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Binding Affinity and Functional Significance of NIT2 and NIT4 Binding Sites in the Promoter of the Highly Regulated *nit-3* Gene, Which Encodes Nitrate Reductase in *Neurospora crassa*

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In the filamentous fungus *Neurospora crassa*, both the global-acting regulatory protein NIT2 and the pathway-specific regulatory protein NIT4 are required to turn on the expression of the *nit-3* gene, which encodes nitrate reductase, the first enzyme in the nitrate assimilatory pathway. Three NIT2 binding sites and two NIT4 binding sites have been identified in the 1.3-kb *nit-3* promoter region via mobility shift and footprinting experiments with NIT2–β-galactosidase and NIT4–β-Gactosidase fusion proteins. Quantitative mobility shift assays were used to examine the affinity of individual NIT2 binding sites for the native NIT2 protein present in *N. crassa* nuclear extracts. In vivo analysis of *nit-3* promoter 5′ deletion constructs and individual NIT2 and NIT4 binding-site deletions or mutations revealed that all of the NIT2 and NIT4 binding sites are required for the full level of expression of the *nit-3* gene. A cluster of two NIT2 and two NIT4 binding sites located more than 1 kb upstream of the translational start site is required for *nit-3* expression, and one NIT2 binding site and one NIT4 site, which are immediately adjacent to each other, are of particular functional importance. A significant NIT2-NIT4 protein-protein interaction might occur upon their binding to nearby sites.

The filamentous fungus *Neurospora crassa* is able to utilize secondary nitrogen sources such as nitrate, purines, and various amino acids when preferred primary nitrogen sources such as ammonia, glutamine, or glutamate are not available (14, 15). A series of unlinked structural genes which encode various nitrogen catabolic enzymes must be turned on by global-acting as well as pathway-specific regulatory factors for secondary nitrogen source assimilation. When nitrate is present as the sole nitrogen source, nitrogen catabolic repression is lifted, and nitrate serves as an inducer for expression of nitrate assimilation pathway genes, *nit-3* and *nit-6*, which encode nitrate reductase and nitrite reductase, respectively. Expression of *nit-3* and *nit-6* is turned on by the global positive-acting nitrogen regulatory factor NIT2 and the pathway-specific regulatory protein NIT4.

NIT2 is a 110-kDa regulatory protein with a single Cys-X_2-Cys-X_{17}-Cys-X_2-Cys-type zinc finger DNA binding motif (5, 6). Three NIT2 binding sites have been identified in the *nit-3* promoter; one is located near the start site for the *nit-3* gene, whereas the other two binding sites are far upstream, approximately 1 kb away from the *nit-3* gene (7). NIT2 DNA binding is dependent on core GATA sequence elements (2). NIT4 is a 120-kDa positive-acting regulatory protein with a single GAL4-like Zn(II)Cys_6-type zinc finger DNA binding motif (20). Two NIT4 binding sites have also been identified in the *nit-3* promoter region (4). In addition to the proximal NIT2 binding site, a cluster of two NIT2 and two NIT4 binding sites is located far upstream of the *nit-3* gene, as diagrammed in Fig. 1.

In this study, we report the results of quantitative in vitro electrophoretic mobility shift assays (EMSAs) to estimate the relative affinities of the three individual NIT2 binding sites for the native NIT2 protein present in nuclear extracts of *N. crassa*. We also report in vivo analyses of *nit-3* promoter 5′ deletions and the effects of deletion or mutation of individual NIT2 and NIT4 sites on *nit-3* expression in order to understand the physiological significance of the distinct regulatory protein binding elements.

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MATERIALS AND METHODS

***N. crassa* nuclear extract preparation.** One liter of 1× Vogel's medium was inoculated with wild-type conidia from a 7-day culture and grown overnight at 30°C. The overnight culture yielded about 20 g (wet weight) of mycelia. Nuclei were isolated from mycelia as described by Hautala et al. (10). Nuclear extracts were obtained by sonicating the nuclei in buffer A, applying the lysate to a heparin-agarose column, and eluting the preparation with buffer A containing 0.5 M KCl. The sample was diluted with buffer A without KCl to a final KCl concentration of 50 mM and then concentrated in a Centricon-10 concentrator (Amicon, Inc., Lexington, Mass.).

