Characterization of *nit-2*, the Major Nitrogen Regulatory Gene of *Neurospora crassa*

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The *nit-2* gene is the major nitrogen-regulatory gene of *Neurospora crassa*, and under conditions of nitrogen limitations, it turns on the expression of various unlinked structural genes which specify nitrogen-catabolic enzymes. The *nit-2* gene was subcloned as a 6-kilobase (kb) DNA fragment from a cosmid that carried approximately a 40-kb *N. crassa* DNA insert. The *nit-2* gene was localized in a DNA segment of approximately 3.5 kb and was shown to correspond to a unique DNA sequence located on linkage group 1. Several *N. crassa nit-2* transformants were characterized and were found to possess significantly different levels of the regulated enzyme nitrate reductase. Northern blot analysis of RNA from various strains was carried out to determine whether the *nit-2* gene was expressed constitutively or was itself subject to regulation. The results revealed that the *nit-2* gene is transcribed to give a single large mRNA of approximately 3.5 kb. Expression of the *nit-2* gene is regulated such that its transcript is present at a substantially higher level in cells which are limited for nitrogen than in cells growing under nitrogen-repressed conditions. However, the *nit-2* gene is not controlled by autogenous regulation. The *nit-2* gene was transcribed only at a low level in *nmr-1* and in *gln-1b*, under both nitrogen-repressed and derepressed conditions, suggesting that these unlinked loci may exert a positive regulatory effect on *nit-2*.

In the fungus *Neurospora crassa*, the expression of enzymes in various global areas of metabolism is subject to regulation. Thus, *N. crassa* has been exploited as a model lower eucaryote for studies of genetic regulation of complex control circuits (12). The nitrogen control circuit comprises a set of genes which encode enzymes that permit the organism to utilize a number of secondary nitrogen sources, e.g., nitrate, purines, or amino acids, when preferred nitrogen sources such as ammonia and glutamine are not available. These nitrogen-related enzymes include nitrate and nitrite reductase and the purine catabolic enzymes, as well as enzymes such as extracellular protease, L-amino acid oxidase, and phenylalanine ammonia lyase, which are involved in the use of proteins and amino acids as nitrogen sources. Both induction and repression by metabolic effectors and major and minor control genes govern the expression of the various unlinked structural genes of the nitrogen circuit. The *nit-2* gene is the major nitrogen-regulatory gene of *N. crassa*, and under conditions of nitrogen derepression, it appears to turn on in a positive manner the expression of the structural genes. Pathway-specific induction by substrates or intermediates is also required for the synthesis of particular sets of enzymes within the nitrogen circuit. Thus, the synthesis of nitrate and nitrite reductase requires the lifting of nitrogen catabolite repression as signalled by the *nit-2* gene product and the simultaneous induction by nitrate, which is mediated by the minor control gene *nit-4*.

Mutants with mutations in *nit-2* were first obtained because they could not use nitrate or nitrite for growth. However, Reinert and Marzluf (15) found new *nit-2* mutants within a group that had been selected for the inability to utilize purines, which indicated that *nit-2* has a more general role in nitrogen metabolism. Subsequently, it became clear that *nit-2* is the major nitrogen-regulatory gene and is central

to the expression of many different nitrogen-related enzymes of separate pathways (12). *nit-2* mutants display a pleiotropic loss of many nitrogen-related enzymes, which are restored in parallel in revertants, suggesting that it acts in a positive manner. The isolation of an amber nonsense *nit-2* mutant that is suppressible by *SSU-1*, a tRNA suppressor gene, implies that this locus encodes a regulatory protein (13). By using contrasting alleles that encode electrophoretic variants of L-amino acid oxidase, we demonstrated that a *nit-2*⁺ gene in one nucleus could turn on the expression of structural genes in the same nucleus and in other nuclei of a heterokaryon (3). Thus it appears that a *nit-2*-encoded regulatory protein acts in a positive fashion to activate the expression of a large family of unlinked nitrogen structural genes. Although it has been recognized that the *nit-2* gene has a major role in nitrogen regulation, it has not been possible to specify the exact nature of the control mechanism. In one proposed model, it was suggested that the *nit-2* gene is expressed in a constitutive fashion to yield an active regulatory protein whose activity is inhibited upon binding glutamine (7). However, it is plausible that the *nit-2* gene itself is regulated, and at least two distinct models incorporating this feature have been presented (4, 6). In one case, it was hypothesized that a second nitrogen control gene, designated *nmr-1*, encodes a repressor protein; upon binding glutamine, the repressor was suggested to bind at a control site adjacent to *nit-2*, thereby preventing its expression (4). An alternative mechanism has been proposed in which the enzyme glutamine synthetase also plays a primary regulatory role; upon binding glutamine, glutamine synthetase was visualized as undergoing a change in oligomeric structure which allowed it to bind at the *nit-2* gene and prevent the expression of this gene (6).

