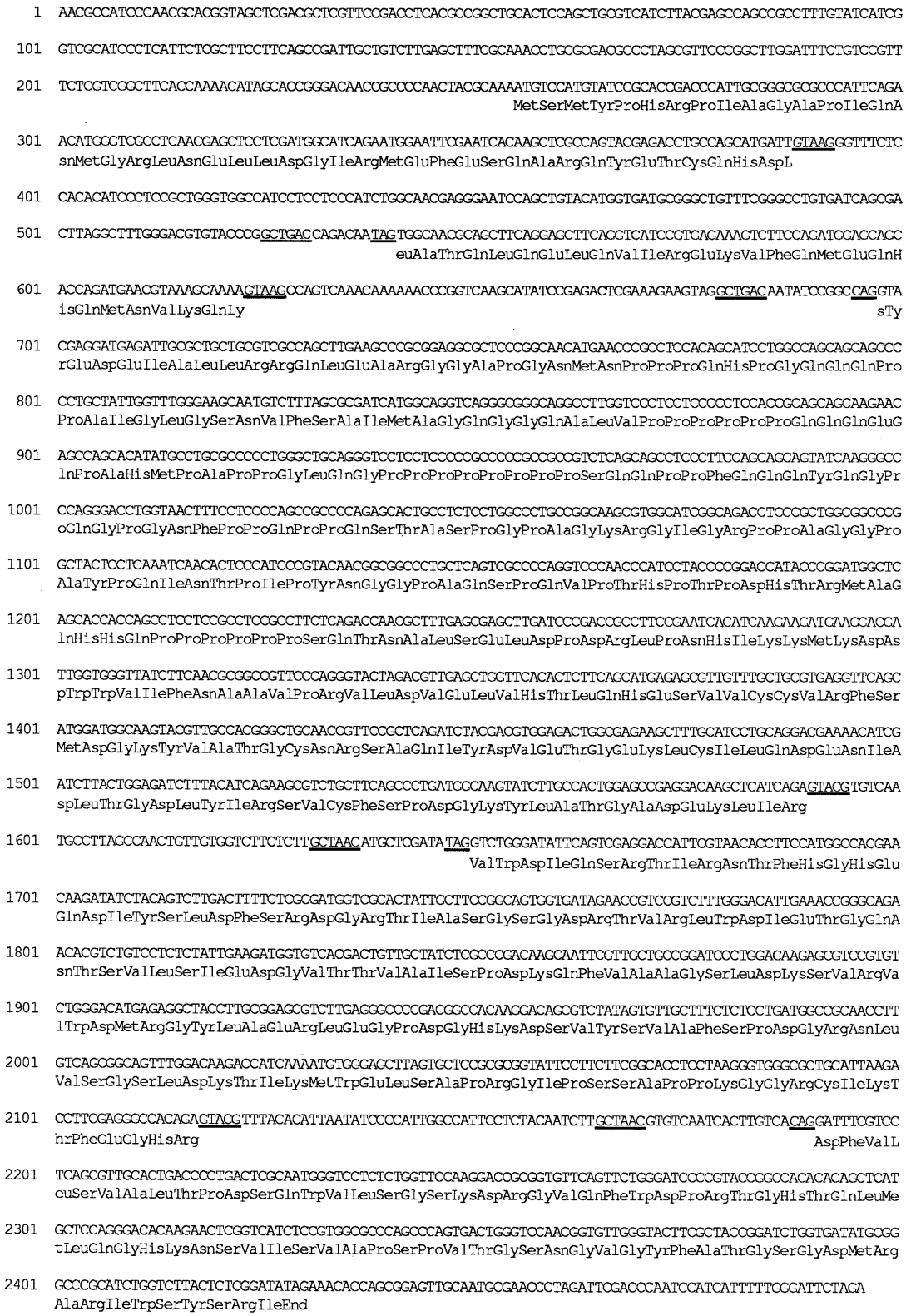

FIG. 5. Nucleotide sequence of *rco-1*. A 2,496-bp DNA sequence containing *rco-1* is shown. Also shown is the predicted sequence of the corresponding RCO1 polypeptide. Underlined DNA sequences indicate conserved splice junctions and lariat sequences of the four introns.

