The Isolation and Characterization of nrc-1 and nrc-2, Two Genes Encoding Protein Kinases That Control Growth and Development in Neurospora crassa

Gregory O. Kothe and Stephen J. Free

Department of Biological Science, State University of New York, Buffalo, New York 14260-1300

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ABSTRACT

Using an insertional mutagenesis approach, a series of Neurospora crassa mutants affected in the ability to control entry into the conidiation developmental program were isolated. One such mutant, GTH16-T4, was found to lack normal vegetative hyphae and to undergo constitutive conidiation. The affected gene has been named nrc-1, for nonrepressible conidiation gene 1. The nrc-1 gene was cloned from the mutant genomic DNA by plasmid rescue, and was found to encode a protein closely related to the protein products of the Saccharomyces cerevisiae STE11 and Schizosaccharomyces pombe by2 genes. Both of these genes encode MAPKK kinases that are necessary for sexual development in these organisms. We conclude the nrc-1 gene encodes a MAPKK kinase that functions to repress the onset of conidiation in N. crassa. A second mutant, GTH16-T17, was found to lack normal vegetative hyphae and to constitutively enter, but not complete, the conidiation program. The affected locus is referred to as nrc-2 (nonrepressible conidiation gene 2). The nrc-2 gene was cloned and found to encode a serine-threonine protein kinase. The kinase is closely related to the predicted protein products of the S. pombe kad5, and the S. cerevisiae YNR047w and KIN82 genes, three genes that have been identified in genome sequencing projects. The N. crassa nrc-2 gene is the first member of this group of kinases for which a phenotype has been defined. We conclude a functional nrc-2-encoded serine/threonine kinase is required to repress entry into the conidiation program.

UN DER nutrient-sufficient conditions, the filamentous fungus Neurospora crassa proliferates through the extension and branching of multinucleate vegetative hyphal cells. In response to nutrient deprivation, desiccation and light cues, N. crassa initiates an asexual developmental program called conidiation (for a review of conidiation see Springer 1993). The availability of a readily usable carbon and energy source, such as glucose, is the major determinant controlling the onset of conidiation (Ricci et al. 1991). In addition to being glucose-deprived, the fungus must be exposed to the air in order for conidiation to occur (Springer and Yanofsky 1989). Conidiation does not occur in submerged cultures. The presence of light is also an important determinant in controlling the differentiation process. N. crassa cultures that are grown in complete darkness produce conidia later, and in reduced numbers, than cultures grown with illumination (Lauter and Russo 1991). In addition to these environmental influences, the endogenous clock of the organism affects the conidiation process, with conidiation occurring during the subjective morning. Clock-controlled genes expressed during the subjective morning have been found to encode proteins that are clearly being expressed as part of the conidiation program (Bell-Pedersen et al. 1996).

Conidiation begins when aerial hyphae emerge and extend away from the underlying vegetative mycelium. These aerial hyphae differ morphologically from the vegetative hyphae in that they have a smaller diameter and an increased frequency of septa (crosswalls). The aerial hyphae contain cell-type-specific proteins that are absent in vegetative hyphae (Berlin and Yanofsky 1985). As differentiation proceeds, the aerial hyphae form branches and enter into a stage of development marked by the formation of minor constrictions near the hyphal tips. While in the minor constriction stage of conidiation, the aerial hyphae may resume hyphal elongation, or may enter the major constriction phase. The major constriction phase of conidiation is characterized by the polarized budding of the hyphal tips to produce chains of proconidia with well-defined interconidial constrictions. Once the major constriction phase of development has begun, cellular extension occurs exclusively in a budding mode. As the conidiophores mature, the major and minor interconidial constrictions become more pronounced. At the completion of conidiation, the interconidial constrictions develop into septa that facilitate separation of individual conidia. When exposed to air currents, the conidia function as airborne spores.

Expression of the Neurospora conidiation program is responsive to the extracellular levels of glucose, the circadian rhythm and the presence of blue light. The
clock-controlled gene 1 (ccg-1) (Loros et al. 1989) is one of the earliest expressed genes in the N. crassa conidiation program and its expression is regulated by all three of these environmental cues. It has been isolated as a glucose-repressible gene, as a clock-controlled gene and as a blue-light-inducible gene (McNally and Free 1988; Loros et al. 1989; Dunlap and Loros 1990; Arpaia et al. 1995). McNally and Free (1988) cloned, sequenced and characterized the gene (then named grg-1) as a highly expressed glucose-repressible gene. The availability of glucose is a major determinant controlling ccg-1 expression and ccg-1 mRNA levels increase over 500-fold following a transfer from glucose-sufficient to glucose-insufficient medium (Wang et al. 1994). The ccg-1 gene was also isolated and characterized as a clock-controlled gene by Loros et al. (1989) and Dunlap and Loros (1990). Under conditions of glucose limitation, conidiation occurs during the subjective morning portion of the circadian clock. The levels of ccg-1 transcript are regulated over a 5–10-fold range by the circadian clock, with the highest levels of transcript being present in the morning, when conidiation occurs (Loros et al. 1989). Arpaia et al. (1995) cloned ccg-1 as a blue-light-inducible gene and showed that ccg-1 expression, like the expression of a number of other ascomycete conidiation genes, is induced by blue light.

This report describes the use of an insertional mutagenesis-based approach to isolate N. crassa mutants affected in the regulation of the N. crassa ccg-1 gene. Most of the mutants isolated were also affected in the ability to regulate entry into conidiation. Some of the mutants had the morphological characteristics of mycelia blocked at various stages of conidiation. Two of these mutants have been characterized. The affected genes, nonrepressible conidiation gene 1 (nrc-1) and nonrepressible conidiation gene 2 (nrc-2), have been cloned, sequenced and identified as likely to encode protein kinases.