It is obvious that a more complete understanding of the nitrogen control circuit and the role of the proposed regulatory elements required that representative structural and

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control genes be isolated and their behavior be examined at the molecular level. Vollmer and Yanofsky (18) have recently introduced the use of a cosmid library in vector pSV50 that carries a benomyl-resistant marker for the rapid isolation of *N. crassa* genes via complementation by the sib selection protocol. A cosmid carrying approximately a 40-kilobase (kb) *N. crassa* DNA insert which complements the *nit-2* mutant and simultaneously transforms it to benomyl resistance was obtained from this library (V. Stewart and S. J. Vollmer, *Gene*, in press). Here we present a detailed characterization of the *nit-2* gene, which is transcribed to give a single large mRNA. We also provide results which clearly demonstrate that expression of the *nit-2* gene itself is highly regulated, and we assess the importance of the *nmr-1* gene and of glutamine synthetase in its control.

MATERIALS AND METHODS

Nucleic acid isolation. *N. crassa* DNA was isolated by a modification of the procedure of R. L. Metzenberg and T. J. Baisch (*Neurospora Newsl.* 28:20–21, 1981). *N. crassa* RNA was isolated by the procedure described by Reinert et al. (16). Poly(A)⁺ RNA was isolated from total RNA by oligo(dT)-cellulose chromatography (1). Plasmid DNA was isolated by the method described by Birnboim and Doly (2).

Transformation. Transformation of *Escherichia coli* was carried out by the method of Mandel and Higa (10). Preparation of *N. crassa* spheroplasts and their transformation were performed as described by Vollmer and Yanofsky (18).

Southern and Northern blots. Southern and Northern transfers were performed as described by Maniatis et al. (11). The baked filters for Southern blots were prehybridized in sealed bags with a solution containing 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.5% sodium dodecyl sulfate, 5× Denhardt solution, and 100 µg of denatured salmon sperm DNA per ml. After at least 1 h of incubation at 65°C, a ³²S-labeled denatured DNA probe was added. Nitrocellulose filters for Northern blots were incubated in 6× SSC–0.25% dry milk–50% formamide for 4 h, and then the desired ³²P-labeled denatured DNA probe was added. After hybridization was complete, the filter was washed in 2× SSC–0.1% sodium dodecyl sulfate at room temperature twice for 15 min each and then in 0.1× SSC–0.1% sodium dodecyl sulfate at 65°C for 1 h with at least one change of the solution. DNA to be used as probes was radioactively labeled by nick translation (17).

Analysis of *nit-2* transformants. Because transformants are invariably heterokaryotic, they must be converted to homokaryons before any analysis can be meaningful. A total of 20 *nit-2* transformants were selected for study, but all failed to cross with a *nit-2* mutant of the opposite mating type. Therefore serial transfers were carried out in an attempt to produce homokaryons. Colonies which arose from single conidia that had been plated onto nitrate medium were picked for each of the primary transformants. This plating procedure was repeated four times in serial transfers, after which 50 colonies from the final isolates were picked from ammonium plates and tested for growth on nitrate. If the isolate were homokaryotic, all colonies should grow on ammonium and on nitrate. We found that only 3 transformants from the original 20 yielded definite homokaryons. These three homokaryotic *nit-2*⁺ transformants were then studied by Southern blot analysis and analyzed for nitrate reductase by using the assay described previously (13).