EMSA. Three 0.5-kb *Eco*RV-*Xba* fragments were cut out from the wild type and mutant *Eco*RV-*Bam*HI subclones of the *nit-3* promoter region. Fragments 1, 2, and 3 contain wild-type NIT2 binding sites A and B, site B, and site A, respectively, and fragment 4, an *Xba-Xho* DNA fragment, contains NIT2 binding site E, as shown in Fig. 2B. These DNA fragments were end labeled with ^{32}P by 3′ fill-in with DNA polymerase I Klenow fragment. The ^{32}P-labeled DNA probes were incubated with nuclear extract containing native NIT2 protein and electrophoresed under conditions described previously (7). The gels were dried and scanned with a Betascope 603 blot analyzer (Betagen, Boston, Mass.). The results are presented as the fraction of each probe which was retarded as a DNA-protein complex and represent a quantitative measure of NIT2 protein binding to each DNA fragment examined.

5′ deletions of the *nit-3* promoter. Five *nit-3* promoter 5′ deletion constructs, pDEn3DD, pDEn3SK, pDEn3KKW, pDEn3KKM, and pDEn3PK, were made by cloning a series of restriction fragments that constitute truncations of the *nit-3*

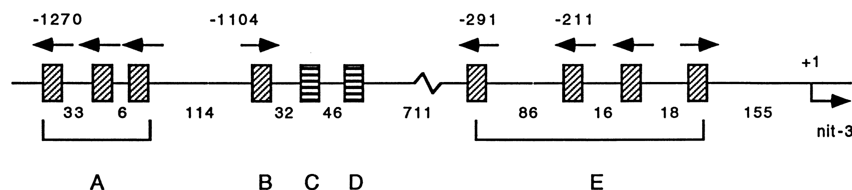

FIG. 1. Schematic diagram of the *nit-3* promoter region with three NIT2 binding sites, A, B, and E, and two NIT4 binding sites, C and D. ▧, GATA core element in each NIT2 binding site; ▤, NIT4 binding site C or D. Arrowheads above the boxes show the orientation of each GATA element, → for 5′-GATA and ← for 5′-TATC- on the coding strand. The numbers above the boxes indicate the locations of the binding sites relative to the translational start site. The numbers below and between the boxes indicate the distances, in base pairs, between binding sites.

promoter into the pDE1 vector as diagrammed in Fig. 3. These constructs were transformed into a *nit-3* repeat-induced point mutation (RIP) mutant to determine the effects of deletions of the promoter region on nitrate reductase expression.

Constructs with mutated NIT2 or NIT4 binding sites. Twelve binding-site mutation constructs were also made to study the contribution of individual binding sites upon nitrate reductase expression, as diagrammed in Fig. 4. pBSn3RK contained the wild-type *nit-3 Eco*RV-*Kpn*I subclone with the full-length *nit-3* promoter. pBSn3RK/BCDE, pBSn3RK/ACDE, pBSn3RK/ABDE, pBSn3RK/ABCE, and pBSn3RK/ABCD contained *nit-3 Eco*RV-*Kpn*I with NIT2 and NIT4 binding sites A through E mutated, respectively. pBSn3RK/CDE had NIT2 sites A and B mutated. pBSn3RK/BCD had NIT2 sites A and E mutated, and pBSn3RK/ACD had NIT2 sites B and E mutated. pBSn3RK/CD had all three NIT2 binding sites, A, B, and E, mutated. pBSn3RK/ABE had both NIT4 binding sites C and D deleted. pBSn3PK, which contained only the *nit-3 Pst*I-*Kpn*I subclone and was missing the entire promoter region and the transcriptional start site, served as a negative control. The *nit-3* wild-type 0.6-kb *Spe*I-*Pst*I fragment was also cloned into the pBluescript vector as the template for NIT2 site E mutagenesis. All four GATA elements in site E were mutated into TATAs. The mutated site E was put into the *nit-3 Eco*RV-*Kpn*I subclone by cutting out the wild-type *Spe*I-*Pst*I fragment and substituting the mutated *Spe*I-*Pst*I fragment. All mutations were verified by sequencing.

