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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[1] and Stephen J. Free

Department of Biological Sciences, 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 byr2* 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 YNRO47w* 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.

UNDER 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

Corresponding author: Stephen J. Free, Department of Biological Science, 370 Cooke Hall, SUNY/Buffalo, Buffalo, NY 14260-1300. E-mail: free@acsu.buffalo.edu

[1] *Present address:* Institute of Molecular Biology, University of Oregon, Eugene, OR 97403-1229.

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 GTH16 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). GTH16, 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 conidia production: To assess the production of conidia, GTH16-T4, RLM57 and 74-OR23-1VA hyphae were inoculated in 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° in the dark. The plates were then placed under constant illumination at 25° for an additional 24 hr prior to assessing conidia 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 H_2O. 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 *Pst*I, which does not cut within the pRAL-1 plasmid. Following the 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 endonuclease 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 Genpulser apparatus (Bio-Rad Laboratories, Hercules, CA) (Vann 1995). 110 μl of GTH16-T4 conidia were mixed with 2 μl of 1 μg/μl pBARKS1 (Pall and Brunelli 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°. The bottles were swirled to distribute the cells, and then 10-ml portions of the top agar were overlaid onto individual Vogel's (ammo-

nium-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 at 30°. 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^RIP* mutants: *N. crassa* strain RLM57 (*al-2*; *arom-9*; *inv*; *qa-2*; *a*) was transformed with pT17-1 by electroporation of conidia, using a Bio-Rad Genpulser apparatus. The transformation was carried out as described above except that Vogel's minimal medium was used to select transformants. Colonies that had reached the surface of the agar by 72 hr of incubation were excised and transferred to individual cornmeal agar plates, where they formed protoperithecia. The transformant protoperithecia were fertilized with conidia from *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 (Kothe *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 (Kothe *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 *arom-9* genes. These genes encode dehydroshikimases and the double mutant is an aromatic amino acid auxotroph (Giles *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 *Bam*HI 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 only 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 conidiator, 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 vegetative 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 conidiophores. 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 those 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

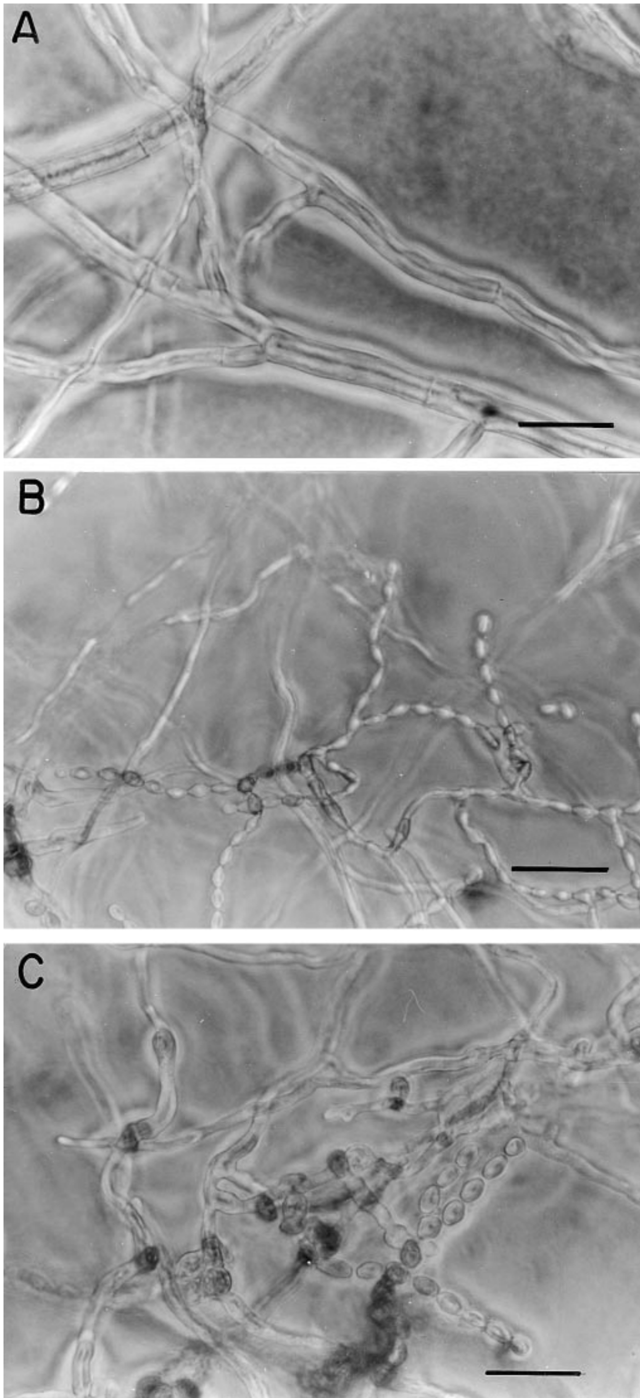

Figure 1.—(A) Wild-type *N. crassa* (74-OR23-1VA), 24 hr after inoculation on Vogel's/glucose agar medium, showing thick, regularly septated vegetative hyphae. (B) GTH16-T4 (*nrc-1* mutant) after 24 hr of growth on the same medium, showing thin, meandering hyphae with minor and major constrictions and 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.

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*$^+$), RLM57 (*nrc-1*$^+$) 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 GTH16-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*+) 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.

