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Molecular cloning and analysis of the regulation of *Nit-3*, the structural gene for nitrate reductase in *Neurospora crassa*

(nitrogen metabolism)

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ABSTRACT **The *nit-3* gene of *Neurospora crassa* encodes the enzyme nitrate reductase and is regulated by nitrogen catabolite repression and by specific induction with nitrate. The *nit-3* gene was isolated from a cosmid-based genomic library by dual selection for benomyl resistance and for the ability to complement a *nit-3* mutant strain using the sibling-selection procedure. The *nit-3* gene was subcloned as a 3.8-kilobase DNA fragment from a cosmid that carried an ≈40-kilobase *N. crassa* DNA insert. A restriction fragment length polymorphism analysis revealed that the cloned segment displayed tight linkage to two linkage-group-4 markers that flank the genomic location of *nit-3*. RNA gel blot analyses of RNA from wild-type and various mutant strains were carried out to examine the molecular mechanism for regulation of *nit-3* gene expression. The *nit-3* gene was transcribed to give a large mRNA of ≈3.4 kilobases, the expected size to encode nitrate reductase. The *nit-3* gene was only expressed in wild-type cells subject to simultaneous nitrogen derepression and nitrate induction. A mutant of *nit-2*, the major nitrogen regulatory gene of *Neurospora*, did not have detectable levels of *nit-3* gene transcripts under the exact conditions in which these transcripts were highly expressed in wild type. Similarly, a mutant of *nit-4*, which defines a minor positive-acting nitrogen control gene, failed to express detectable levels of the *nit-3* transcript. Nitrate reductase gene expression was partially resistant to nitrogen repression in a mutant of the *nmr* gene, which appears to be a regulatory gene with a direct role in nitrogen catabolite repression. Results are presented that suggest that the enzyme glutamine synthetase does not serve any regulatory role in controlling nitrate reductase gene expression.**

In the fungus *Neurospora crassa* the synthesis of enzymes in global areas of metabolism is subject to genetic regulation. Thus, *Neurospora* has been utilized as a model lower eukaryote to investigate complex regulatory circuits (1–3). The nitrogen control circuit of *Neurospora* has been studied extensively and is comprised of a set of genes that encode enzymes that enable this fungus to use various secondary nitrogen sources, e.g., nitrate, nitrite, purines, or amino acids, when preferred nitrogen sources such as ammonia or glutamine are unavailable (1). These nitrogen-related enzymes include nitrate and nitrite reductase, several purine catabolic enzymes, as well as various enzymes, including L-amino acid oxidase, a general amino acid permease, and an extracellular protease involved in the use of proteins and amino acids as nitrogen sources (1, 4–8). The various nitrogen-related structural genes of the nitrogen circuit are unlinked, yet their expression is controlled in a parallel fashion by major and minor control genes as well as metabolic inducers and repressors. The *nit-2* gene is the major nitrogen regulatory gene; it appears to mediate nitrogen catabolite repression in *Neurospora* by turning on the various unlinked structural genes during conditions of nitrogen limitation (1, 8). The *nit-2* gene appears to encode a regulatory protein that diffuses freely throughout the nucleus (9, 10). 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 signaled by the *nit-2* gene product and simultaneous induction by nitrate, which appears to be mediated by the minor control gene *nit-4* (1).

Nitrate reductase is one of the best-studied nitrogen-regulated enzymes of *Neurospora*; it is a dimeric enzyme of M_r 228,000 that is composed of two identical polypeptide subunits (11). Nitrate reductase possesses a molybdenum-containing cofactor, which is also present in other molybdenum enzymes including xanthine dehydrogenase (12). Mutants in a large number of unlinked genes of *Neurospora* lead to the failure to use nitrate as a nitrogen source. The *nit-3* gene encodes the major polypeptide of nitrate reductase and is unlinked to *nit-6*, which encodes nitrite reductase. Mutants of the *nit-2* major control gene and the *nit-4* minor control gene lack both nitrate and nitrite reductase, whereas mutants in at least four other loci are missing the molybdenum cofactor and thus lack nitrate reductase activity (12). The induction of nitrate reductase involves *de novo* enzyme synthesis (11), and indirect studies have suggested that the synthesis of nitrate reductase may be transcriptionally controlled (13, 14). *gln-1b*, a mutant of *gln-1* (the structural gene for glutamine synthetase), was found to be partially resistant to nitrogen repression of nitrate reductase, suggesting a possible regulatory role for glutamine synthetase, perhaps in the negative control of the *nit-2* gene (14, 15). Mutants of a second gene, *nmr-1* also display constitutive synthesis of nitrate reductase and several related enzymes (10, 16–18), which has led to the suggestion that *nmr-1* exerts negative control over *nit-2* (17).