MATERIALS AND METHODS

Insertional mutagenesis: Construction of the ccg-1/tyrosinase reporter gene construct and generation of the GTH 16 reporter strain have been previously reported (Kothe et al. 1993). Insertional mutagenesis was performed as described by Kothe and Free (1996).

Strains and culturing conditions: RLM57, a strain with an al-2; arom-9; inv; qa-2; a genotype was used in a number of the experiments, including the heterokaryon mating experiments. RLM57 was obtained from R. L. Metzenberg (Stanford University, Stanford, CA). GTH 16, the strain used for the insertional mutagenesis, was derived from RLM57 by transformation with a plasmid containing a hygromycin resistance marker and the chimeric ccg-1/tyrosinase gene (Kothe et al. 1993). N. crassa strain 74-OR23-1VA (wild type) was used for all crosses. The fungus was cultured on Vogel’s minimal medium supplemented with 2% glucose (Davis and de Serres 1970). Sorbose medium consisted of Vogel’s minimal medium supplemented with 2% sorbose, 0.05% glucose and 0.05% fructose. Solid media contained 2% agar. Strains having qa-2 and arom-9 mutations were cultured on Vogel’s medium supplemented with 5X aromatic amino acids (Davis and de Serres 1970). All crosses were carried out on corn meal agar medium (no. 0386-01; Difco, Detroit) at room temperature. Growth rate was assessed by placing an inoculum near the edge of a 100-mm circular Petri dish containing Vogel’s glucose medium and measuring the growth of the fungus across the surface of the agar as a function of time.

Microscopic analysis: Microscopic analyses were done with a phase contrast microscope (model AFM; Nikon, Garden City, NY). Cultures were inoculated onto slides which had been overlaid with Vogel’s glucose agar medium.

Assessing conidial production: To assess the production of conidia, GTH 16-T4, RLM57 and 74-OR23-1VA hyphae were inoculated into the center of three petri plates (100-mm circular dish) containing Vogel’s glucose agar medium supplemented with aromatic amino acids and allowed to grow for 48 hr at 30°C in the dark. The plates were then placed under constant illumination at 25°C for an additional 24 hr prior to assessing conidial production. The production of conidia in the center of the petri plates was assessed by excising 6 cm in diameter disks from the center of the dishes and placing them in 50-ml conical tubes containing 25 ml of H2O. After vortexing to dislodge and disperse the conidia, an aliquot of the water was removed and conidial density was determined with a hemocytometer. Conidial production at the periphery of plates was similarly accomplished by carrying out the same manipulations on 2-cm-wide rings excised from the edge of the petri dishes. Similar experiments were done to assess production of conidia in the dark.

Nucleic acid manipulations: Southern blots and cloning procedures were performed as described by Sambrook et al. (1989). Radiolabeled probes were generated using a multiprime labeling kit (RPN 1600y; Amersham, Arlington Heights, IL). The pMOcosX cosmid library of N. crassa genomic DNA (Orbach 1994) was obtained from the Fungal Genetics Stock Center (Kansas City, KS).

Plasmid rescue and sequencing: Plasmid rescue was performed essentially as described by Kang and Metzenberg (1993). Ten micrograms of chromosomal DNA from the nrc-1 mutant was digested with PstI, which does not cut within the pRAL-1 plasmid. Following digestion, the DNA was treated with DNA ligase. The ligated DNA was precipitated with ethanol and resuspended in 15 μl of TE buffer. Five microliters of the preparation was used to transform E. coli strain K802 by electroporation, and transformants were selected on LB medium containing 30 μg/ml chloramphenicol. Plasmid DNA was prepared from chloramphenicol-resistant E. coli transformants and characterized by restriction and nucleotide digestions. Sequencing of plasmid and cosmid DNAs was carried out by an automated DNA sequencing facility at SUNY/Buffalo.

Transformation of the nrc-1 mutant with cosmid G15:C5: Transformation of nrc-1 conidia was performed by electroporation using a Genpulsar apparatus (Bio-Rad Laboratories, Hercules, CA) (Vann 1995). 110 μl of GTH 16-T4 conidia were mixed with 2 μl of 1 μg/μl pBARKS1 (Pall and Brusell 1993), and 5 μl of 1 μg/μl G15:C5 cosmid DNA. The G15:C5 cosmid contains an intact copy of the nrc-1 gene. A control was prepared using 2 μg of pBARKS1 plasmid DNA and no cosmid DNA. The transformations were carried out in cuvettes with a 0.2-cm gap, using the following parameters: 25 microfarads, 600 ohms, 1.5 kilovolts. Immediately after each pulse, the conidia were resuspended in the cuvettes using 1 ml of ice-cold 1 m sorbitol. The cells were kept on ice for 10 min, at which point they were transferred to 100-ml pyrex bottles containing 50 ml of Vogel’s (ammonium-free)/1 m sorbitol/sorbose-top agar which were being kept at 50°C. The bottles were swirled to distribute the cells, and then 10-ml portions of the top agar were overlaid onto individual Vogel’s (ammo-
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dium-free)/sorbose plates containing 300 μg/ml BASTA (Hoechst-Roussel Agri-Vet Company, Somerville, NJ; Pall 1993). The plates were incubated for three days in the dark (30°C). Colonies that reached the surface of the agar by 72 hr of incubation were excised and transferred to Vogel’s glucose slants.