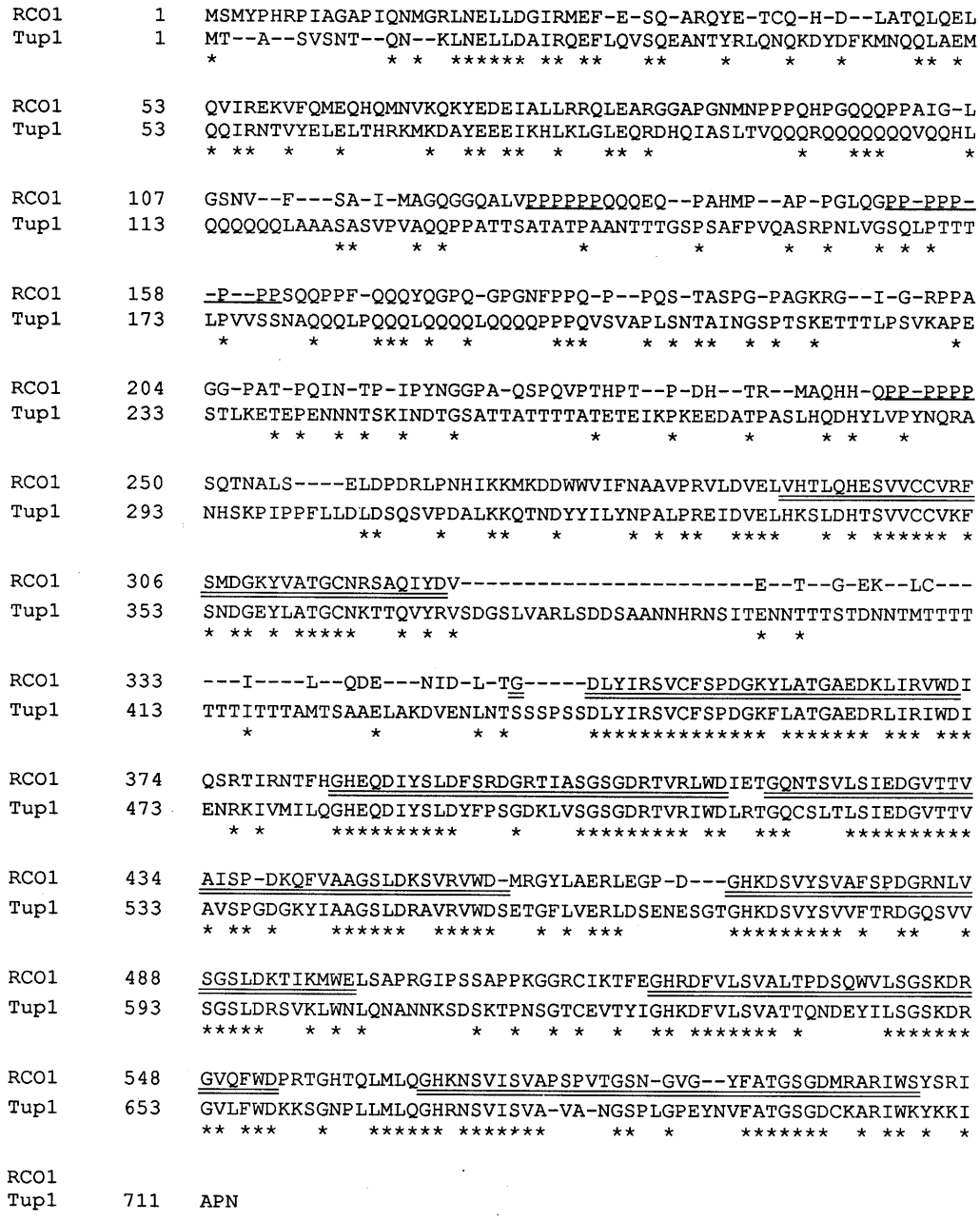

FIG. 6. Amino acid sequence comparison between RCO1 and Tup1. Asterisks mark identical residues. Dashes indicate gaps introduced to maximize the alignment. Double-underlined amino acid sequences identify the seven WD-repeat motifs of RCO1 and Tup1. Single underlined amino acid sequences identify the three proline-rich segments of RCO1.

be in a regulatory gene (or genes) concerned with conidiation. Four of the five mutants were found to be altered at a single locus, which we designated *rco-1*. Since these four mutants arose independently, it is possible that mutations in only a small number of genes can lead to expression of *con-10* during mycelial growth and block conidiation. A previous mutational analysis with this experimental approach yielded eight overexpressing mutants, four of which were conidiation defective (30). It is not known how many complementation groups are represented by these mutants or whether any are alleles at the *rco-1* locus. However, at least two of the previously isolated conidiation-defective mutants displayed phenotypes not observed in the four *rco-1* mutants (30). One of these *rco* mutants has been shown to have an alteration in a gene distinct from *rco-1*, designated *rco-3* (31). *rco-3* has been cloned and sequenced, and its characteristics suggest that its product, RCO3, may be concerned with carbon source sensing (31). Our genetic analyses indicate that *rco-1* does not correspond to any previously known gene that influences morphology or conidiation.