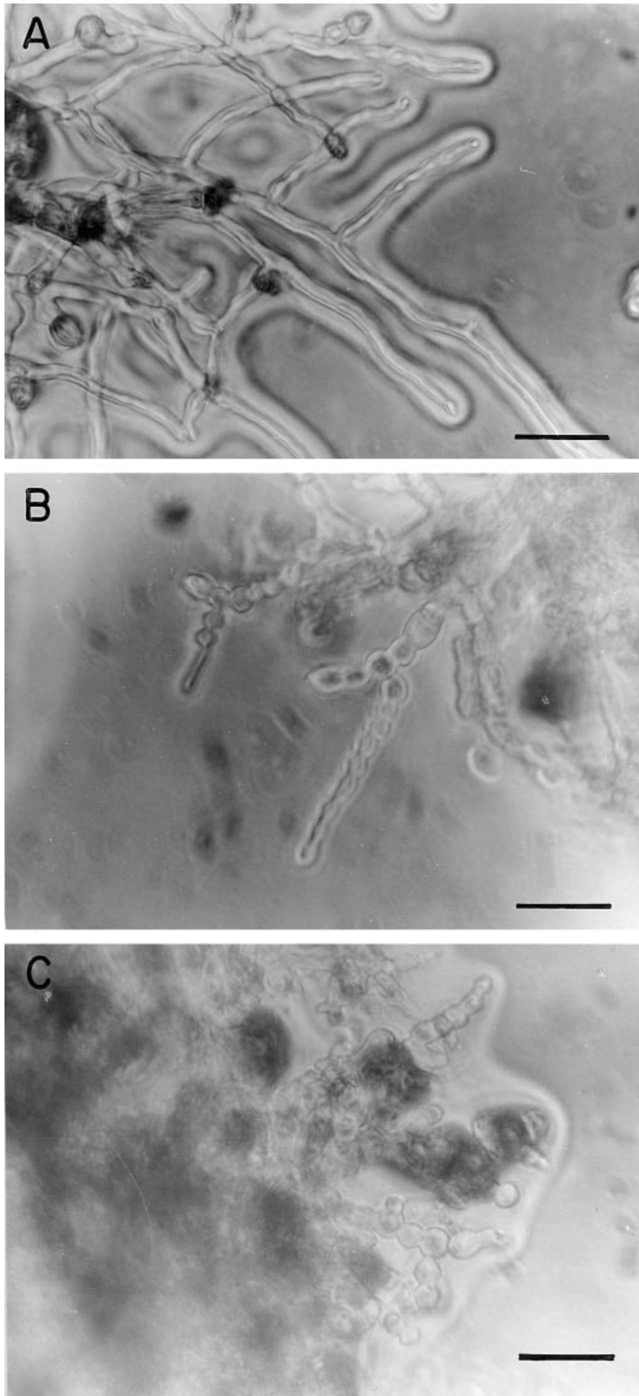

Figure 2.—(A) Wild-type *N. crassa* (74-OR23-1VA) 48 hr after inoculation on sorbose minimal medium, showing an increased frequency of branching from vegetative hyphae. (B) GTH16-T17 (*nrc-2* mutant) 48 hr after inoculation on the same medium, showing the production of conidiophores. (C) RIP-generated mutant (*nrc-2rip-18-6*) 48 hr after inoculation on the same medium, showing the conidiophore phenotype seen in the originally isolated GTH16-T17. Bars, 5 mm.

Sexual development in *nrc-1* and *nrc-2* mutants: To ascertain whether the *nrc-1* and *nrc-2* genes are required for sexual differentiation, we made matings between mutant and wild-type isolates. The *nrc-1* and *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-*

TABLE 1

GTH16-T4 (*nrc-1* mutant) constitutively produces conidia

Strain (genotype)	Light or dark	Number of conidia in the center of the petri dish (conidia × 10^6/dish)	Number of conidia at the periphery of the petri dish (conidia × 10^6/dish)
GTH16-T4 (*nrc-1*)	Light	234	NA[a]
RLM57 (*nrc-1*$^+$)	Light	0	406
74-OR23-IVA (*nrc-1*$^+$)	Light	2	620
GTH16-T4 (*nrc-1*)	Dark	328	NA
RLM57 (*nrc-1*$^+$)	Dark	1	14
74-OR23-IVA (*nrc-1*$^+$)	Dark	2	5

The production of conidia in the center of a petri plate and at the periphery of the plate was used to assess whether GTH16-T4 and wild-type strains regulated conidia production in response to the availability of glucose and illumination (see text and materials and methods). The number of conidia produced per petri dish is given. The results shown are the average of three separate trials.

[a] Since GTH16-T4 has a semicolonial growth pattern and doesn't reach the periphery of the plate, a conidia count from the edge of the plate was not applicable (NA).

***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 *Pst*I, ligating the digested DNA, and transforming *E. coli* strain K802 (Kang and Metzenberg 1993; Orbach *et al.* 1988). *Pst*I 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.

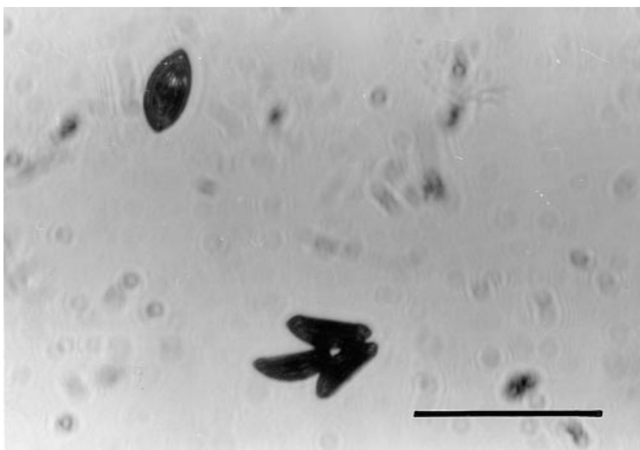

Figure 3.—Ascospores derived from a cross of GTH16-T4 (*nrc-1* mutant) as the male parent with 74-OR23-1VA (wild-type strain) as the female parent. A normal ascospore is seen in the upper left corner, and three ascospores with a "flattened" appearance are seen clustered together in the bottom of the photograph. Bar, 10 mm.