Isolation of nrc-2 bp mutants: N. crassa strain RLM57 (al-2; aron-9; inv; qa-2; a) was transformed with pT17-1 by electroporation of conidia, using a Bio-Rad Genpulser apparatus. The integration events occurred almost randomly in the vegetative hyphae characteristic of wild-type N. crassa strain 74-OR23-1VA. Ascospores from twelve of these crosses were germinated on sorbose plates supplemented with 5X aromatic amino acids, and inspected for their phenotype.

Nucleotide sequence accession numbers: The nucleotide sequence data for the nrc-1 and nrc-2 genes can be found in the GenBank nucleotide sequence database under accession numbers AF034090 and AF034260.

RESULTS

Isolation and characterization of the nrc-1 and nrc-2 mutants: To isolate mutants affected in the ability to regulate conidia development and subsequently to clone the mutant genes, we employed insertional mutagenesis and plasmid rescue (Arganoza et al. 1994; Kang and Metzenberg 1993). N. crassa is well suited for insertional mutagenesis because transformation occurs largely through the ectopic integration of the transforming DNA into chromosomes (Fincham 1989). These integration events occur almost randomly in the genome, and frequently result in the disruption of genes. The integrated plasmids, along with sequences from the disrupted genes flanking the insertion sites, are recovered by transforming E. coli with chromosomal DNA fragments isolated from the mutants.

The strategy used in isolating mutants affected in regulating conidiation relies on the use of a N. crassa reporter strain, GTH16, which harbors multiple copies of a ccg-1 tyrosinase chimeric reporter gene (Kothé et al. 1993). The chimeric reporter gene consists of the ccg-1 upstream regulatory region, start of transcription site and 5' UTR sequences fused to the tyrosinase coding region and 3' UTR sequences. Tyrosinase is a phenol oxidase and catalyzes the only enzymatic step in the biosynthesis of the black pigment melanin. The ccg-1 regulatory region confers conidiation-specific expression on the downstream tyrosinase gene and causes GTH16 to turn black under conditions that promote conidial development, but not when growing on glucose or sorbose agar media (Kothé et al. 1993). Mutants that are unable to repress the expression of the chimeric gene are readily identified on a sorbose agar medium by visually screening for the presence of melanin.

In addition to having multiple copies of the chimeric gene, GTH16 has mutations in the qa-2 and aron-9 genes. These genes encode dehydroshikimases and the double mutant is an aromatic amino acid auxotroph (Gilles et al. 1985). Insertional mutagenesis was performed using the pRAL-1 plasmid, which contains a functional copy of the qa-2 gene, and thus confers prototrophy (Akins and Lambowitz 1985). The pRAL-1 plasmid was linearized by digestion with BamHI prior to transformation to make it easier to localize the crossover points between the plasmid and the genomic DNA and to decrease the frequency of tandem plasmid insertions. In a screening of 50,000 transformants, 18 consistently dark staining mutants were identified. Two of these mutants, GTH16-T4 and GTH16-T17, were chosen for further analysis because they had unique morphological phenotypes.

GTH16-T4 produces abundant, short conidiophores close to the surface of a Vogel’s glucose agar medium. Although the conidiophore chains are shorter than wild-type conidiophores, they are morphologically normal. Wild-type N. crassa produces conidia in abundance on an air/water interface and only after most of the available glucose has been utilized, which typically occurs 72 to 96 hr postinoculation. The mutant could be classified as a constitutive or non-repressible conidinator, because it produces mature conidiophores within 24 hr of growth on glucose-sufficient agar medium and in glucose-sufficient shaken liquid culture. Based on its phenotypic characteristics, the affected locus of this mutant was named nrc-1. Another striking characteristic of the mutant is that it lacks normal vegetative hyphae. Instead of producing the thick, straight, evenly septated hyphae characteristic of wild-type N. crassa (Figure 1A), the nrc-1 mutant produces thin hyphae that meander, often in a corkscrew-like manner (Figure 1B). These hyphae are indistinguishable from wild-type aerial hyphae, and continuously give rise to conidia. The nrc-1 mutant also exhibits much more invasive growth on solid medium than the wild type, with a higher proportion of its hyphae growing downward beneath the surface of the agar. As a result of this abnormal cell morphology, GTH16-T4 grows in a semicolonial mode, with a radial growth rate of 1.0 mm/hr, compared to the wild-type rate of 6.0 mm/hr.

Microscopic analysis of GTH16-T17 growing on the surface of agar medium or in shaken liquid culture revealed that this mutant also exists as thin, meandering hyphae that closely resemble wild-type aerial hyphae and completely lacks normal vegetative hyphae (Figure 1C). The mutant hyphae contain the minor and major constrictions characteristic of aerial hyphae. Although some of these hyphae generate chains of proconidia, most of the cells remain as aerial hyphae. Within these chains of proconidia that do form, the septa that delineate individual conidia do not fully mature and the conidia remain attached together. Unlike wild-type conidia, which are readily dispersed in air, GTH16-T17 conidia, whether produced on an agar medium or in

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**Figure 1A:** A representative colony of the wild-type N. crassa strain 74-OR23-1VA shows characteristic radial growth and formation of thick conidiophores on the surface of the agar medium. **Figure 1B:** The nrc-1 mutant produces thin, meandering hyphae that resemble wild-type aerial hyphae. **Figure 1C:** GTH16-T17 hyphae have both aerial and conidial morphologies, with septa that are not fully matured, resulting in attached conidia.
submerged culture, have to be wetted and vigorously agitated to be dispersed. Thus, in addition to being unable to repress entry into the conidiation program, GTH16-T17 is unable to complete conidial differentiation. The mutant could be described as having a conidial-separation defect, a designation frequently used for mutants that are unable to complete the conidiation program.