A more complete understanding of the nitrogen control circuit and the role of the proposed regulatory elements requires that representative structural and control genes be isolated and their behavior examined at the molecular level. Vollmer and Yanofsky (19) constructed a *Neurospora* genomic library in pSV50, an efficient cosmid vector that they developed; pSV50 carries a benomyl-resistance marker that permits the rapid isolation of *Neurospora* genes by way of complementation as described in the "sib-selection" protocol (19). We report here the isolation of a single cosmid from this library that carries a 40-kilobase (kb) *Neurospora* DNA insert that both complements the *nit-3* mutant strain and transforms it to benomyl resistance. Here we characterize the *nit-3* gene and show that it encodes a transcript of the expected size based on the subunit molecular weight of nitrate reductase. We also describe results that directly demonstrate that the expression of *nit-3* is transcriptionally regulated and assess the role of each putative regulatory gene in the control of nitrate reductase synthesis.

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MATERIALS AND METHODS

Strains and Growth Conditions. *N. crassa* wild-type 74OR23-1A and the mutant strains *nit-2* a (allele nr37), *nit-3* A (allele 14789), *nit-4* A [Fungal Genetics Stock Center (FGSC) no. 2993], *gln-1b* A (FGSC no. 4536), and *nmr-1* A (allele V2M304) were obtained from the Fungal Genetics Stock Center, Kansas City, KS. Flasks containing Vogel's minimal medium (20) with any required supplements were inoculated with conidial suspensions of wild-type or mutant strains and grown with shaking at 30°C for 24 hr, then the mycelia were harvested. The mycelia were washed and then transferred to fresh Vogel's minimal medium lacking nitrogen supplemented with 25 mM L-glutamine (nitrogen-repressed), with 0.5 mM L-glutamine (nitrogen-derepressed), with 50 mM sodium nitrate (nitrogen-derepressed/nitrate-induced), or with 25 mM L-glutamine plus 50 mM sodium nitrate (nitrogen-repressed/nitrate-induced) to provide contrasting metabolic conditions. After incubation with shaking at 30°C in the second medium for 4 to 6 hr, the mycelia were harvested and frozen at −80°C until they were used.

Nucleic Acid Isolation. *Neurospora* DNA was isolated by a modification of the procedure of Metzenberg and Baisch (21). Neurospora RNA was isolated as described by Reinert *et al.* (22), and poly(A)$^+$ RNA was isolated from total RNA by oligo(dT)-cellulose chromatography (23). Plasmid DNA was isolated by the method of Birnboim and Doly (24).

Transformation. Transformation of *Escherichia coli* was performed by the method of Mandel and Higa (25). Preparation of *Neurospora* spheroplasts and their transformation was conducted as described by Vollmer and Yanofsky (19). A *Neurospora* genomic library constructed by Vollmer and Yanofsky (19) in cosmid pSV50 and organized in microtiter plates was obtained from the Fungal Genetics Stock Center. Plasmid pIBI, a vector with a multiple cloning region and promoters for T3- and T7-specific RNA polymerases, was obtained from International Biotechnologies (New Haven, CT).