Subcloning experiments. Plasmid pYHF252 contains an *EcoRI*–*AvaI* *N. crassa* DNA fragment that carries the entire

FIG. 1. Restriction map of plasmid pNit-2. Plasmid pNit2 contains a 6-kb *N. crassa* *EcoRI* DNA fragment cloned into pUC8 (□). The approximate location of the *nit-2* gene, ascertained as described in the text, is indicated by the arrow. The apparent direction of transcription as indicated by the arrow was indicated by a preliminary in vitro coupled transcription-translation experiment (results not shown). The lower half of the figure shows the location of the left edge of the *N. crassa* DNA insert carried in six different plasmids which were obtained by deleting in from the *EcoRI* site and subcloning as described in Materials and Methods. Symbols: +, transforms the *nit-2* mutant; –, fails to transform the *nit-2* mutant. A line representing 1 kb of DNA is shown.

nit-2 gene inserted into the multiple cloning region of pIBI25 (International Biotechnologies, Inc.). Deletions were generated by use of DNA polymerase I in the *N. crassa* insert of pYHF252 starting at the *EcoRI* site with the Cyclone system (International Biotechnologies, Inc.) as specified by the manufacturer. The various derived plasmids are described in Fig. 1.

RESULTS

Identification of the *nit-2* gene. The cosmid which transforms the *nit-2* mutant contains an *N. crassa* DNA fragment of at least 40 kb. After the cosmid DNA was digested with either *HindIII* or *EcoRI*, the cleaved DNA mixtures were able to transform the *nit-2* mutant to *nit-2*⁺ (by using only single selection for the ability to grow on nitrate), indicating that neither of these enzymes cuts within the *nit-2*⁺ gene. Restriction of the cosmid DNA with *EcoRI* yielded six distinct fragments. These six different *EcoRI* fragments were individually subcloned into the *EcoRI* site of plasmid pUC8, and each was then tested for the ability to transform the *nit-2* mutant of *N. crassa*. One subclone that contains an approximately 6-kb *EcoRI* DNA insert yielded many *nit-2*⁺ transformants, whereas the other subclones failed to transform the *nit-2* mutant. The positive subclone was designated plasmid pNit-2, and a restriction map was derived for it (Fig. 1). The approximate location of the *nit-2* gene within the 6.1-kb cloned *N. crassa* DNA fragment was determined by

examining the transformation of the *nit-2* mutant with pNit-2 DNA that had been digested with various restriction enzymes. Digestion with *Pvu*II, *Kpn*I, *Sac*II, *Pst*I, and *Bam*HI inactivated the *nit-2* gene, whereas transformants were still readily obtained when the DNA was restricted with *Ava*I or *Eco*RV.

To further confirm and refine the location of the *nit-2* gene in pNit-2, a series of additional subcloning experiments were carried out. A plasmid which carries the large *Eco*RI-*Pst*I *N. crassa* DNA fragment failed to transform the *nit-2* mutant, whereas a second plasmid pYHF252, which contained the longer *Eco*RI-*Ava*I fragment, gave a positive result, demonstrating that the right end of the gene lies between the *Pst*I site and the *Ava*I site (Fig. 1). To define the left end of the *nit-2* gene, a series of six plasmids were then constructed which represent a nested set of deletions extending in from the left *Eco*RI site (Fig. 1); all of these plasmids have an identical right end, terminating at the *Ava*I site. When tested for their ability to transform the *nit-2* mutant, four of these plasmids gave no transformations, whereas the other two were strongly positive. The results shown in Fig. 1 demonstrate that the left edge of the *nit-2* gene is defined by two plasmids, designated 22 and 76, and lies within a DNA segment of approximately 0.2 kb situated to the left of the *Pvu*II site. These results demonstrate that the *nit-2* gene is contained within a DNA sequence of approximately 3.5 kb (Fig. 1).

To confirm that the DNA insert present in plasmid pNit-2 is actually *N. crassa* DNA and that it contains the *nit-2* gene, a restriction fragment length polymorphism analysis was performed. The results demonstrate a tight linkage between the cloned sequence and the two markers which identify linkage group 1, the resident site for *nit-2* (Fig. 2). These results confirm that pNit-2 carries a unique segment of *N. crassa* DNA derived from linkage group 1 and strongly imply that it indeed contains the *nit-2*⁺ gene.