Examination of GTH16-T17 growth and morphology on sorbose agar suggests that the mutant constitutively enters the early stages of the conidiation program. Sorbose is a glucose analog that causes *N. crassa* vegetative hyphae to undergo frequent branching and the fungus to grow in a tight colonial form. Although wild-type *N. crassa* hyphae growing on the surface of sorbose agar medium undergo frequent branching, they have the morphological characteristics of vegetative hyphae (Figure 2). When grown on sorbose agar, GTH16-T17 cells enter into a budding mode of cellular growth and give rise to chains of interconnected buds. These chains of interconnected buds have a striking resemblance to newly formed chains of proconidia (Figure 2). Because GTH16-T17 is unable to repress entry into the conidiation program, the affected locus has been named *nrc-2*.

*nrc-1* is required to repress conidiation on nutrient-sufficient medium and in the dark: To demonstrate that the *nrc-1* mutant is unable to repress conidiation, an assay procedure was developed to examine the production of conidia on an agar medium as a function of nutrient availability and in response to light. As described in materials and methods, the assay involved inoculating the center of petri dishes containing Vogel's/glucose medium supplemented with aromatic amino acids with 74-OR23-IVA (*nrc-1*<sup>1</sup>), RLM57 (*nrc-1*<sup>1</sup>) and GTH16-T4 (*nrc-1*) hyphae. The production of conidia was then followed as a function of time and position on the petri dish. When inoculated on the agar medium, the wild-type hyphae rapidly grew across the surface of the plate until they reached the plate's edge. Then, in response to the depletion of glucose and in the presence of light, the fungus produced an abundance of conidia at the periphery of the dish. The production of conidia by GTH 16-T4 (*nuc-1*) follows a different temporal and spatial pattern. The *nrc-1* mutant rapidly produced conidia in the middle of the agar plate. It produced between 100- and 1000-fold more conidia in the middle of the plate than the wild-type (*nrc-1*<sup>1</sup>) strains, which almost completely repressed conidiophore production until they reached the edge of the plate and entered a state of nutrient deprivation (Table 1). Similar experiments were carried out on cultures maintained in constant darkness. Wild-type strains repressed conidia production under these conditions. In contrast, dark grown cultures of the *nrc-1* mutant were not repressed for conidiation (Table 1). Thus conidiation occurs constitutively in the *nrc-1* mutant and the asexual developmental program is no longer regulated by glucose and light level.

![Figure 1](image_url)

**Figure 1.**—(A) Wild-type *N. crassa* (74-OR23-IVA), 24 hr after inoculation on Vogel's/glucose agar medium, showing thick, regularly septated vegetative hyphae. (B) GTH16-T4 (*nrc-1* mutant) after 24 hr of growth on the same medium, showing thin, meandering hyphae with minor and major constrictions and forming conidiophores. (C) GTH16-T17 (*nrc-2* mutant) 24 hr after inoculation on the same medium, showing thin meandering hyphae with minor and major constrictions. Developing conidiophores are clearly evident in the preparation shown. Bars, 5 mm.
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The nrc-2 mutants were female sterile because they were unable to make protoperithecia, the N. crassa female mating structure. Since these mutants constitutively entered the conidiation program and lacked the vegetative hyphae from which protoperithecia arise, the female sterile phenotype is not surprising. The mutants could, however, participate in a mating when used as the conidial (male) partner. Crosses with wild type in which the nrc-2 mutant was the conidial partner were normal and resulted in the production of morphologically normal perithecia and ascospores. Such crosses were found to produce a 1:1 ratio of nrc-2 and wild-type progeny, suggesting that a single mutant locus was responsible for the nrc-2 phenotype.

Examination of crosses in which a nrc-1 mutant was used as a male (conidial) partner and wild type as the female demonstrated that the nrc-1 mutation affected ascospore development. The perithecia generated from these matings were morphologically normal and gave rise to melanized ascospores. However, after being ejected, half of the ascospores develop a “flattened” appearance (Figure 3). When ascospores from nrc-1 matings were heat activated the “flattened” ascospores failed to germinate. The normal ascospores were viable and gave rise exclusively to wild-type progeny. We deduced that the inviable “flattened” ascospores had the nrc-1 mutation and that when inherited from the male (conidial) parent nrc-1 has an ascospore lethal phenotype.

The question of whether the nrc-1 mutation has an ascospore lethal phenotype when inherited from the protoperithecial (female) parent can be addressed by using a heterokaryon isolate as the female parent. Heterokaryons are isolates in which two different types of haploid nuclei inhabit a common cytoplasm. Heterokaryon formation between GTH16-T4 and RLM57 (a qa-2, arom-9 mutant) produced isolates with wild-type vegetative morphology, indicating that the nrc-1 mutation is recessive in heterokaryons. These heterokaryons produced morphologically normal protoperithecia on crossing medium. Matings in which a heterokaryon was used as the female partner and a wild-type isolate (74-OR23-1VA) served as the male partner were normal in terms of perithecial morphology. The vast majority of the ascospores produced in such crosses were morphologically normal, but a number of ascospores with a “flattened” appearance were produced. All of the single ascospore progeny generated from the viable ascospores produced in these crosses had wild-type morphology, indicating that the mutant ascospores were inviable. Taken together with the results in which nrc-1 was used as the male partner in the mating, this indicates that developing ascospores require a functional copy of the nrc-1 gene. Neither the male nor the female nuclei within the dikaryotic tissue that gives rise to the ascus can provide the gene product to the differentiating ascospores. The nrc-1 mutant can therefore be classified as an autonomous ascospore lethal.