Southern and RNA Gel Blots. Southern and RNA gel transfers were performed as described by Maniatis *et al.* (26). After baking, the filters for Southern blots were prehybridized in sealed bags with a solution containing 0.25% dry milk and 6× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate pH 7.0). After at least 1 hr of incubation at 65°C, a ^{32}P-labeled denatured DNA probe was added. RNA samples were electrophoresed in 1% agarose/2.2 M formaldehyde gels. The nitrocellulose filters for RNA gel blots were incubated in a solution of 6× SSC, 0.25% dry milk, and 50% (vol/vol) formamide for 4 hr prior to the addition of a ^{32}P-labeled denatured DNA probe. After hybridization was complete, each filter was washed in 2× SSC/0.1% $NaDodSO_4$ at room temperature for two 15-min periods, and then at least twice in 0.1× SSC/0.1% $NaDodSO_4$ at 65°C over a 1-hr period. DNA to be used as the probe was radioactively labeled by nick-translation (27). Autoradiography was carried out with Kodak XAR-5 film at −70°C.

RESULTS

Cloning of the *nit-3* Gene. The *nit-3* locus of *N. crassa* encodes the enzyme nitrate reductase; *nit-3* mutants completely lack nitrate reductase activity and cannot use nitrate for growth. We screened the cosmid library constructed by Vollmer and Yanofsky (19) to identify a cosmid that would complement a *nit-3* mutant. This library is ordered and arranged into 32 pools of 96 clones, each of which carries a large *Neurospora* genomic DNA fragment cloned into cosmid vector pSV50. This vector carries the dominant marker for benomyl resistance that allowed dual selection for benomyl resistance and for growth with inorganic nitrate as the sole nitrogen source. Protoplasts of the *nit-3* mutant strain were transformed with DNA from each of the 32 cosmid pools to identify a clone that would complement the *nit-3* mutation and simultaneously transform the strain to benomyl resistance. A single cosmid pool yielded positive results, giving rise to benomyl-resistant colonies that grew on nitrate plates; these colonies appeared after ≈8 days at 30°C. The *nit-3* mutant was then transformed with DNA from each of 8 subpools derived from the positive pool identified in the first round. One of these subpools gave *nit-3*$^+$ benomyl-resistant transformants. DNA was then prepared from each of the 12 single *E. coli* transformants isolated from the positive subpool of the second round of selection. A single cosmid was found that transforms the *nit-3* mutant at a high frequency; the other 11 cosmids did not transform *nit-3*.

Subcloning the *nit-3* Gene. The cosmid that complemented the *nit-3* mutation carries a *Neurospora* DNA fragment of at least 40 kb. DNA from this cosmid was digested individually with six different restriction endonucleases, and the cleaved DNA mixtures were then tested for the ability to transform *nit-3* (using only a single selection for growth on nitrate). After digestion with *Pst* I, *Eco*RI, or *Hin*dIII, the cosmid DNA still transformed *nit-3*, whereas digestion with *Bam*HI, *Bgl* II, or *Xho* I destroyed this ability, suggesting that these later three enzymes cut within the gene. Gel analysis revealed that *Eco*RI cleaved the cosmid to give six fragments >3 kb, the minimum size sufficient to contain an intact *nit-3* gene, and four or more fragments of smaller size. Two of the larger *Eco*RI fragments corresponded to vector sequences, leaving four *Neurospora Eco*RI DNA fragments of 3.8, 5.1, 8.0, and 10.2 kb that could carry a functional *nit-3* gene. Each of these fragments was subcloned into plasmid vector pIBI-31 and individually tested for the ability to transform *nit-3*; the subclone that contains the 3.8-kb EcoRI fragments yielded many *nit-3* transformants, whereas the other subclones completely failed to transform *nit-3*. The *nit-3*-positive plasmid with the 3.8-kb insert was designated pNit-3. A restriction map of pNit-3 is shown in Fig. 1. The *Neurospora* insert contains restriction sites for endonucleases *Bam*HI, *Bgl* II, and *Xho* I, as anticipated.