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 *Pst*I-*Sal*I fragment from pT4-1. This probe was used to screen the pMOcosX cosmid 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,

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     CTTCGCGTATGACCGCCAGTACGACTCACCCCTGCGACATGTCGAACTTCTTCGCGGCGGACTTCGCCGTCACTTAGTTCTGACTACAAC   90

     AGACAAGCCCGGCGAGGCTATGTGCAACAACAGGGCTATGGCAATCAACCTACACAAGCTGCTCCCTCGCGGTTCCCCATGTCTCCTCCT   180

     GAGTCCCACCCCGGCCGATTGGTACAAGCCCACACTAGGAACAACTCCAGCATGGATGGTTCGCTGATGGCTGCGCTGCCACAGGGTCAG   270
  1                                                    MetAspGlySerLeuMetAlaAlaLeuProGlnGlyGln

     GATGTCATTCGCGTCATCTCCACAGGTGGCGTAACCAAGGTTGTTAAGATTGCCGAGTGCAACACCTGTGAAGAGGTTATGCGTGTTACC   360
 14  AspValIleArgValIleSerThrGlyGlyValThrLysValValLysIleAlaGluCysAsnThrCysGluGluValMetArgValThr

     CTACGTAAGTTTGGCCTGCGGGAGGACCATGAAAGGAATTACTGTTTCTGGGTACTGGCCGGCGTTGATCCGGACCCAAACCAATGCCGC   450
 44  LeuArgLysPheGlyLeuArgGluAspHisGluArgAsnTyrCysPheTrpValLeuAlaGlyValAspProAspProAsnGlnCysArg

     CGCCTCGGGGATACGGAGCTGTGGAGAGTCATCAAGGACCACACTCGCCCAGAGCGCAACCGCCTGATTCTCAGGCGTGTTCCTTCTGGA   540
 74  ArgLeuGlyAspThrGluLeuTrpArgValIleLysAspHisThrArgProGluArgAsnArgLeuIleLeuArgArgValProSerGly

     GAGCCGGGCAAGGCAGAACTCGAAAGAGCAGCTGCCATTGCGATGGAGGAAGCACAGCAAACACATAGGCCTCCAATTGAGCCCAGCGAT   630
104  GluProGlyLysAlaGluLeuGluArgAlaAlaAlaIleAlaMetGluGluAlaGlnGlnThrHisArgProProIleGluProSerAsp

     AAGCGGAGTCAGCTGAAGCTGCAAAAGGTGCTGGGAGTGGGCTGGGAAGACCTCCAACAACAGCCTCCGCTGTCTCCCATGTCCTATCAG   720
134  LysArgSerGlnLeuLysLeuGlnLysValLeuGlyValGlyTrpGluAspLeuGlnGlnGlnProProLeuSerProMetSerTyrGln

     GACCGAGAAAGAAACGTCTCAAACGCTGCTAGGGATCTAGAGCGGCCAGCACCTCTGGAAACCCCCAGGGCCATGCCTCGCCGTACACAA   810
164  AspArgGluArgAsnValSerAsnAlaAlaArgAspLeuGluArgProAlaProLeuGluThrProArgAlaMetProArgArgThrGln

     GCACTTCGTCAGTTCGGTGGGCTGAGACCGCCTAGCGAGCTTATCGCCTCGGATCTTACGAGCTACTTTCCAGATCACTCTCGTGAAGCT   900
194  AlaLeuArgGlnPheGlyGlyLeuArgProProSerGluLeuIleAlaSerAspLeuThrSerTyrPheProAspHisSerArgGluAla

     ATTGACAGGACGGCCCGTTTGTCCATGAGACGCTCGGCACGTCTGAGTAGAGTGAACCATCGTCTGAGTGTTGCCAGCACCCTTAGCTTT   990
224  IleAspArgThrAlaArgLeuSerMetArgArgSerAlaArgLeuSerArgValAsnHisArgLeuSerValAlaSerThrLeuSerPhe

     GCCTCTAGCATACAAGACGCTCCGCCGATCCCTACCATCGCAGACAGCTGGTTGACCGCTTCCAACCAGATTGCCAAGGTACGCCCGCGT   1080
254  AlaSerSerIleGlnAspAlaProProIleProThrIleAlaAspSerTrpLeuThrAlaSerAsnGlnIleAlaLysValArgProArg

     GATGTGCTGCCAAGGGCGCCTCACGGGTATAGAGATTCTGTGGCTTCATCCGTGCTTGATACGCTACAAGAAGAAGGCTCCCCAACCGAG   1170
284  AspValLeuProArgAlaProHisGlyTyrArgAspSerValAlaSerSerValLeuAspThrLeuGlnGluGluGlySerProThrGlu

     CCTAACCGGAGATCATTCGTTCCATTTTCGGACAGCGGCTCGGATACAGCTGCAGTTAGCGTCATTGATCCCGATGGTAACATAGTCAGA   1260
314  ProAsnArgArgSerPheValProPheSerAspSerGlySerAspThrAlaAlaValSerValIleAspProAspGlyAsnIleValArg

     CACAGTTACTACGACAGCGGCACCAACAACTCGGCCGATTCCGCGGTAATACAGGAAGCACTTGCTGAAGATGGCGAGGATGCAGCTGAC   1350
344  HisSerTyrTyrAspSerGlyThrAsnAsnSerAlaAspSerAlaValIleGlnGluAlaLeuAlaGluAspGlyGluAspAlaAlaAsp