Analysis of *nit-2* transformants. A total of 20 *nit-2* transformants were selected for analysis of the manner in which the transforming DNA had integrated. Since the primary transformants obtained with *N. crassa* are invariably heterokaryotic, each transformant was crossed with a *nit-2* mutant to obtain homokaryons. However, all of these crosses proved to be infertile, despite several independent crossing attempts. For this reason, homokaryons for the transformed *nit-2*⁺ gene were obtained with serial transfers as described in Materials and Methods, although only three definite homokaryons were obtained. Southern blot analysis of these three transformants revealed that in addition to the resident *nit-2* band, each of them contained two *Eco*RI fragments that hybridized with plasmid pNit-2 DNA (Fig. 3). In each case, one of the additional bands also hybridized with just *nit-2* DNA, whereas the other extra band contained only pUC8 sequences (Fig. 3). These results indicate that each of these *nit-2* transformants occurred via the integration of the transforming DNA at a secondary site rather than at the resident locus. This type of behavior in transformation has been noted for the *qa-2* gene (5) and the *am* gene (8) of *N. crassa*. The level of nitrate reductase expressed in these *nit-2* transformants is compared with that in the wild-type strain and the *nit-2* mutant recipient strain in Table 1. The synthesis of nitrate reductase was regulated normally in each of the *nit-2* transformants and required nitrogen derepression and nitrate induction. However, the amount of nitrate reductase varied dramatically among these transformants: one transformant had almost twice the level of the enzyme present in the wild-type control, whereas another transform-

FIG. 2. Restriction fragment length polymorphism analysis of the *N. crassa* DNA fragment carried by pNit-2. DNA isolated from 18 progeny of a cross of Mauriceville-1c-A and RLM 33-1a was digested with *Bam*HI, and after separation on agarose gels, Southern blots were probed with nick-translated ³²S-labeled pNit-2 DNA. The polymorphism being monitored is the presence or absence of the DNA band indicated by the arrow. This polymorphism displays a tight linkage to two markers (MT [mating type] and 5S gene 12) that identify linkage group I. The presence of the Oak Ridge (O) or the Mauriceville (M) trait for each marker in the progeny is shown. The numbers above each lane identify the individual progeny from the cross as described by Metzberg et al. (R. L. Metzberg, J. N. Stevens, E. U. Selker, and E. Morzycka-Wroblewska, *Neurospora* Newsl. 31:35-39, 1984), except that number 4416 is the Mauriceville parent. The identity of the 5S gene 12 was not reported for 4429 and 4430 (Metzberg et al., newsletter).

ant had just 62% of the normal level. These results suggest that the site of integration of the *nit-2* gene affects the level of its expression, which in turn leads to the production of different amounts of the regulated enzyme nitrate reductase.

Regulation of *nit-2* gene expression. A number of genetic studies have implied that the *nit-2* gene is a major nitrogen-regulatory gene and that under conditions of nitrogen limitation, it is responsible for turning on the expression of an entire family of unlinked structural genes which encode enzymes of nitrogen catabolism. However, it has never been clear whether the *nit-2* gene is expressed constitutively or might itself be subject to nitrogen regulation. Therefore, it was of considerable interest to directly examine the expression of the *nit-2* gene in cells grown under both nitrogen-repressed and -derepressed conditions. Poly(A)⁺ RNA was isolated as described in Materials and Methods from cells grown under repressed and derepressed conditions and used in Northern blot analysis to detect and quantify the amount of the *nit-2* gene transcript present in cells grown under these contrasting conditions. Using plasmid pNit-2 DNA as a

FIG. 3. Southern blots of *Eco*RI-digested DNA of the wild-type strain (lane 1), the *nit-2* mutant strain used as a recipient for transformation (lane 2), and homokaryons for transformants 6 (lane 3), 12 (lane 4), and 15 (lane 5). The upper panel shows a blot that was probed with ^{35}S -labeled plasmid pNit-2 DNA. The blot shown in the lower panel was probed with the *N. crassa* *Eco*RI fragment of pNit-2 that was isolated by electroelution from an agarose gel and labeled with ^{35}S via nick translation. Each transformant contains the expected resident *nit-2* gene and one additional *nit-2* gene that has integrated at a secondary site.

probe, we detected a single RNA band of approximately 3.5 kb, which we concluded is the *nit-2* gene transcript, since only one transcript is evident and its size corresponds to that expected for the *nit-2* gene and, moreover, it is subject to nitrogen control (see below).