The nrc-1 gene encodes a homolog of the Saccharo-
myces cerevisiae STE11 and Schizosaccharomyces pombe byr-2 gene products: In order to clone the nrc-1 gene, sequences flanking the pRAL-1 insertion site were isolated with a plasmid rescue procedure. Plasmid rescue was carried out by digesting chromosomal DNA from the nrc-1 mutant GTH16-T4 with the restriction endonuclease PstI, ligating the digested DNA, and transforming E. coli strain K802 (Kang and Metzenberg 1993; Orbach et al. 1988). PstI does not cut within the pRAL-1 plasmid sequences, and the plasmids that are subsequently recovered contain N. crassa genomic DNA from the region flanking the insertion site. Thirteen E. coli transformants were recovered from the plasmid rescue experiments. A restriction endonuclease analysis revealed that all thirteen plasmids were identical. One of the rescued plasmids, pT4-1, was selected for further analysis.

The genomic DNA included in pT4-1 was sequenced. The sequencing strategy included using primers designed to anneal to regions of pRAL-1 near the crossover points to sequence from pRAL-1 into the N. crassa DNA. The strategy also involved subcloning fragments from pT4-1 into a Bluescript vector to facilitate sequencing of the DNA, and making oligonucleotide sequencing primers specific to genomic DNA sequences within pT4-1 to use in sequencing reactions. To verify that the sequences in pT4-1 were not rearranged and to obtain further sequence information, additional sequencing was done in cosmids containing the nrc-1 gene. To isolate these cosmids, a radiolabeled probe was generated to a genomic DNA-containing PstI-Sall fragment from pT4-1. This probe was used to screen the pMOCosX cosmids library (Orbach 1994) and five nrc-1-containing cosmids were identified (G8:E5, G11:G2, G15:C5, G16:C2 and X15:A5). Sequencing of part of one cosmid (G15:C5) showed that 3 bp of genomic DNA had been deleted from the site at which the insertion occurred in pT4-1. The sequence of the genomic DNA showing the site of pRAL-1 insertion is given in Figure 4.

Sequencing of the pT4-1 and cosmid DNAs revealed the presence of an uninterrupted open reading frame encoding a 666-amino-acid protein with a molecular weight of 74 kD (Figure 4). The open reading frame starts with an AUG in a sequence context suggesting it is a N. crassa start-of-translation site (Edelmann and Staben 1994). Database searches revealed that the predicted protein is a homolog of the S. cerevisiae STE11 and S. pombe byr2 gene products, both of which are MAPKK kinases (Rhodes et al. 1990; Wang et al. 1991). An alignment of these proteins is shown in Figure 5. Sequence comparisons using the UWCGC BESTFIT (University of Wisconsin Genetics Computer Group,
Figure 4.—Nucleotide and encoded amino acid sequences from the nrc-1 gene. The nucleotide sequence and the encoded amino acid sequence from the nrc-1 gene are given. The underlined nucleotides indicate the insertion point of the pRAL-1 plasmid in GTH16-T4. Comparison of the sequence of the disrupted gene in pT4-1 with that of the undisrupted gene in cosmid G15-C5 indicated that these 3 bp had been deleted during the insertion event. The nucleotide numbers are given in the right margin and the amino acid numbers in NRC-1 are given in the left margin.
Isolation of the nrc-2 gene by plasmid rescue: Plasmid rescue from the nrc-2 mutant was accomplished by digesting chromosomal DNA from GTH16-T17 with PstI, ligating the digested DNA, and then transforming E. coli strain K802 (Kang and Metzenberg 1993; Orbach et al. 1988). Plasmid DNA was prepared from STE11 and byr2 gene products, respectively. Conservation is highest within the carboxyl terminus, which contains the protein kinase catalytic site (Rhodes et al. 1990). Sequence comparisons of the 275 amino acids at the C terminus of the N. crassa kinase revealed 59% and 58% sequence identity to the Ste11p and byr2 proteins. The nrc-1 mutant allele present in GTH16-T4 is disrupted by the pRAL-1 plasmid sequences near the beginning of the highly conserved carboxyl terminal region and would be expected to produce a truncated protein lacking protein kinase function.
over point showed similarity to a variety of protein kinase genes. Cosmids containing the nrc-2 gene were identified by using a radioactively-labeled PstI-Sall fragment from pT17-1 to screen the pMOcosX N. crassa cosmid library (Orbach 1994). Two cosmids, X7:E9 and X9:E6, were identified as containing the genomic copy of the nrc-2 gene. The X9:E6 cosmid was used to obtain a complete sequence of the gene. This revealed a putative coding region that has three introns and that encodes a 623-amino-acid protein with a molecular weight of 68 kD (Figure 6). The start-of-translation and the intron boundaries were identified by the presence of sequence over point showed similarity to a variety of protein kinase genes. Cosmids containing the nrc-2 gene were identified by using a radioactively-labeled PstI-Sall fragment from pT17-1 to screen the pMOcosX N. crassa cosmid library (Orbach 1994). Two cosmids, X7:E9 and X9:E6, were identified as containing the genomic copy of the nrc-2 gene. The X9:E6 cosmid was used to obtain a complete sequence of the gene. This revealed a putative coding region that has three introns and that encodes a 623-amino-acid protein with a molecular weight of 68 kD (Figure 6). The start-of-translation and the intron boundaries were identified by the presence of sequence
elements that are conserved among N. crassa genes (Edelmann and Staben 1994). As shown in Figure 6, 17 bp of nrc-2 sequence were deleted as pRAL-1 was inserted into the genome. Database searches revealed that this gene is closely related to the S. pombe kad5 gene, and the KIN82 and YNR047w genes of S. cerevisiae. KIN82, YNR047w and kad5 all encode putative serine-threonine protein kinases. These putative serine-threonine kinases were identified during genome sequencing projects as ORFs with extensive sequence homology to serine-threonine protein kinases. These putative kinases are most closely related to CAM+ dependent protein kinases. A sequence alignment of the encoded amino acid sequences from nrc-2 and these genes is shown in Figure 7. Sequence comparisons using the UWCGC BESTFIT computer program revealed that the predicted nrc-2 protein product has a 390-amino-acid carboxyl terminus with 71%, 61%, and 59% sequence identity to the predicted carboxyl termini of the kad5, Ynr047wp, and Kin82p proteins, respectively. This conserved carboxyl terminus contains the protein kinase catalytic domain. The mutant nrc-2 allele found in GTH16-T17 is disrupted by the insertion of the pRAL-1 plasmid in the middle of this highly conserved carboxyl terminal region and would be expected to lack kinase function. The region upstream of the sequence shown in Figure 6 has some additional putative start-of-translation sites and introns, which, if used in vivo, would encode a larger protein product. However, these putative upstream coding regions do not show strong amino acid sequence homology to other coding regions.