     AAGGAGCTTCAAACTTTTCTGGCGGGCGACGCTTGGGACGACAGTATGTGGATGAAGGGTTCTCTTATCGGCCAGGGTTCATTTGGCTCC   1440
374  LysGluLeuGlnThrPheLeuAlaGlyAspAlaTrpAspAspSerMetTrpMetLysGlySerLeuIleGlyGlnGlySerPheGlySer

     GTGTACCTCGCCCTACATGCCATCACTGGTGAACTGCTCGCCGTGAAGCAGGTCGAGACACCTGCGCCTGGTGCAGACAGTAAGAACGAT   1530
404  ValTyrLeuAlaLeuHisAlaIleThrGlyGluLeuLeuAlaValLysGlnValGluThrProAlaProGlyAlaAspSerLysAsnAsp

     GCCCGCAAGAAGAGCATGATTGAAGCGCTCAAGCGCGAGATCACCCTCCTTCGTGACCTCCAACATCCCAATATCGTGCAGTACCTGGGC   1620
434  AlaArgLysLysSerMetIleGluAlaLeuLysArgGluIleThrLeuLeuArgAspLeuGlnHisProAsnIleValGlnTyrLeuGly

     TGCAGCTCATCCGCGGAATATCTCAACATTTTCCTCGAATACGTTCCCGGTGGTTCCGTGCAGACCATGCTCGACCAATACGGTGCCCTC   1710
464  CysSerSerSerAlaGluTyrLeuAsnIlePheLeuGluTyrValProGlyGlySerValGlnThrMetLeuAspGlnTyrGlyAlaLeu

     CCTGAATCACTCGTTCGCAGCTTCGTTCGCCAAATCCTCCAGGGCCTCTCCTACGTCCACAACCGTGACATCATTCACCGCGACATCAAG   1800
494  ProGluSerLeuValArgSerPheValArgGlnIleLeuGlnGlyLeuSerTyrValHisAsnArgAspIleIleHisArgAspIleLys

     GGCGCCAACATCCTTGTCGACAACAAAGGTACCATCAAAATCTCCGATTTCGGCATCTCCAAGAAACTCGAAGCCACCAACATCCTCAAC   1890
524  GlyAlaAsnIleLeuValAspAsnLysGlyThrIleLysIleSerAspPheGlyIleSerLysLysLeuGluAlaThrAsnIleLeuAsn

     GGCGCCAACAACAACAAGCACCGTCCCTCACTGCAAGGCTCCGTCTTCTGGATGGCTCCTGAGGTAGTCAAACAAACCAGTTACACCCGC   1980
554  GlyAlaAsnAsnAsnLysHisArgProSerLeuGlnGlySerValPheTrpMetAlaProGluValValLysGlnThrSerTyrThrArg

     AAAGCCGACATCTGGTCACTCGGCTGCCTGGTGGTCGAGATGATGACGGGTACCCACCCGTTTCCCGATTGCACCCAGTTGCAGGCCATC   2070
584  LysAlaAspIleTrpSerLeuGlyCysLeuValValGluMetMetThrGlyThrHisProPheProAspCysThrGlnLeuGlnAlaIle

     TTCAAGATTGGCGGCTCCAAGGCCTCGCCGACGATCCCGGATAACGCTAGCGAGGAGGCTAAGCAGTTCCTTGCGCAGACATTCGAGATT   2160
614  PheLysIleGlyGlySerLysAlaSerProThrIleProAspAsnAlaSerGluGluAlaLysGlnPheLeuAlaGlnThrPheGluIle

     GATCATAATAAGCGGCCGAGCGCAGATGAGCTGATGTTGAGCCCGTTCTTGACGCCTGTGCCCGGGACATAGGAGTCTTCCAATAAAGTG   2250
644  AspHisAsnLysArgProSerAlaAspGluLeuMetLeuSerProPheLeuThrProValProGlyThrEnd