A single large transcript of approximately 3.5 kb was detected in the RNA isolated from wild-type cells grown under both repressed and derepressed conditions (Fig. 4). Moreover, the amount of the *nit-2* transcript was significantly greater in the RNA from derepressed cells than in the RNA from repressed cells. Similarly, when a *nit-2* mutant was examined, its nonfunctional *nit-2* gene was expressed at a considerably higher level in derepressed cells than in nitrogen-repressed cells. These results clearly demonstrate that the *nit-2* gene is subject to regulation and is expressed at

an enhanced level in cells which are limited for nitrogen; they also suggest that *nit-2* gene expression is not autogenously regulated by its own product.

An unlinked gene designated *nmr-1* has been proposed to have a nitrogen-regulatory function and to possibly control the expression of *nit-2* (4). The *nmr-1* mutant strain has a wild-type phenotype, except that it displays a constitutive synthesis of nitrate reductase and several other nitrogen-related enzymes (3, 4, 14). Therefore, *nmr-1* might be expected to lead to a parallel constitutive expression of *nit-2*. We therefore carried out an identical Northern blot analysis of *nit-2* gene expression in the *nmr-1* mutant. The results shown in Fig. 4 revealed that the *nit-2* gene was expressed only at a low level in *nmr-1* under both repressed and derepressed conditions; in fact, the content of the *nit-2* transcript in *nmr-1* was only approximately 30% of the repressed wild-type level. This result was surprising but suggests that the product of *nmr-1*⁺ may in some way control *nit-2* gene expression in a positive manner.

It has also been suggested that the enzyme glutamine synthetase, encoded by *gln-1* may have a role in nitrogen regulation, because one mutant, known as *gln-1b*, is deficient in glutamine synthetase but also displays a constitutive synthesis of nitrate reductase (6). An identical Northern blot analysis was carried out to determine the level of *nit-2* gene transcription in *gln-1b* mutant cells under repressed and derepressed conditions. The *nit-2* gene was found to be expressed at only the low level characteristic of N-repressed wild-type cells in either nitrogen-repressed or derepressed *gln-1b* cells (results not shown); this finding suggests that

FIG. 4. Northern blot analysis of RNA isolated from various *N. crassa* strains grown under nitrogen-repressed and derepressed conditions. Equal amounts of poly(A)⁺ RNA were run in each lane for the blot shown in the upper panel and one-third that amount of RNA for each lane of the lower panel. After electrophoresis, the RNAs were blotted to nitrocellulose. The blot shown in the upper panel was probed with ^{32}P -labeled pNit-2 DNA, and that in the lower panel was probed with ^{32}P -labeled plasmid pNC2 DNA (which contains the *N. crassa* *trp-1* gene). The lanes represent RNA isolated from wild-type, derepressed (lane 1); wild-type, repressed (lane 2); *nit-2* mutant, derepressed (lane 3); *nit-2* mutant, repressed (lane 4); *nmr-1* mutant, repressed (lane 5); *nmr-1* mutant derepressed (lane 6). The size of the *trp-1* transcript is 2.2 kb. Densitometer scans revealed that the signal for derepressed wild-type or *nit-2* cells (lanes 1 and 3) was approximately three times greater than that for the corresponding repressed cells (lanes 2 and 4).

TABLE 1. Nitrate reductase activity of the wild type, the *nit-2* recipient strain, and homokaryons of pNit-2 transformants^a

Strain	Sp act	% activity of wild type
Wild type	6.0	100
<i>nit-2</i> ⁻	0.0	0
Transformant 6	3.71	62
Transformant 12	10.9	182
Transformant 15	6.11	102

^a Nitrate reductase activity was assayed by using extracts of induced cells as described in Materials and Methods and is expressed in nanomoles of nitrite produced per milligram of protein per minute.

glutamine synthetase might also have a positive effect on *nit-2* gene expression.