Effect of *rco* mutations on fertility and growth. Our *rco-1* mutants could not serve as female parents in sexual crosses. Conceivably *rco-1* encodes a shared regulatory factor that functions in both the conidiation and sexual pathways. Alterna-

TABLE 2. Examination of the progeny of crosses of *rco-1 hyg*^{R} transformants by the wild type

Cross	% Germination	No. of ascospore progeny with phenotype[a]:		
		cw/wt	ccw/slow	Other
223	67	17	8 (30)	2
224	81	21	17 (44)	1
225	75	20	10 (28)	6
226	87	21	10 (29)	4
227	84	32	9 (21)	1
228	82	24	14 (34)	3
229	82	28	8 (20)	5

[a] cw, clockwise; ccw, counterclockwise, hypal growth; wt or slow, wild-type or slow growth rate, respectively. Values in parentheses represent percentage of viable spores with the phenotype given.

tively, release of repression of mycelial functions may block the normal progression of developmental events required for completion of the sexual cycle to the protoperithecial stage. Of relevance is the finding that *con-10*, *con-6*, and *con-8* are expressed in the developmental pathways leading to the formation of all three types of spores (60). Thus, *rco-1* appears to regulate genes that are expressed in both the asexual and sexual spore pathways. Similarly, the *stuA* and *medA* genes of *Aspergillus nidulans* are required both for normal development of asexual spores and for fertility (36). Fungi may generally share some specific developmental regulators in different developmental pathways. There are numerous comparable examples in other organisms, such as *Notch* of *Drosophila melanogaster* and *lin-12* of *Caenorhabditis elegans* (19), in which regulators of cell fate are called upon at different times in different tissues to coordinate events in development.

The reversed spiral growth phenotype of the *rco-1* mutants may be related to phenomena observed in two other fungi, *Phycomyces blakesleeanus* and *S. cerevisiae. P. blakesleeanus* exhibits unidirectional spiral growth of the sporangiophore prior to production of the sporangium. Then, upon formation of the sporangium, spiral growth reverses its direction (8). *S. cerevisiae* exhibits a reversal of the polarity of bud formation in haploid and diploid cells (10, 18). The above two phenomena appear to be cell type dependent in determination of polarity of cell elongation and bud formation, respectively. Since *rco-1* mutants are defective in asexual and sexual development, it is possible that reversal of spiral growth directionality is due to a defect in regulation of cell type determination. Mutants of *N. crassa* have been isolated in which wild-type spiral growth is enhanced (2).

Patterns of *con* gene expression in the *rco-1* mutants. Northern analyses with mycelial RNA from the different *rco-1* mutants showed that the expression pattern of each mutant differed from that of the wild type. In addition, the individual *rco-1* mutant alleles displayed somewhat different patterns of *con* gene expression (Fig. 1). Considerable variation was observed in the levels of some *con* mRNAs in RNA preparations from the same mutant, particularly for *con-6* and *con-10*, suggesting that expression is not constant for each gene under the test conditions employed. *rco-1*^{4} generally revealed the highest expression of the four *con* genes examined (Fig. 1). *rco-1*^{1} had elevated levels of *con-6*, *con-8*, and *con-10* mRNAs. *rco-1*^{1} had elevated levels of only *con-8* and *con-10* mRNAs. Mutants *rco-1*^{2} and *rco-1*^{3}, which are blocked late, at the conidial separation stage, generally formed moderate levels of *con-10* and *eas* mRNAs (no *con-10* mRNA was detected in the sample analyzed in Fig. 1), but low levels of *con-6* and *con-8* mRNAs.

In previous regulatory studies of the promoter region of *con-10*, it was observed that deletions removing segments of the 520 bp preceding the transcription start site had marked effects on this gene's expression (11). One promoter segment, from −517 to −353, was shown to be responsible for mycelial repression, a second promoter segment, from −281 to −217, was responsible for activation during conidiation, a third segment, from −353 to −265, mediated dark repression, and two enhancer elements contributed to overall *con-10* expression. Since RCO1 is a homolog of the regulatory protein Tup1 of *S. cerevisiae*, RCO1 could be expected to participate directly or indirectly in regulatory events that are responsible for such diverse processes as mycelial repression and/or dark repression. Tup1 is known to mediate repression of genes concerned with a variety of processes (16, 38, 52, 73). The promoters for *con-6*, *con-8*, and *eas* have sequences in common with sequences present in the segments of the *con-10* promoter that are believed to mediate mycelial repression and dark repression (4, 11). Thus, a common element with the consensus sequence GGGAGCT is located in the mycelial repression regulatory regions of *con-10* and *eas* (4, 11). Identical or similar sequences are present in the promoter regions of *con-6* and *con-8* (11, 46, 47). As we have shown, each of the four genes *con-6*, *con-8*, *con-10*, and *eas* is aberrantly transcribed during mycelial growth in strains bearing one or more of the *rco-1* alleles (Fig. 1). In addition, in other mycelial expression studies performed with the intact natural copy of *con-10*, *con-10* was expressed in the dark in strains with mutant alleles *rco-1*^{1} or *rco-1*^{4}, whereas it was not expressed in the dark in the wild type. Most importantly, *con-10* expression was increased appreciably in the two *rco-1* mutants upon exposure to light (26). These findings suggest that in the *rco-1* mutants, mycelial expression of *con-10'-'hph* and *con-10* may result from the inability of mutant RCO1 to mediate repression at the GGGA GCT-containing segment of the *con-10* promoter. It remains to be determined if there are other sites of RCO1-mediated repression in the promoters of the *con* genes or *eas.*