     TGAGAGTTTTCGTATGTCAGAAATTGACATAAGAGGAGGATTTAATCAGGGCTTGAATTGTAGTGACAAAAAACATGTGTATGTGTTGCT   2340

     ACTCATCTGTGAAGTTTGTCTGTCTGTCACGCTGTCTGTTTACGCTG  2387
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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.

```
NRC-1   MDGSLMAALPQGQDVIRVISTGGVTKVVKIAEC...NTCEEVMRVTLRKFGLREDHERNY   57
byr2    .........FPRPCILRFIACNGQTRAVQSRGDY.....QKTLAIALKKFSLEDASK...  109
Ste11p  ATLSMNSELIPEKHCVIFILNDGSAKKVNVNGCFNADSIKKRLIRRLPHELLATNSNGEV  165

NRC-1   CF....WVLAGVDPDPNQCRRLGDTELWRVIKDHTRPERNRLILRRVPSGEPGKAELERA  113
byr2    ......FIVCVSQSSR..IKLITEEEFKQICFNSSSPERDRLIIVPKEKPCPSFEDLRRS  161
Ste11p  TKMVQDYDVFVLDYTKNVLHLLYDVELVTICHANDRVEKNRLIFVSKDQT.PSDKAISTS  224

NRC-1   AAIAMEEAQQTHRPPIEPSKDRSQLKLQKVLGVGWEDLQQQPPLSPMSYQDRERNVS.NA  172
byr2    WEIELAQPA..............ALSSQSSLSPKLSSV........LPTSTQKRSVRSNN  199
Ste11p  KKLYL...R..............TLSALSQVGPSSSNL........LA...QNKGISHNN  256

NRC-1   ARDLERPARLETPRAMPRRTQALRQFGGLRPPSELIASDLTSYFPDHSREAIDRTARLSM  232
byr2    AKPFESY.....................QRPPSELINSRISDFFPDHQPKLLEKTISNSL  238
Ste11p  AEGKLRIDNTEKDR........IRQIFNQRPPSEFISTNLAGYFPHTDMKRLQKTMRESF  308

NRC-1   RRSARLSRVNHRLSVASTLSFASSIQDAPPIPTIADSWLTASNQIAKVRPRDV..LPRAP  290
byr2    RRNLSIRTSQGHNLG....NFGQEILPRSSRRARPSELVCPLSSLRISVAEDVNRLPRID  294
Ste11p  RHSARLSIAQRRPLSAESNNIGDILLKHSN..AVDMALLQGLDQTRLSSKLDTTKIPKLA  366

NRC-1   HGYRDSVASSVLDTLQEEGSPTEPNRRSFVPFSDSGSDTAAVSVIDPDGNIVRHSYYDSG  350
byr2    RGFDPPLTVSSTQRISRPPSLQKSITMVGVEPLYQSNGNEKSSKYNVFSESA.HGNHQVL  353
Ste11p  HK..........................................................  368

NRC-1   TNNSADSAVIQEALAEDGEDAADKELQTFLAGDAWDDSM.........WMKGSLIGQGSF  401
byr2    SFSPGSSPSFIEQPSPISPTSTTSEDTNTLEEDTDDQSI........KWIRGALIGSGSF  405
Ste11p  ..RPEDNDAISNQLELLSVESGEEEDHDFFGEDSDIVSLPTKIATPKNWLKGACIGSGSF  426

NRC-1   GSVYLALHAITGELLAVKQVET.................................PAPGA  428
byr2    GQVYLGMNASSGELMAVKQV.................................ILDSVSE  432
Ste11p  GSVYLGMNAHTGELMAVKQVEIKNNNIGVPTDNNKQANSDENNEQEEQQEKIEDVGAVSH  486

NRC-1   DSKNDARKKSMIEALKREITLLRDLQHPNIVQYLGCSSSAEYLNIFLEYVPGGSVQTMLD  488
byr2    SK...DRHAKLLDALAGEIALLQELSHEHIVQYLGSNLNSDHLNIFLEYVPGGSVAGLLT  489
Ste11p  PKTNQNIHRKMVDALQHEMNLLKELHHENIVTYYGASQEGGNLNIFLEYVPGGSVSSMLN  546

NRC-1   QYGALPESLVRSFVRQILQGLSYVHNRDIIHRDIKGANILVDNKGTIKISDFGISKKLEA  548
byr2    MYGSFEETLVKNFIKQTLKGLEYLHSRGIVHRDIKGANILVDNKGKIKISDFGISKKLEL  549
Ste11p  NYGPFEESLITNFTRQILIGVAYLHKKNIIHRDIKGANILIDIKGCVKITDFGISKKL..  604

NRC-1   TNILNGANNNKHRPSLQGSVFWMAPEVVKQTSYTRKADIWSLGCLVVEMMTGTHPFPDCT  608
byr2    NSTSTKTGGA..RPSFQGSSFWMAPEVVKQTMHTEKTDIWSLGCLVIEMLTSKHPYPNCD  607
Ste11p  .SPLNKKQNK..RASLQGSVFWMSPEVVKQTATTAKADIWSTGCVVIEMFTGKHPFPDFS  661

NRC-1   QLQAIFKIGGSKASPTIPDNASEEAKQFLAQTFEIDHNKRPSADELMLSPFLTPVPGT    666
byr2    QMQAIFRIGE.NILPEFPSNISSSAIDFLEKTFAIDCNLRPTASELLSHPFVS         659
Ste11p  QMQAIFKIGT.NTTPEIPSWATSEGKNFLRKAFELDYQYRPSALELLQHPWLDAHII     697
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Figure 5.—A comparison of the amino acid sequences in the NRC-1, with the byr2 and Ste11p MAPKK kinases. An alignment of the amino acid sequences found in NRC-1, byr2 and Ste11p was made using the UWCGC program PILEUP (University of Wisconsin Genetics Computer Group, Madison, WI). The numbers on the right refer to the amino acid numbers in the proteins. The amino terminal 66 amino acids of byr2 and the amino terminal 105 amino acids of Ste11p are not included in the analysis.

Madison, WI) computer program indicated that over the entire length of the coding region the predicted *nrc-1* protein shares 44% and 41% sequence identity with *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.

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 *Pst*I, ligating the digested DNA, and then transforming *E. coli* strain K802 (Kang and Metzenberg 1993; Orbach *et al.* 1988). Plasmid DNA was prepared from thirteen *E. coli* transformants and a restriction enzyme analysis indicated all thirteen transformants harbored the same plasmid. One of the plasmid isolates, pT17-1, was selected for further characterization.