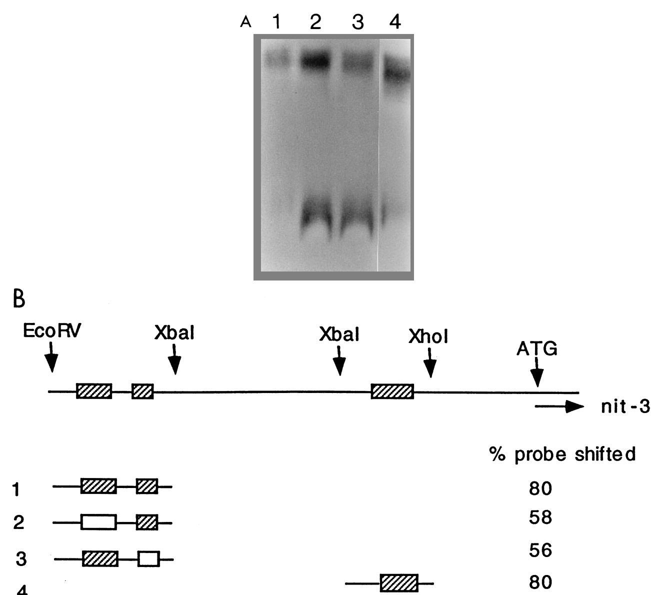

FIG. 2. EMSA with NIT2 binding sites in the *nit-3* promoter. (A) The affinity of the NIT2 protein for four DNA fragments defined in panel B, which contain different NIT2 binding sites, was examined by the mobility shift technique. Lanes 1, 2, 3, and 4 contained *Eco*RV-*Xba*I fragments from pn3RK/AB with both NIT2 sites A and B, pn3RK/B with NIT2 site B, pn3RK/A with NIT2 site A, and an *Xba*I-*Xho*I fragment from pn3RK/E containing NIT2 site E, respectively, which were incubated with *N. crassa* nuclear extract containing native NIT2 protein prior to EMSA. (B) Schematic diagram of the *nit-3* promoter. DNA fragments 1 through 4 with restriction sites indicated were used as probes in the EMSA analysis shown in panel A. Hatched boxes represent NIT2 binding sites; open boxes represent mutated, nonfunctional NIT2 binding sites. The results of quantitative EMSA analysis shown in panel A are reported as the percentage of each DNA fragment that was shifted.

***N. crassa nit-3* RIP *his-3* double mutant.** To transform the constructs with deleted or mutated *nit-3* promoters into *N. crassa* without targeting back to the resident gene locus by homologous recombination, deletion or mutation of the entire *nit-3* gene and its promoter region was essential. The *N. crassa nit-3* mutant strain 14789A carries a point mutation in the 3′ end of the *nit-3* protein-coding region (16) and could undergo homologous recombination with the transforming mutant *nit-3* gene and therefore was not suitable as the host for mutant promoter analysis. RIP was used to obtain a mutant strain damaged throughout the entire promoter and coding regions of the *nit-3* gene (18). Furthermore, to avoid the position effects when transforming DNAs integrate at various ectopic locations in the genome, it is preferable to target each construct to the same genomic location. An *N. crassa nit-3 his-3* double mutant strain was therefore generated as the host for transformation analysis of *nit-3* mutant constructs. This host strain, which contains a disrupted *nit-3* gene obtained by the RIP process and a *his-3* gene with a point mutation at 3′ end, was constructed as follows.

A 4.5-kb *nit-3 Dra*II-*Dra*II fragment covering the full-length *nit-3* structural gene, 3′ flanking sequences, and 5′ flanking sequences including the promoter region to be studied was cloned into the pDE vector and transformed into *his-3* mutant strain Y234M723 spheroplasts. The pDE vector contains a 5′-truncated *his-3* gene, which cannot work by itself but can recombine with the *his-3* mutant (allele Y234M723) which carries a point mutation in the 3′ end of the coding region. Twelve transformants showed the *his-3* wild-type phenotype. One transformant, T1, was crossed with wild-type strain 74a; 100 ascospores from this cross were tested, among which 27 displayed a *nit-3* mutant phenotype. Two *nit-3* RIP *hit-3*$^+$ progeny, C2a and C3a, were crossed with the *his-3* mutant to derive the desired double-mutant strain; among 72 progeny examined, 23 showed the *nit-3 his-3* double-mutant phenotype. A strain designated RIP15A (*nit-3* RIP *his-3*) was chosen to be the host for mutant *nit-3* promoter transformations.

***N. crassa* growth conditions.** The growth medium consisted of 1× Vogel's minimum medium without nitrogen, 1.5% sucrose, and 0.01 μg of biotin per ml, with the addition of either 25 mM L-glutamine or 20 mM potassium nitrate for nitrogen repression or nitrate induction, respectively. The *nit-3* mutant strain cannot grow on nitrate and was provided with glutamine, and the *nit-3 his-3* double-mutant strain was grown on medium containing glutamine and 1 mM histidine.