Hanks et al. (1988) identified eleven conserved subdomains within the catalytic region of protein kinases. All eleven of these conserved subdomains are present in the nrc-2 protein product. All of the invariant amino acids identified by Hanks et al. (1988) exist in the predicted NRC-2 kinase, with the exception of a single substitution in subdomain VII. Within this subdomain the sequence L/IXDFG is normally found, with the D and G residues being invariant. In the predicted NRC-2 protein product, the sequence LSDFD is found. This LSDFD sequence is also found in the homologous regions of the putative kad5 and YNR047w protein products.

**Demonstrating that the disruptions in the nrc-1 and nrc-2 genes are responsible for the mutant phenotypes:** Southern blot analysis revealed that the nrc-2 mutant, GTH 16-T17, had a single pRAL-1 plasmid inserted into its genome. To demonstrate that the inserted plasmid was responsible for the nonrepressible conidiation/ lack of vegetative hyphae phenotype, GTH 16-T17 was mated with 74-OR23-1VA, a wild-type N. crassa isolate. The segregation of the pRAL-1 plasmid sequences as well as the segregation of the mutant phenotype was then followed in single ascospore progeny. A Southern blot analysis was carried out on twenty single ascospore progeny and the pRAL-1 sequences were observed to cosegregate with the mutant phenotype in all cases (data not shown). This demonstrates that the pRAL-1 disruption of the nrc-2 gene, or a closely linked mutation, is responsible for the mutant phenotype.

In order to definitely demonstrate that the disruption of the nrc-2 gene by the pRAL-1 plasmid was responsible for the mutant phenotype, the RIP (repeat-induced point mutation) phenomenon was used to generate null mutants in the nrc-2 gene. This phenomenon is associated with the N. crassa mating process (Selker 1990). During the premeiotic phase of mating, the fungus scans the genomic DNA of the male and female pronuclei and introduces multiple mutations in both copies of duplicated DNA sequences that are greater than 1 kb in length. The mutations are exclusively G/C to A/T transitions. Because the RIP process introduces a large number of mutations within the duplicated DNAs, it can be used to generate null mutations in a cloned gene (Selker et al. 1989).

The pT17-1 plasmid has 1,227 bp of uninterrupted nrc-2 sequence. To generate isolates with nrc-2 sequence duplications, pT17-1 was used to transform the aromatic acid auxotroph RLM57. It was possible to select directly for transformants because pT17-1 has a functional copy of the qa-2 gene, which confers prototrophy. The transformants had the endogenous copy of the nrc-2 gene, as well as the nrc-2 sequences contained in pT17-1. To generate the RIP-induced mutations in the nrc-2 gene, twelve transformants were crossed as females with the wild-type strain 74-OR23-1VA. Single ascospore progeny from these crosses were isolated and characterized. Progeny exhibiting the nrc-2 mutant phenotype were isolated from seven of the twelve crosses. These nrc-2 mutant progeny were morphologically indistinguishable from GTH16-T17 (Figure 3). These results definitively demonstrate that the disrupted nrc-2 gene identified in pT17-1 is responsible for the mutant phenotype.

Since the nrc-1 mutant has an autonomous ascospore lethal phenotype, it is not possible to recover mutant progeny from a genetic cross between GTH 16-T4 and a wild-type isolate. Thus, it is not possible to demonstrate by Southern blot analysis that the pRAL-1 disrupted copy of the nrc-1 gene cosegregates with the mutant phenotype. However, examination of the viable wild-type progeny produced by such a mating showed that all of the wild-type progeny had the normal, nondisrupted copy of the nrc-1 gene (data not shown). This strongly implies that the disrupted nrc-1 allele is segregating with the ascospore lethal/ lack of vegetative hyphae/ constitutive conidiation phenotype and that the phenotype is due to the disrupted nrc-1 gene, or to a gene closely linked to it.