Restriction Fragment Length Polymorphism Analysis. To confirm that the DNA insert present in plasmid pNit-3 is actually *Neurospora* DNA and that it contains the *nit-3* gene,

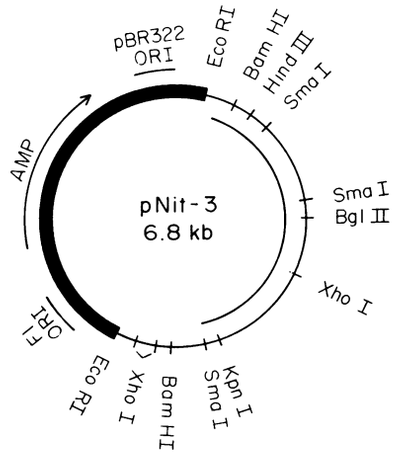

FIG. 1. Restriction map of plasmid pNit-3. pNit-3 contains a *Neurospora* 3.8-kb *Eco*RI DNA fragment cloned into plasmid pIBI31. The vector sequences are shown as a heavy line, with the origin of replication (ORI) for F1 phage and plasmid pBR322 and with the ampicillin resistance gene (AMP). In addition to the restriction sites shown, the *Neurospora* DNA fragment contains one *Apa* I site and one *Sph* I site, both near the lower *Bam*HI site. The approximate location of the *nit-3* gene is indicated by the inner line.

a restriction fragment length polymorphism analysis was performed (28). The results (Table 1) show that the cloned DNA segment is tightly linked to two linkage-group-4 markers, *cot-1* and 5S gene *4*, that flank the *nit-3* gene. These results confirm that pNit-3 carries a unique segment of *Neurospora* DNA derived from linkage group 4 and strongly imply that it indeed contains the *nit-3*$^+$ gene.

Regulation of *nit-3* Gene Expression. The induction of nitrate reductase requires *de novo* enzyme synthesis, and several indirect studies have suggested that *nit-3* gene expression may be transcriptionally regulated (13, 14). It was of considerable interest to directly analyze the transcriptional pattern of the *nit-3* gene with the use of the cloned gene as a hybridization probe. Poly(A)$^+$ RNA was isolated from cells grown under various metabolic conditions and used in RNA gel blot analysis to detect and quantify the amount of the *nit-3* gene transcript present in cells grown under these contrasting conditions. Using plasmid pNit-3 DNA as a probe, two RNA bands were detected (Fig. 2). One of these RNAs is 0.9 kb long and was present in wild-type cells grown under all nutritional conditions tested. This constitutively expressed RNA serves as a useful internal standard to ensure that each lane of gels used for RNA gel blot analyses was loaded with the same amount of poly(A)$^+$ RNA and that the RNA was not degraded. These points were also verified by testing the RNA samples in separate RNA gel blots for the amount of the constitutively expressed β-tubulin mRNA (using plasmid pSV50 as a probe); these independent blots revealed a single transcript of the correct size and confirmed the estimated amount and undegraded condition of each RNA preparation (results not shown). The second transcript detected by hybridization with pNit-3 was 3.4 kb long and was present only in wild-type cells that were derepressed for nitrogen and simultaneously induced with nitrate. We have concluded that this 3.4-kb RNA represents the *nit-3* gene transcript, because it is of the correct size to encode the nitrate reductase subunit and because its content increased dramatically, at least 50-fold, under the precise conditions of nitrogen limitation and induction that result in a similar increase in the level of nitrate reductase protein and enzyme activity in wild-type cells (13, 14). The *nit-3* transcript was either completely absent or present in extremely small amounts in wild-type cells that had been incubated under conditions of nitrogen repression, of nitrogen derepression, or of nitrogen repression and nitrate induction (Fig. 2, lanes 1–3), whereas its content increased greatly in cells that were subjected to a combination of nitrogen derepression and nitrate induction (Fig. 2, lane 4).