The *N. crassa* genomic DNA contained in pT17-1 was sequenced with a strategy similar to that used for sequencing pT4-1. Computer database searches revealed that the *N. crassa* genomic DNA sequences at the cross-

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     GGGCCCCCCCCGTCTTTCCCCCTTCCTCCCATCCACAAGTCGCATCATGGACTCGTCTTCATGAGCATTACATCACAAGGATGCCGTCCA   90
  1                                                                                 MetProSerT

     CAAAGAACGCCAACGGCGAAGGCCATTTCCCCTCGCGCATCAAGCAATTCTTCCGCATCAATTCCGGCTCCAAAGACCACAAGGACCGCG   180
  4  hrLysAsnAlaAsnGlyGluGlyHisPheProSerArgIleLysGlnPhePheArgIleAsnSerGlySerLysAspHisLysAspArgA

     ATGCCCACACCACCTCCAGCTCCCACGGCGGAGCACCACGCGCCGACGCCAAGACGCCCTCCGGCTTCCGTCAATCCCGTTTCTTCAGCG   270
 34  spAlaHisThrThrSerSerSerHisGlyGlyAlaProArgAlaAspAlaLysThrProSerGlyPheArgGlnSerArgPhePheSerV

     TCGGCCGCCTTCGCAGCACCACCGTCGTGAGCGAGGGCAATCCGCTCGACGAATCCATGAGTCCGACCGCACACGCGAACCCGTATTTTG   360
 64  alGlyArgLeuArgSerThrThrValValSerGluGlyAsnProLeuAspGluSerMetSerProThrAlaHisAlaAsnProTyrPheA

     CCCACCAGGGCCAACCGGGCCTGCGCCATCACAATGACGGCTCGGTTCCGCCCAGCCCGCCCGACACGCCCTCACTCAAGGTCGACGGCC   450
 94  laHisGlnGlyGlnProGlyLeuArgHisHisAsnAspGlySerValProProSerProProAspThrProSerLeuLysValAspGlyP

     CCGAAGGCTCGCAGCAGCCCACGGCGGCCACAAAAGAAGAGCTTGCGAGGAAACTGAGGAGAGTCGCCAGTGCTCCCAACGCTCAAGGCC   540
124  roGluGlySerGlnGlnProThrAlaAlaThrLysGluGluLeuAlaArgLysLeuArgArgValAlaSerAlaProAsnAlaGlnGlyL

     TGTTTTCAAAGGGTCAGGGCAACGGCGACCGCCCTGCCACGGCCGAACTCAGCAAGGAGCCGCTCGAGGAGAGCAAGGATTCCAACACCG   630
154  euPheSerLysGlyGlnGlyAsnGlyAspArgProAlaThrAlaGluLeuSerLysGluProLeuGluGluSerLysAspSerAsnThrV

     TCGGTTTCGCAGAGCAAAAGCCCAACAACGATTCCTCCACAAGTCTGGCTGCGCCCGATGCCGATGGTCTCGGAGCGCTGCCTCCTCCCA   720
184  alGlyPheAlaGluGlnLysProAsnAsnAspSerSerThrSerLeuAlaAlaProAspAlaAspGlyLeuGlyAlaLeuProProProI

     TCCGTCAGTCGCCGCTCGCCTTCCGCCGAACATACAGCTCCAACTCCATCAAGGTCCGCAACGTTGAGGTCGGCCCCCAGAGTTTCGACA   810
214  leArgGlnSerProLeuAlaPheArgArgThrTyrSerSerAsnSerIleLysValArgAsnValGluValGlyProGlnSerPheAspL

     AGATCAAGTTGATTGGCAAGGGCGATGTCGGCAAGGTATATCTAGTCAAGGAGAAGAAGAGCGGGAGGTTATATGCCATGAAGGgtacgt   900
244  ysIleLysLeuIleGlyLysGlyAspValGlyLysValTyrLeuValLysGluLysLysSerGlyArgLeuTyrAlaMetLysV

     cttcgcgtctgttcggtacagtgtctcaccccttactaacaaagcaacacagTTCTGAGCAAGAAGGAAATGATCAAGCGTAACAAGATC   990
272                                                      alLeuSerLysLysGluMetIleLysArgAsnLysIle

     AAGAGAGCCTTGGCCGAGCAGGAAATCCTCGCGACAAGCAACCATCCCTTCATCGTGACCCTGTATCATTCCTTCCAATCCGAGGACTAC   1080
285  LysArgAlaLeuAlaGluGlnGluIleLeuAlaThrSerAsnHisProPheIleValThrLeuTyrHisSerPheGlnSerGluAspTyr

     CTTTACCTTTGCATGGAATACTGCAGCGGTGGCGAGTTCTTCAGAGCTCTACAGACACGTCCCGGCAAGTGTATCCCCGAGGACGATGCT   1170
315  LeuTyrLeuCysMetGluTyrCysSerGlyGlyGluPhePheArgAlaLeuGlnThrArgProGlyLysCysIleProGluAspAspAla

     CGCTTCTACGCCGCAGAGGTGACTGCTGCGCTTGAATACCTGCATCTTATGGGATTCATCTACCGGGATCTCAAGCCAGAGAgtacgttt   1260
345  ArgPheTyrAlaAlaGluValThrAlaAlaLeuGluTyrLeuHisLeuMetGlyPheIleTyrArgAspLeuLysProGluA

     ccacttttcccacctttcatcatgccgatcatttcgcataactaactgtcgatcatcacagATATTCTACTGCATCAGTCTGGACATATC   1350
372                                                               snIleLeuLeuHisGlnSerGlyHisIle

     ATGCTTTCAGATTTCGATCTGTCCAAGCAGTCCGACCCGGGCGGCAAGCCAACCATGATTATTGGCAAGAACGGCACGAGTACGTCGTCG   1440
382  MetLeuSerAspPheAspLeuSerLysGlnSerAspProGlyGlyLysProThrMetIleIleGlyLysAsnGlyThrSerThrSerSer

     TTGCCGACCATCGATACCAAGTCATGCATAGCAAACTTCCGCACAAACTCCTTTGTGGGCACAGAAGAGTATATCGCACCGGAAGTGATT   1530
412  LeuProThrIleAspThrLysSerCysIleAlaAsnPheArgThrAsnSerPheValGlyThrGluGluTyrIleAlaProGluValIle

     AAGGGCAGTGGGCATACTAGTGCAGTCGACTGGTGGACGCTCGGAATCCTTATCTACGAAATGCTGTACGGTACTACCCCCTTCAAGGGT   1620
442  LysGlySerGlyHisThrSerAlaValAspTrpTrpThrLeuGlyIleLeuIleTyrGluMetLeuTyrGlyThrThrProPheLysGly

     AAGAACCGTAACGCAACGTTCGCCAACATCCTCAGGGAAGATATTCCCTTCCCTGACCATGCCGGCGCGCCGCAAATATCAAAgtgagtc   1710