Comparison of Tup1 and RCO1. The RCO1 protein shows homology to the yeast regulatory protein Tup1. Tup1 is associated with Ssn6 in a large complex that does not itself bind DNA, but is thought to interact with transcription factors already bound to DNA, thereby mediating their ability to repress transcription. There is increasing evidence that Tup1 has separate domains that are concerned with different events in several cellular processes (23, 65). This may explain why *tup1* mutants are highly pleiotropic. Since RCO1 has high homology with Tup1, it is likely that RCO1 also has specific domains with separate functions. This may explain why some of the *rco-1* mutants have different phenotypes. The 80 N-terminal residues of RCO1 show high homology with the 72 N-terminal residues of Tup1 (Fig. 6). This segment of Tup1 is likely to form an α-helix (66). The N-terminal segment of RCO1 is also predicted to be largely α-helical (nnpredict [references 22 and 33 and data not shown]). Since the N-terminal segment of Tup1 serves as the binding domain for Ssn6, the 80 N-terminal residues of RCO1 may serve a similar role. The predicted RCO1 sequence contains a proline-rich region (Fig. 6). Proline-rich regions (PRRs) are found in both prokaryotic and eukaryotic proteins (for a review, see reference 71). The functions of most PRRs are unknown. Some are believed to serve as binding regions for proteins concerned with essential cellular functions. For example, the proline-rich domain of mSos1 in *Mus musculus* is known to bind to the Src homology 3 (SH3) domain (48). In Tup1, a glutamine- and alanine-rich region replaces the proline-rich region of RCO1 (69). This region of Tup1 participates in repression of *SUS2* and *ANB1* gene ex-

pression (66). Tup1 contains seven copies of the β-transducin motif, also called the WD40 repeat (38, 69). These repeats in their respective proteins are involved in regulatory mechanisms that control many cell functions, including cellular differentiation (for a review, see reference 39). Like Tup1, the predicted RCO1 polypeptide has seven WD40 repeat sequences, averaging 68.0% identity between the two proteins (Fig. 6). This structural similarity between RCO1 and Tup1 suggests that RCO1 may serve as a mediator of transcriptional repression in *N. crassa.*

How might an *rco-1* mutation block development? It is perhaps surprising that mutations that cause aberrant expression of developmentally regulated genes during vegetative growth, presumably by relieving their repression, appear to block developmental processes that are essential for conidiation and protoperithecium formation. Several explanations are possible. Conceivably, the *rco-1* mutations prevent the normal sequence of events that are necessary for proper development in these two pathways. For example, the *rco-1* mutations may allow activation of some genes necessary for some stages of conidiation, but may not permit activation of expression of other genes essential for completion of conidiation. Thus only an incomplete set of conidiation genes may be turned on in vegetative tissue as a result of an *rco-1* mutation. A second possibility is that the *rco-1* gene product is primarily responsible for regulating a class of genes essential for a separate developmental pathway, such as protoperithecium formation. From what is known about the gene used in our selection, *con-10*, this is reasonable since, as mentioned, *con-10* is expressed in all three sporulation pathways (60). By inappropriately turning on some genes concerned with conidiation, that pathway may be rendered defective.

The observation that the putative null mutants of *rco-1* created by RIP have a phenotype similar to those of *rco-1*^{2} and *rco-1*^{3}, the two UV-induced alleles with the least severe phenotype with respect to conidiation, is consistent with the idea that RCO1, like Tup1, is a component of a macromolecular complex. Conceivably, the *rco-1*^{1} and *rco-1*^{4} polypeptides, although functionally defective, can "poison" the macromolecular complex, resulting in a more severe phenotype than is observed with null mutants which do not make the protein. Thus the Ssn6 homolog of *N. crassa*, by itself or when complexed with other factors, may be capable of functioning at a reduced capacity, whereas, when complexed with the defective RCO1 of *rco-1*^{1} or *rco-1*^{4}, it may be rendered nonfunctional or less active.

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