Northern blots for the same RNAs obtained from these various strains under nitrogen-repressed and derepressed conditions were probed with the *N. crassa trp-1* gene as a control to ensure that identical amounts of RNA were actually loaded into each lane. The results (Fig. 4) demonstrate that a single *trp-1* transcript of the expected size is present in similar amounts in all lanes and reveal no obvious problems with RNA degradation.

DISCUSSION

We report here a basic characterization of the cloned *nit-2* gene, the major nitrogen-regulatory gene of *N. crassa*. The fact that this cloned sequence efficiently transforms the *N. crassa nit-2* mutant and shows tight linkage via restriction fragment length polymorphism analysis to the resident chromosome of *nit-2* confirms that it does in fact carry the *nit-2* gene. The gene was localized to a segment of unique *N. crassa* DNA of approximately 3.5 kb in length. Northern blot analysis demonstrated that the *nit-2* gene is expressed to give a single large poly(A)⁺ mRNA of approximately 3.5 kb.

The *nit-2* gene is the major nitrogen control gene of *N. crassa* and during conditions of nitrogen limitation, it appears to turn on the expression of an entire series of unlinked structural genes which specify nitrogen-catabolic enzymes. It has never been clear whether the *nit-2* gene is expressed constitutively or is itself subject to genetic regulation, although several models have been proposed that suggest different ways in which *nit-2* might be expressed (4, 6, 7). The results presented here clearly demonstrate that expression of the *nit-2* gene is indeed controlled and that the cellular content of the *nit-2* transcript is significantly increased during conditions of nitrogen limitation. This certainly suggests that *nit-2* gene expression is itself regulated by other control genes, as suggested in several models for nitrogen regulation (3, 4, 6). Although we believe that the increased amount of *nit-2* mRNA is due to increased *nit-2* transcription during nitrogen-derepressed conditions, we cannot yet exclude the possibility that its stability might be increased when the nitrogen source becomes limiting. The extent of apparent derepression of *nit-2* transcription is approximately 3-fold, whereas certain nitrogen-regulated enzymes are derepressed to a much greater extent, e.g., nitrate reductase is derepressed at least 40-fold. This suggests that additional signals act during derepression, beyond that of increased *nit-2* gene expression, within the nitrogen circuit.

DeBusk and Ogilvie (4) proposed that the unlinked gene known as *nmr-1* encodes a repressor protein that in the presence of sufficient glutamine repressed the expression of *nit-2*. This model would lead to the prediction that the *nit-2* gene is transcribed at the higher level characteristic of the derepressed state in the *nmr-1* mutant in the presence or absence of ample glutamine. The results presented here revealed that *nit-2* in fact was expressed only at the basal repressed level in *nmr-1* cells, whether they are repressed or derepressed. This finding does implicate the *nmr-1* locus in the control of *nit-2* transcription or the stability of *nit-2* mRNA but suggests that it acts in a positive rather than a negative fashion.

In another model, the enzyme glutamine synthetase has been implicated as a regulatory element in the nitrogen control circuit (6). In the presence of glutamine, the active multimeric form of glutamine synthetase was imagined to act

as a repressor of *nit-2* gene expression. This suggestion was based upon the finding that a mutant with a particular mutation of the structural gene of glutamine synthetase, *gln-1b*, displayed constitutive synthesis of nitrate reductase in the presence or absence of exogenous glutamine. We present results that suggest that transcription of the *nit-2* gene only occurs at the low repressed level in the *gln-1b* mutant, whether the mutant is growing under nitrogen-repressed or derepressed conditions. These results suggest that glutamine synthetase may have a regulatory role but indicate that its control of *nit-2* is in a positive rather than a negative manner. Recent studies have demonstrated that an entire set of regulatory gene products, which even include a special sigma subunit for RNA polymerase, are involved in nitrogen control in *E. coli*. The complexity demonstrated for nitrogen regulation in *E. coli* suggest that the nitrogen control circuit of *N. crassa* could be far more complicated than previously imagined. A detailed analysis of the *nit-2* gene, its control, and the interaction of the *nit-2*-encoded regulatory protein with the various nitrogen-related structural genes is now feasible and should reveal many new insights concerning eucaryotic gene regulation.

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