472  LysAsnArgAsnAlaThrPheAlaAsnIleLeuArgGluAspIleProPheProAspHisAlaGlyAlaProGlnIleSerAs

     tctcatacttgtcaacgctttctactctgactacttgctaaccttgaatcatcagCCTTTGCAAATCACTCATTCGCAAGTTGCTTATCA   1800
499                                                          nLeuCysLysSerLeuIleArgLysLeuLeuIleL

     AGGACGAGAACCGTCGTCTAGGTGCCCGTGCTGGTGCATCCGACATTAAGACTCATCCCTTCTTCAGGACGACACAATGGGCTCTGATCC   1890
511  ysAspGluAsnArgArgLeuGlyAlaArgAlaGlyAlaSerAspIleLysThrHisProPhePheArgThrThrGlnTrpAlaLeuIleA

     GTCATATGAAGCCGCCCATTGTCCCGAACCAAGGTCGCGGCATTGATACACTGAACTTCCGCAACGTCAAGGAAAGTGAGAGCGTGGACA   1980
541  rgHisMetLysProProIleValProAsnGlnGlyArgGlyIleAspThrLeuAsnPheArgAsnValLysGluSerGluSerValAspI

     TCAGCGGGTCCAGGCAGATGGGCCTCAAAGGGGAACCACTTGAAAGCGGGATGGTAACACCAGGCGAGAATGCCGTTGATCCATTCGAAG   2070
571  leSerGlySerArgGlnMetGlyLeuLysGlyGluProLeuGluSerGlyMetValThrProGlyGluAsnAlaValAspProPheGluG

     AGTTCAACAGCGTTACATTACATCACGATGGGGATGAAGAGTACCACTCTGATGCTTACGAGAAACGATAAGGGAGTTTCGGGAGGTCCC   2160
601  luPheAsnSerValThrLeuHisHisAspGlyAspGluGluTyrHisSerAspAlaTyrGluLysArgEnd

     AACTCCTGGTCGTTTCTATTGAGGGCATTTTCAGCTGGTGAGGATCGCAATCAATCGATGGCTCCGTCATCTCTCGCTCCTCTGCCTTGC   2250
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Figure 6.—Nucleotide and encoded amino acid sequence from the *nrc-2* gene. The nucleotide sequence and the encoded amino acid sequence from the *nrc-2* gene are given. Intron sequences are in small case type. Sequences underlined within introns represent 5′ and 3′ recognition splice sites. The sequence underlined in the second exon represents the region where the pRAL-1 plasmid was inserted in the *nrc-2* mutant. The underlined 17 bp were deleted during the insertion event. The nucleotide numbers are given in the right margin and the amino acid numbers in NRC-2 are given in the left margin.

over point showed similarity to a variety of protein kinase genes. Cosmids containing the *nrc-2* gene were identified by using a radioactively-labeled *Pst*I-*Sal*I 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 *YNRO47w* genes of *S. cerevisiae. KIN82*, *YNRO47w* 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 cAMP-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, Ynro47wp, 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, GTH16-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, GTH16-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 amino 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^{RIP}* 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 GTH16-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

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NRC-2        MPSTKNANGEGHFPSRIKQFFRINSGSKDHKDRDAHTTSSSHGGAPRA............   48
kad5         MNELHDGESSEEGRINVEDHL.....EEAKKDDTGHWKHSGTAKPSKF............   43
Ynr047w SPSTPIMPSQNSNNSSSTSAIRPNNYRHHSGSQGFSSNNPFRERAGTVRSSNPYFAYQGLPTHAM  263
Kin82p  IPNEAICSTPNEISGSS................................SPDAELFTFDMPTDPS  183

NRC-2   .......................DAKTPSGFRQSRFFSVGRLRSTTVVSE............GNP   78
Kad5    .......................RAFIRLHFKDSRKFAFSRKKEKELTSE............D..   71
Ynr047w SSHDLDEGFQPYANGSGIHFLSTPTSKTNSLTNTKNLSNLSLNDIKENEEVQEFNNEDFFFHDIP  328
Kin82p  SFH...................TPSSPSYIAKDSRNLSNGSLNDINENEELQNFH......RKIS  223

NRC-2   LDESMSPTAHANPYFAHQGQPGLRHHNDGSVPPSPPDTPSLKVDGPEGSQQPTAATKEELARKLR  143
Kad5    .....SDAANQSPSGAPESQTE..............EESDRKIDGTGSSAEGGDGSGTDSISVIK  117
Ynr047w KDLSLKDTLNGSPSRGSSKSPTITQTFPSIIVGFDNEYEEDNNNDKHDEKEEQQTTTDNKTRNLS  393
Kin82p  ENGSASPLANLSLSNSPIDSP............................RKNSETRKDQIPMNIT  260

NRC-2   RVASAPNAQGLFSKGQGNGDRPATAELSKEPLEESKDSNTVGFAEQKPNNDSSTSLAAPDADGLG  208
Kad5    K........SFFKSGRKKKDVPKSRNVSR........SNGADTSVQREKLKDIFSPHGKEKE...  163
Ynr047w PTKQNGKATHPRIKIPLRRAASEPNGLQLASATSPTSSSARKTSGSSNINDKIPGQSVPPPNSFF  458
Kin82p  P..............RLRRAASEPFN...........................TAKDGLMREDYI  284

NRC-2   ALPPPIRQSPLAFRR.....TYSSNSIKVRNVEVGPQSFDKIKLIGKGDVGKVYLVKEKKSGRLY  268