The ascospore lethal phenotype also precludes using the RIP phenomenon to determine if the mutant phenotype is due to the disruption in the nrc-1 gene. Thus, the ability of a wild-type copy of the nrc-1 gene to complement the mutant phenotype was used to demonstrate
Figure 7.—A comparison of the amino acid sequences encoded by the N. crassa nrc-2, the S. pombe kad5 and the S. cerevisiae YN-047w and KIN82 genes. The encoded amino acid sequences were aligned using the UWCGC program PILE-UP. The amino acid sequences shown extend through the entire amino acid coding regions of NRC-2 and kad5. The numbers on the right refer to the amino acid numbers in the proteins. An amino terminal region of 198 amino acids and a carboxyl terminal region of 15 amino acids are not shown for the Ynr-047wp protein. Similarly, an amino terminal region of 150 amino acids and a carboxyl terminal 18-amino acid sequence are not included for the Kin82p protein.

that the nrc-1 gene functions to control entry into the conidiation program. A cotransformation experiment was carried out in which GTH16-T4 conidia were simultaneously transformed with a cosmid having an intact copy of the nrc-1 gene and the pBARKS1 plasmid, which confers resistance to the antibiotic BASTA (Pall and Brunelli 1993). Of fifty BASTA-resistant transformants, five were found to have reverted to a wild-type phenotype. Southern blot analysis revealed that the revertants had obtained an intact copy of the nrc-1 gene from the cosmid. Analysis of nonrevertant transformants showed that they contained only the disrupted gene. This provides clear evidence that the mutant phenotype is due to the disruption of the nrc-1 gene.

DISCUSSION

The tyrosinase-based mutant isolation system: N. crassa mutants affected in the ability to regulate entry into the conidiation program were isolated using a tyrosinase-based screening procedure. A chimeric gene was prepared by fusing the ccg-1 promoter and upstream DNA regulatory elements to the N. crassa tyrosinase reporter gene (Kothe et al. 1993). The ccg-1 sequences

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provided a means for the conidiation-specific regulation of the tyrosinase gene. Because tyrosinase is the only enzymatic step in the synthesis of the black pigment melanin, mutant cells expressing the chimeric gene were readily identified.

In addition to the tyrosinase-based screening procedure, two other N. crassa chimeric gene-based mutant isolation procedures have been developed. Madi et al. (1994) used chimeric genes containing the con-10 and con-6 gene sequences in translational fusions with the hygromycin resistance gene to isolate mutants affected in regulating the expression of conidiation-specific genes. These chimeric genes provided a means for the conidiation-specific expression of the hygromycin resistance gene. Mutants were isolated by selecting for cells having resistance to hygromycin under conditions that normally repress expression of the conidiation program. Using this procedure, mutants affected in the rco-1 and rco-3 genes were identified and characterized. The rco-1 gene encodes a homolog of the yeast TUP1p transcription factor involved in regulating glucose-repressible genes (Yamashiro et al. 1996). The rco-3 gene codes for a glucose transporter (Madi et al. 1997). The isolation of these genes highlights the glucose-repressible nature of the N. crassa conidiation program.

Carattoli et al. (1995) used a chimeric gene containing the light-regulated al-3 promoter fused to the N. crassa mtr gene. The mtr gene encodes a neutral amino acid permease that, if expressed, makes the cell sensitive to the toxic amino acid analog p-fluorophenylalanine. When a mtr mutant cell is transformed with the chimeric gene, the resultant transformant was resistant to p-fluorophenylalanine in the dark but sensitive to the toxic amino acid analog in the presence of light. Mutants affected in the ability to regulate light-dependent gene expression were isolated by selecting for cells that were resistant to p-fluorophenylalanine in the light (Carattoli et al. 1995).

The tyrosinase-based procedure differs from the hygromycin and mtr-based systems in that it is a screening procedure and not a selection procedure. The selective procedures allow an investigator to look at a greater number of mutagenized cells than could be done with a screening procedure. However, because of their slow growth rates, the rnc-1 and rnc-2 mutants could be easily missed in a selection regimen. The screening procedure has the advantage of providing the investigator with an estimate of the levels of chimeric gene expression.

A second important difference between the mutant isolation procedure described herein and the chimeric gene-based procedures described by Madi et al. (1994) and Carattoli et al. (1995) is the use of insertional mutagenesis to tag the mutant genes. Because the rnc-1 and rnc-2 genes had been tagged by the inserted pRAL-1 plasmid, the genes were readily isolated with the plasmid rescue procedure.

**The rnc-1 gene and its characterization:** MAP kinase cascades have been implicated in controlling cellular growth and developmental processes in a variety of eukaryotic organisms. These protein kinase cascades are typically initiated by activation of cell surface receptors. They function by having an upstream activation event that leads to the phosphorylation of a MAPKK kinase. This activates the MAPKK kinase, which then phosphorylates a MAP kinase. This phosphorylation activates the MAP kinase, which then phosphorylates a MAP kinase on a tyrosine and a closely neighboring threonine to activate it. The activated MAP kinase then goes on to phosphorylate various nuclear, membrane-associated, cytosolic, and cytoskeletal target proteins (for reviews see Cobbs and Goldsmith 1995; Seger and Krebs 1995; Blenis 1993).

We have isolated the N. crassa rnc-1 gene and found it to encode a homolog of the S. cerevisiae STE11, and S. pombe byr2 gene products, both of which function as MAPKK kinases (Rhodes et al. 1990; Wang et al. 1991). The closely related Ste11p and byr2 proteins function in yeast pheromone-responsive MAP kinase signal transduction pathways to regulate the yeast mating process (reviewed by Kurjan 1993). The research reported herein demonstrates that the rnc-1 gene is necessary to repress asexual development in N. crassa. It may do so as a component of a MAP kinase cascade that functions to promote vegetative growth (hyphal extension), while repressing the conidiation program (a budding phase of the life cycle).