Table 1. Restriction fragment length polymorphism analysis of *nit-3*

Strain	Origin of trait		
	cot-1	DNA polymorphism	5S gene *4*
RLM 1-33	O	O	O
4412	M	M	O
4413	M	M	M
4414	M	M	M
4415	M	M	M
4416	M	M	M
4417	M	M	M
4418	M	M	M
4419	M	O	O
4420	M	M	O
4421	O	O	O
4422	O	O	O
4423	O	O	M
4424	O	M	M
4425	O	O	M
4426	O	O	O
4427	O	O	M
4428	O	M	M
4429	O	M	M
4430	O	O	M

DNA isolated from 18 progeny of a cross of Mauriceville-1C-A with RLM 33-1a was digested with *Bam*HI, and after separation on agarose gels, Southern blots were probed with ^{32}P-labeled DNA of the cosmid that complements *nit-3*. The polymorphism analyzed is a *Bam*HI fragment of 18 kb in the RLM 33-1a strain (Oak Ridge genetic background) that is replaced by a smaller, 14-kb *Bam*HI fragment in the Mauriceville strain. This polymorphism showed tight linkage to the linkage-group-4 markers *cot-1* and 5S gene *4*, known to flank the *nit-3* locus, as shown in the table. The presence of the Oak Ridge (O) or Mauriceville (M) trait for each marker in the individual progeny is shown. The strain number identifies the individual progeny from the cross described by Metzenberg *et al.* (28), except that 4416 is the Mauriceville parent and RLM 33-1a is the Oak Ridge parental strain.

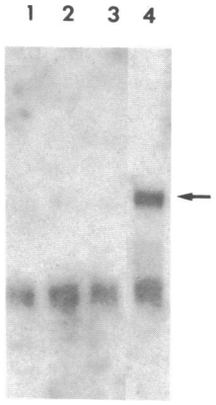

FIG. 2. RNA gel blot analysis of *nit-3* gene expression in wild-type cells. Equal amounts of polyadenylylated RNA were electrophoresed in each lane of gels, blotted to nitrocellulose, and probed with ^{32}P-labeled pNit-3 DNA. This probe reveals the 3.4-kb *nit-3* transcript (identified by the arrow) and a 0.9-kb constitutively expressed RNA, which demonstrates that equal amounts of RNA were loaded into each lane. Lanes: 1, RNA, from nitrogen-repressed cells; 2, RNA from nitrogen-repressed/nitrate-induced cells; 3, RNA from derepressed cells; 4, RNA from derepressed/nitrate-induced cells.

***nit-3* Gene Expression in Regulatory Mutants.** Two well-defined regulatory genes have been shown to control the expression of nitrate reductase (1). One of these, *nit-2* is a major nitrogen control gene of *Neurospora* and appears to mediate nitrogen derepression of numerous nitrogen-related genes, whereas the other, *nit-4*, is a minor control gene that apparently is responsible for nitrate induction of nitrate reductase and of nitrite reductase. Both *nit-2* and *nit-4* appear to be positive-acting regulatory genes because mutants in either of these genes cause a null phenotype and lack both nitrate and nitrite reductase. RNA gel blot analysis of the expression of the *nit-3* gene revealed that the *nit-4* mutant lacked any detectable *nit-3* mRNA under nitrogen-repressed and nitrogen-derepressed/nitrate-induced conditions (Fig. 3). Similarly, the *nit-2* mutant strain lacked any detectable *nit-3* transcript under these same growth conditions (Fig. 3). Thus, both the *nit-2* and *nit-4* mutants lack *nit-3* mRNA under the identical metabolic condition that led to a great increase in the content of the *nit-3* transcript in wild-type cells (Fig. 3). These results imply that the *nit-2* and the *nit-4* regulatory gene products each act at the transcriptional level to turn on the expression of *nit-3*.

Additional Control Features. It has been proposed that the gene *nmr-1*, which is unlinked to *nit-2*, *nit-3*, or *nit-4*, has a negative role in the control of nitrate reductase. Mutants of *nmr-1* have a wild-type phenotype except that they appear to