Kad5    ..LAHIKKTVATRAR.....TYSSNSIKICDVEVGPSSFEKVFLLGKGDVGRVYLVREKKSGKFY  221
Ynr047w PQEPSPKISDFPEPRRSRRLRTKSFSNKFQDIMVGPQSFEKIRLLGQGDVGLVFLVREEKTNRVY  523
Kin82p  ALKQPPSLGDIVEPRRSRRLRTKSFGNKFQDITVEPQSFEKIRLLGQGDVGKVYLVRERDTNQIF  349

NRC-2   AMKVLSKKEMIKRNKIKRALAEQEILATSNHPFIVTLYHSFQSEDYLYLCMEYCSGGEFFRALQT  333
Kad5    AMKVLSKQEMIKRNKSKRAFAEQHILATSNHPFIVTLYHSFQSDEYLYLCMEYCMGGEFFRALQR  286
Ynr047w ALKVLSKDEMIKRNKIKRVLTEQEILATSNHPFIVTLYHSFQSEDYLYLCMEYCMGGEFFRALQT  588
Kin82p  ALKVLNKHEMIKRKKIKRVLTEQEILATSDHPFIVTLYHSFQTKDYLYLCMEYCMGGEFFRALQT  414

NRC-2   RPGKCIPEDDARFYAAEVTAALEYLHLMGFIYRDLKPENILLHQSGHIMLSDFDLSKQSDPGGKP  398
Kad5    RPGRCLSENEAKFYIAEVTAALEYLHLMGFIYRDLKPENILLHESGHIMLSDFDLSKQSNSAGAP  351
Ynr047w RKTKCICEDDARFYASEVTAALEYLHLLGFIYRDLKPENILLHQSGHIMLSDFDLSIQATDSKVP  653
Kin82p  RKSKCIAEEDARFYASEVVAALEYLHLLGFIYRDLKPENILLHQSGHVMLSDFDLSIQATGSKKP  479

NRC-2   TMIIGKNGTSTSSLPTIDTKSCIANFRTNSFVGTEEYIAPEVIKGSGHTSAVDWWTLGILIYEML  463
Kad5    TVIQARNAPSAQNAYALDTKSCIADFRTNSFVGTEEYIAPEVIKGCGHTSAVDWWTLGILFYEML  461
Ynr047w VVKGSAQST......LVDTKICSDGFRTNSFVGTEEYIAPEVIRGNGHTAAVDWWTLGILIYEML  712
Kin82p  TMK...DST......YLDTKICSDGFRTNSFVGTEEYLAPEVIRGNGHTAAVDWWTLGILIYEML  535

NRC-2   YGTTPFKGKNRNATFANILREDIPFPDHAGAPQISNLCKSLIRKLLIKDENRRLGARAGASDIKT  528
Kad5    YATTPFKGKNRNMTFSNILHKDVIFPEYADAPSISSLCKNLIRKLLVKDENDRLGSQAGAADVKL  481
Ynr047w FGFTPFKGDNTNETFTNILKNEVSFPNNNE...ISRTCKDLIKKLLTKNESKRLGCKMGAADVKK  774
Kin82p  FGCTPFKGDNSNETFSNILTKDVKFPHDKE...VSKNCKDLIKKLLNKNEAKRLGSKSGAADIKR  597

NRC-2   HPFFRTTQWALIRHMKPPIVPNQGR..GIDTLNFRNVKESESVDISGSRQMGLKGEPLESGMVTP  591
Kad5    HPPFKNVQWALLRHTEPPIIPKLAPIDEKGNPNISHLKESKSLDITHSPQNTQTVEVPLSNL.SG  545
Ynr047w HPFFKKVQWSLLRNQEPPLIPVLSEDGYDFA.KLSSNKKRQTSQDSHKHLDEQEKNMFEERVEYD  838
Kin82p  HPFFKKVQWSFLRNQDPPLIPALNDNGCELPFILSCNK.....HPKRNSVSEQETKMFCEKVAND  657

NRC-2   GE.NAVDPFEEFNSVTLHHDGDEEYHSDAYEKR                                 623
Kad5    AD.HGDDPFESFNSVTVHHEWD                                            566
Ynr047w DEVSEDDPFHDFNSMSLMEQDNNSMIYGNTNSYGKI.........AYTPN                879
Kin82p  DEIDEADPFHDFNSMSLTKKDHNILTYSENYTTEKFYTKQLVQGQGITAH                707
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Figure 7.—A comparison of the amino acid sequences encoded by the *N. crassa nrc-2*, the *S. pombe kad5* and the *S. cerevisiae YNR047w* 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 Ynr047wp 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

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 neutal 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 *nrc-1* and *nrc-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 *nrc-1* and *nrc-2* genes had been tagged by the inserted pRAL-1 plasmid, the genes were readily isolated with the plasmid rescue procedure.

The *nrc-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 MAPK kinase. This phosphorylation activates the MAPK 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; Davis 1993).

We have isolated the *N. crassa nrc-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 *nrc-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 MAPK 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 G_1 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 nrc-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 filament formation, which occurs when haploid *S. cerevisiae* strains become nutrient starved (Roberts and Fink 1994).

This report suggests that the cellular events required for pseudohyphal growth and filament formation in yeast and for the formation of vegetative hyphae in *N. crassa* appear to be regulated by closely related MAP kinase signal transduction pathways. The NRC-1 MAPKK kinase is required for the vegetative hyphal growth phase of the *N. crassa* life cycle. This vegetative hyphal phase of the life cycle is a defining characteristic of the 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 crisp (*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 the 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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