The MAP kinase cascade that functions in the pheromone response pathway in the yeast S. cerevisiae has been well characterized. The cascade includes a MAPKK kinase (Ste11p), a MAP kinase (Ste7p), and two closely related MAP kinases (Fus3p and Kss1p) (Nakayama et al. 1988; Hartwell 1980; Elion et al. 1990). Null mutants in STE11, STE7, or both Fus3 and Kss1 are sterile. Unlike wild-type S. cerevisiae, these mutants fail to respond to mating pheromone by terminating budding growth, arresting in the G1 phase of the cell cycle and undergoing morphological changes that culminate in making an elongated cell (a shmoo). The byr2 gene of the fission yeast S. pombe is structurally and functionally homologous to STE11 and is necessary for sexual conjugation in this organism (Wang et al. 1991).

The finding that the N. crassa rnc-1 gene encodes a homolog of the STE11 and byr2 MAPKK kinases is interesting from the point of view of cellular morphology. The STE11 gene, along with other components of the pheromone response pathway, has also been shown to be necessary for pseudohyphal development in diploid cells (Gimeno et al. 1992; Liu et al. 1993). When diploid S. cerevisiae strains are starved for nitrogen, their cells become elongated, and change from a bipolar to a unipolar budding pattern. This pattern of cellular extension, referred to as pseudohyphal growth, results in formation of a chain of elongated cells. Components of the pheromone response pathway, including Ste11p,
are also required for a similar growth pattern called filamentous fungi and, in N. crassa, it is regulated by a MAPKK kinase closely related to the yeast MAPKK kinases involved in directing yeast pseudohyphal growth and filament formation. Interestingly, activation of the MAP kinase pathway in yeast is involved in the process of sexual differentiation, while activation of the pathway in a filamentous fungus leads to vegetative growth. Thus, it would appear that during evolution the yeast and filamentous fungi have diverged, not in terms of how they control cellular morphology, but rather in terms of how cellular morphology is utilized to define different developmental alternatives.

N. crassa cr-1 (cr-1) mutants share some of the phenotypic characteristics of the nrc-1 mutants. Both types of mutants are constitutive conidiators that produce short conidial chains. The cr-1 gene has been cloned and shown to encode an adenylate cyclase (Kor-Eda et al. 1991). The cr-1 mutant phenotype indicates that in the absence of intracellular cAMP N. crassa alters its cell morphology and enters the conidiation program. The importance of cAMP in regulating N. crassa cellular morphology is further illustrated by the mcb mutant, which has a temperature-sensitive cAMP-dependent protein kinase regulatory subunit (Bruno et al. 1996). At the nonpermissive temperature, the mcb mutant is unable to undergo polarized cell growth to form filamentous hyphae. Furthermore, drugs that affect intracellular cAMP levels have been shown to affect N. crassa morphology (Scott and Solomon 1975). The similarities between the nrc-1 and cr-1 mutant phenotypes raise the possibility that adenylate cyclase and the NRC-1 MAP kinase pathway function in the same signal transduction pathway.

The nrc-1 mutant is unable to produce protoperithecia and is therefore female sterile. However, because of the constitutive conidiation phenotype, it is difficult to assess whether the nrc-1 gene plays a role in the N. crassa sexual developmental program. The inability of the nrc-1 mutant to enter the sexual developmental program might simply be a result of the mutant being unable to exit from the asexual developmental program. Alternatively, the nrc-1 gene might be required for sexual differentiation.

The results reported herein show that the nrc-1 gene is necessary for ascospore development in N. crassa. The nrc-1 mutant has an autonomous ascospore lethal phenotype. Mutant ascospores have a “flattened” morphology and are not viable. Thus, in addition to its role in directing asexual development, the NRC-1 MAPKK kinase plays a role in directing the terminal steps in ascospore differentiation.

The nrc-2 gene and its characterization: Like the nrc-1 mutant, nrc-2 mutants are unable to repress entry into the conidiation program. However, the nrc-2 mutants do not complete conidiation and could be classed as having a conidial-separation defect. Thus, the nrc-2 gene is required at two points in the conidiation program, to regulate entry into the program and to complete asexual differentiation.

The sequence of the nrc-2 gene clearly identifies the predicted gene product as being a serine/threonine kinase. The high level of amino acid sequence identity with the predicted S. pombe Kad5 gene product and the predicted products of the S. cerevisiae Kin82 and YN047w genes suggests these proteins form a closely related group of kinases. The S. pombe and S. cerevisiae genes were identified as part of genome sequencing projects, so the nrc-2 gene is the first member of this group to have an identifiable function. Interestingly, these kinases are closely related to cAMP-dependent protein kinases. The role of the nrc-2 gene in repressing the N. crassa conidiation program suggests that members of this group of kinases may play important roles in regulating cellular functions.

It is unclear whether the NRC-2 kinase functions in the same signal transduction pathway as the NRC-1 MAPKK kinase. Both genes were isolated in a screening procedure designed to isolate mutants that had lost the ability to repress the expression of ccg-1, and mutants affected in the two genes are unable to regulate the entry into the conidiation program. The data would be consistent with two kinases being part of a single signal transduction pathway. However, the data do not preclude the possibility that the nrc-1 and nrc-2 gene products function in two different pathways, both of which would be simultaneously required in order to repress conidiation and the transcription of the ccg-1 gene.

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LITERATURE CITED


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