***N. crassa* genomic DNA isolation and Southern blot analyses.** Small amounts of *N. crassa* DNA were obtained as described by Leach et al. (12). The DNA samples were electrophoresed in a 0.7% agarose gel, and Southern blotting was carried out as described by Sambrook et al. (17). To detect the *nit-3* gene, DNA samples were digested with *Eco*RV, and *nit-3 Dra*II-*Dra*II, *Eco*RV-*Bam*HI, and *Sna*BI-*Spe*I DNA fragments were used as probes. To detect the *his-3* gene, DNA was digested with *Bam*HI, and the *Nde*I-*Xba*I DNA fragment from the pDE vector which contains the *his-3* gene was used as the probe. Southern analysis confirmed that RIP15A carried only a single copy of the *nit-3* gene, i.e., the damaged resident *nit-3* gene. All transformants assayed for nitrate reductase activity were confirmed by Southern blot to have a single copy of the transformed *nit-3* gene construct (located at the *his-3* locus) plus the RIP-generated genomic *nit-3* gene. Representative Southern analysis results of transformants that received 5′ deletion constructs are shown in Fig. 5.

Nitrate reductase assays. Mycelia from 50-ml cultures grown at 30°C with shaking at 150 rpm for 14 h under nitrogen-repressed conditions were harvested via Miracloth and rinsed with water. The mycelial pads were then transferred into nitrogen repression or nitrogen derepression-nitrate induction medium and incubated for an additional 4 h under the same conditions. The mycelial pads were harvested, frozen in liquid nitrogen, and ground with sand and extraction buffer (100 mM phosphate buffer [pH 6.8], 1 mM β-mercaptoethanol, 0.5 mM EDTA, 1% NaCl) on ice. The mixture was then centrifuged, and 100 μl of the supernatant was used for assay of nitrate reductase enzyme activity as described previously (9). Specific activity was calculated from measurements taken at 25 min of reaction time. Protein concentrations were assayed by the Bradford method, using bovine serum albumin as the standard. At least three independent transformants were tested for every construct examined and found to yield consistent results. All protein and enzyme assays were performed in duplicate.