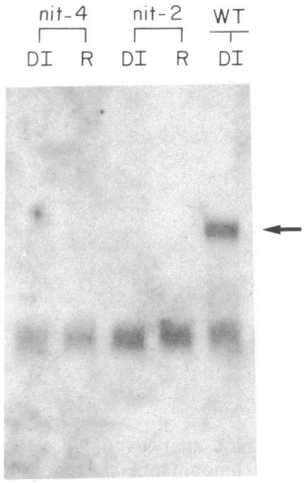

FIG. 3. RNA gel blot analysis of *nit-3* gene expression in wild-type and the regulatory mutant strains, *nit-2* and *nit-4*. The analysis was conducted as described in the legend to Fig. 1. DI, RNA from derepressed, nitrate-induced cells; R, RNA from nitrogen-repressed cells. The arrow identifies the 3.4-kb *nit-3* gene transcript.

be largely insensitive to nitrogen catabolite repression and thus display a partial constitutive synthesis of nitrate reductase and several other nitrogen-related enzymes (10, 16–18). To determine whether *nmr* controls the nitrate reductase structural gene at the level of transcription, we carried out a RNA gel blot analysis of *nit-3* gene expression in the *nmr-1* mutant. The results (Fig. 4) show that under nitrogen-derepressed/nitrate-induced conditions, *nmr-1* cells possessed abundant *nit-3* transcripts, as was found in wild-type cells. However, in contrast to wild-type, *nmr-1* cells also possessed a substantial amount of *nit-3* transcript under nitrogen-repressed/nitrate-induced conditions. This result demonstrates that *nit-3* expression is at least partially resistant to nitrogen repression in *nmr-1* mutant cells.

It has also been suggested that the enzyme glutamine synthetase, encoded by *gln-1*, may have a role in nitrogen regulation, because a particular mutant, known as *gln-1b*, is deficient in glutamine synthetase and also constitutively synthesizes nitrate reductase (15, 16). A RNA gel blot analysis was carried out to determine the *nit-3* gene transcript

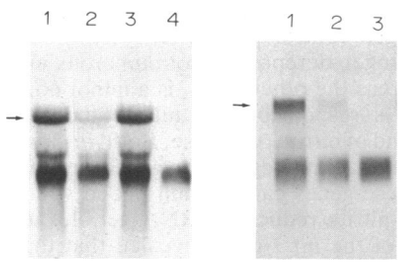

FIG. 4. RNA gel blot analysis of *nit-3* gene expression in wild-type cells and mutant strains, *nmr-1* (allele MS5) and *gln-1b*. The RNA gel blot analysis was carried out as described in Fig. 2. (*Left*) RNA samples were from *nmr-1* and wild-type cells. Lanes: 1, derepressed/nitrate-induced *nmr-1*; 2, nitrogen-repressed/nitrate-induced *nmr-1*; 3, derepressed/nitrate-induced wild-type; 4, nitrogen-repressed/nitrate-induced wild-type. The amount of the *nit-3* transcript in lane 2, when corrected for a lower amount of RNA in that lane, relative to the 0.9-kb constitutive RNA in lane 1, represents ≈20% of the derepressed condition. (*Right*) Different gel in which the RNA samples were all isolated from *gln-1b* mutant cells grown under various conditions. Lanes: 1, derepressed/nitrate-induced; 2, nitrogen-repressed/nitrate-induced; 3, nitrogen-repressed. Arrows identify the 3.4-kb *nit-3* transcript.

content of *gln-1b* mutant cells incubated under various metabolic conditions. Similar to wild-type cells, only a trace of *nit-3* transcript was evident in *gln-1b* cells under nitrogen-repressed/nitrate-induced conditions; the high level characteristic of wild-type cells was present in *gln-1b* cells that were both induced and derepressed (Fig. 4).

DISCUSSION

Several lines of evidence demonstrate that we have cloned the *nit-3* gene from *N. crassa*. The cosmid isolated from the genomic library and subclones derived from it transform a *nit-3* mutant with high efficiency, and a restriction fragment length polymorphism analysis revealed that the cloned DNA segment genetically maps to the precise chromosomal location of the *nit-3* locus. Moreover, the cloned segment hybridizes with a relatively large, 3.4-kb RNA transcript, which is strongly nitrate-inducible and which is of the size required to encode nitrate reductase. Thus, it appears conclusive that *nit-3*, the structural gene for nitrate reductase, has been isolated. A short, constitutively expressed transcript of 0.9 kb was also detected when pNit-3 was used to probe RNA gel blots. Deletion experiments carried out with pNit-3 have shown that this small transcript hybridizes only to the left end of the cloned DNA segment, in the vicinity of the *Xho* I and *Bam*HI sites (Fig. 1), and thus the cloned segment may contain only a portion of this shorter gene. We have not yet excluded the possibility that this small transcript may have some role in the expression or function of nitrate reductase.