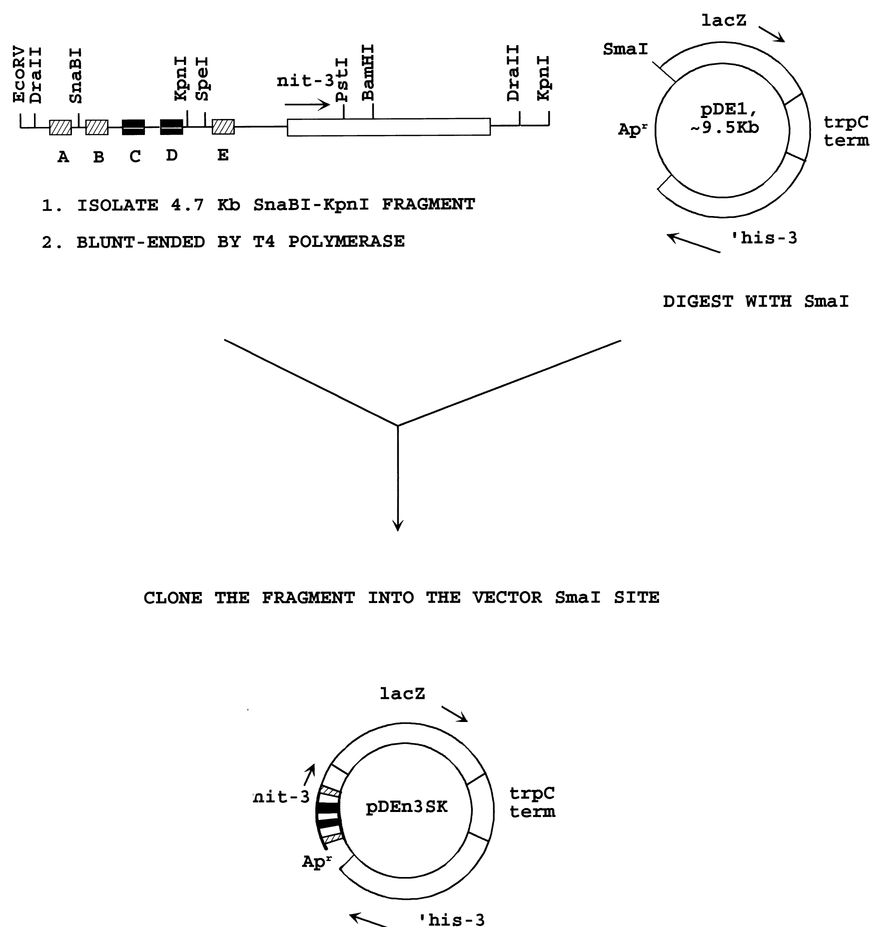

FIG. 3. Construction of 5′ deletions of the *nit-3* promoter. The figure shows construction of pDEn3SK, in which a 4.7-kb *Sna*BI-*Kpn* restriction fragment was isolated and cloned into plasmid pDE1, which contains the truncated *his-3* gene used to target constructs to the *his-3* locus. Other DNA fragments were similarly cloned into pDE1 to obtain a set of 5′ promoter deletion constructs. pDEn3SK contained the 4.7-kb *Sna*BI-*Kpn*I *nit-3* fragment with NIT2 binding site A deleted. Construct pDEn3KKW contained the 4.3-kb *nit-3 Kpn*I-*Kpn*I fragment with only NIT2 binding site E remaining. pDEn3KKM was the same as pDEn3KKW except all GATA elements in the NIT2 binding site E were mutated to TATA. pDEn3DD, which contained the 4.5-kb *nit-3 Dra*II-*Dra*II fragment with all three NIT2 binding sites, A, B, and E, two NIT4 binding sites, C and D, and the full-length *nit-3* structural gene, served as a wild-type positive control. pDEn3PK, which contained *nit-3* 3.4-kb *Pst-Kpn* fragment which lacked the entire promoter plus the transcriptional start site, served as a negative control. term, termination site.

RESULTS

Affinity of NIT2 binding sites within the *nit-3* promoter. Binding sites for the global-acting NIT2 have been located in the promoters of the *N. crassa nit-3*, *alc*, and *lao* genes, which encode nitrogen catabolic enzymes (8, 13). Each site contains two or more GATA core elements, which were found to be essential for NIT2 binding, although bases flanking the GATA sequences seemed to make only minor contributions to binding-site strength (1, 2).

Three NIT2 binding sites (A, B, and E) have been identified in the upstream promoter region of the *nit-3* gene by EMSA and footprinting experiments with a NIT2–β-galactosidase fusion protein (7). Site A contains three GATA elements, site B contains only a single GATA, and site E has four GATA sequences (Fig. 1). Four different DNA fragments were used as probes for EMSAs to compare the affinities of these individual sites for the native NIT2 protein present in *N. crassa* nuclear extracts (Fig. 2). The results of the quantitative EMSAs are shown in Fig. 2A and summarized in Fig. 2B. Fragments 1 and 4 each contain four GATA elements, three of which are clustered (Fig. 1 and 2B), and showed comparable NIT2 binding (80% of the probes shifted). Fragment 3, which contains three GATA core elements (site A), exhibited moderate NIT2 binding (56% of the probe shifted). Fragment 2, which contains only a single GATA element (site B), would not be expected to constitute a strong NIT2 binding site according to previous in vitro studies of NIT2 binding characteristics conducted with an *Escherichia coli*-expressed NIT2–β-galactosidase fusion protein (1, 2). However, surprisingly, fragment 2 showed considerable binding (56%) with the native NIT2 protein present in *N. crassa* nuclear extracts, with an affinity comparable to that observed with the other fragments.

Construction of a *his-3 nit-3* host strain for transformation. To transform manipulated gene constructs with deleted or mutated *nit-3* promoter regions into *N. crassa* without the possibility of obtaining a completely wild-type sequence by homologous recombination, it was necessary to have either a deletion or a completely damaged copy of the entire *nit-3* gene and its promoter region. Furthermore, because the expression of genes inserted at various ectopic locations can be significantly influenced by position effects, it was essential to target each construct to the same genomic location. An *N. crassa nit-3 his-3* double-mutant strain was developed to serve as the host for transformation. This host strain, which contains a *nit-3* gene