Numerous studies have concerned the enzyme nitrate reductase and the regulation of its expression (4, 9, 11–16, 18). Induction of nitrate reductase requires *de novo* synthesis (11), and several indirect studies suggested that nitrate reductase was controlled transcriptionally (13, 14). The availability of the cloned *nit-3* gene allowed us to directly examine the level of specific mRNA for nitrate reductase in wild-type and various mutant strains. The results obtained convincingly demonstrate that the *nit-3* gene is transcriptionally regulated. Thus, nit-3 mRNA was not detectable in wild-type cells under nitrogen repression or not specifically induced with inorganic nitrate. However, upon simultaneous nitrate induction and nitrogen derepression, the amount of *nit-3* transcript present in the wild-type cells increased very dramatically—by a factor of at least 50. These results clearly demonstrate that nitrogen repression of nitrate reductase expression is exerted at the level of transcription and that nitrate induction of the enzyme also takes place at this step. Moreover, neither *nit-2* nor *nit-4* mutant cells possess any detectable nit-3 mRNA, even when nitrogen derepressed and induced. Thus, the activation of *nit-3* expression by the positive-acting *nit-2* and *nit-4* regulatory genes also leads to a dramatic increase in the cellular content of nitrate reductase mRNA. These results strongly imply that each element of metabolic and genetic control that governs *nit-3* expression acts at the level of transcription, although we cannot completely exclude the possibility that a closely related step such as mRNA stability might also be involved. The regulation of gene expression in *Neurospora* has been examined at the molecular level in only a few cases. Metabolic and genetic regulation of the quinic acid catabolic genes (29), cross-pathway control of the *arg-12* gene (30), and developmental regulation of conidiation-specific genes (31) occur at the transcriptional level.

We examined the possibility that two other possible nitrogen control genes, *nmr-1* and *gln-1*, might regulate the transcription of *nit-3*. The results demonstrated that the *gln-1* mutant behaved very much like wild-type cells and did not express the *nit-3* gene to any significant level during nitrogen repression in the presence of inducer. We have concluded that glutamine synthetase does not have any regulatory function in the control of *nit-3* gene transcription. In contrast,

the results that we obtained revealed that *nit-3* gene transcription was largely insensitive to nitrogen repression in the *nmr* mutant strain under conditions where full repression was observed in wild-type cells. Thus, it appears that *nmr* is a control gene that has an important role in nitrogen catabolite repression. *nmr* does not control *nit-2* gene transcription (32), suggesting that the *nmr* gene product might interfere with gene activation by directly binding to the *nit-2* regulatory protein or, alternatively, that it might interact at a cis-acting recognition sequence adjacent to *nit-3* and other nitrogen-controlled structural genes. Obviously, considerable additional work will be necessary to define the exact role which the *nmr* gene plays in the nitrogen regulatory circuit of *Neurospora*.

The isolation of the *nit-3* gene will make possible significant progress and insight concerning nitrogen regulation in *Neurospora*. Of particular interest will be potential upstream control sites of *nit-3* that may serve as recognition elements for the *nit-2* and *nit-4* positive-acting regulatory proteins. The molecular cloning and characterization of the *nit-2* gene (32, 33) is highly significant in this regard since it makes possible a precise definition of the *nit-2*-encoded major nitrogen regulatory protein, as well as the means to obtain useful amounts of this protein. The major nitrogen regulatory gene of *Aspergillus nidulans, areA*, has been cloned (34) so that a detailed comparison of it with the homologous *nit-2* gene of *Neurospora* should soon be possible and can be anticipated to provide insight concerning nitrogen regulation in lower eukaryotes.

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