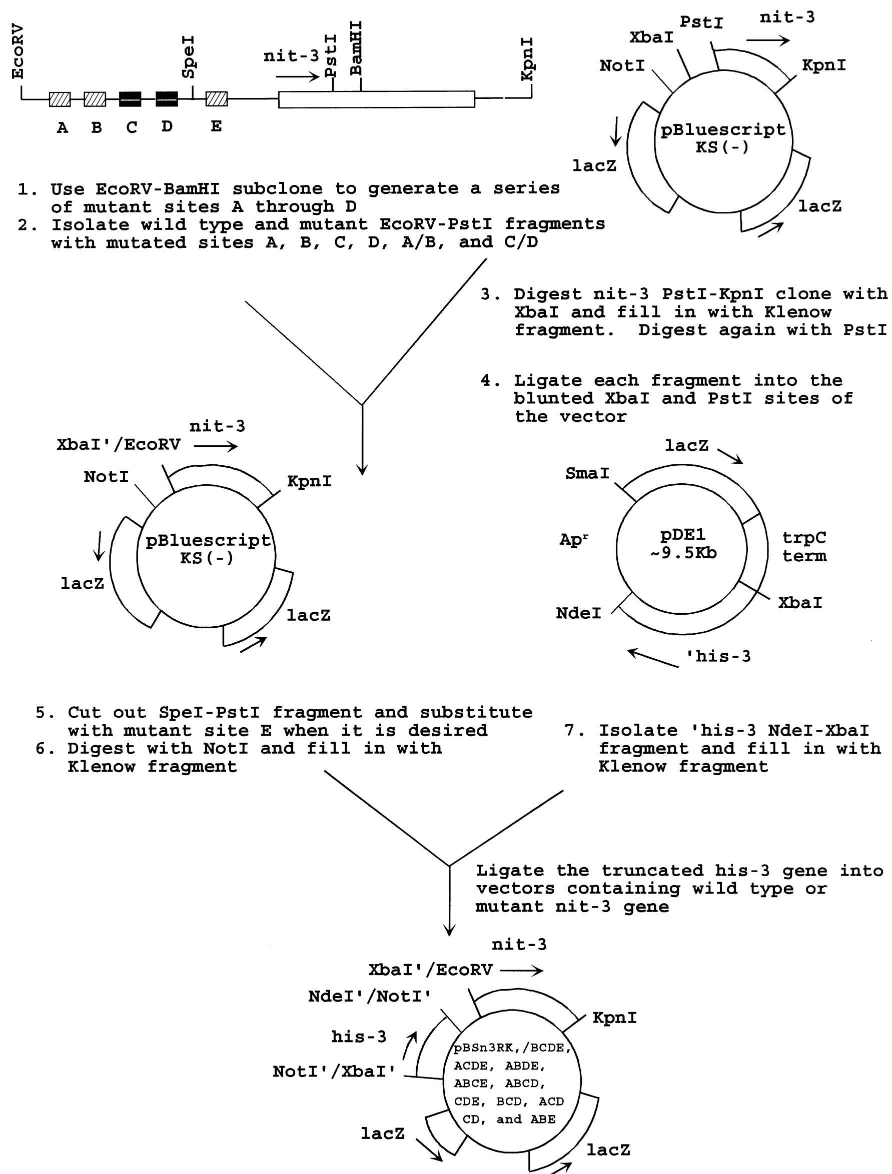

FIG. 4. Construction of mutations in NIT2 and NIT4 binding-site elements. A *nit-3* wild-type 2-kb *Eco*RV-*Bam*HI fragment, which contained NIT2 binding sites A and B and NIT4 binding sites C and D, was cloned into the pBluescript vector and served as the template for a series of site-directed mutations. Four mutagenic primers were designed to mutate all three GATA elements in NIT2 site A and one in site B to TATA and to delete the core sequences in NIT4 sites C (TCCGCGGA) and D (TCCGTGGA), respectively (4). The mutated *Eco*RV-*Bam*HI subclones carrying mutations in sites A through D served as templates for sequential mutations until all possible combination of binding-site mutations were obtained. *Eco*RV-*Pst*I fragments were then cut out from wild-type or mutated *Eco*RV-*Bam*HI subclones carrying one or more nonfunctional sites and cloned into the pBluescript vector containing the *nit-3 Pst*I-*Kpn*I subclone, which was first cut with *Xba*I and blunt ended with DNA polymerase I Klenow fragment and then cut again with *Pst*I. Thus the full-length *nit-3 Eco*RV-*Kpn*I fragment containing the *nit-3* promoter with various mutations of NIT2 and NIT4 binding sites A through D was obtained, with the *Eco*RV site destroyed. The *Nde*I-*Xba*I fragment from the pDE vector containing the 5′-truncated *his-3* gene was blunt ended and cloned into the *Not*I site in the polylinker region of the pBluescript vector carrying the *nit-3 Eco*RV-*Kpn*I subclone. term, termination site.

severely damaged along its entire length by the RIP process (18) and a *his-3* gene with a point mutation at its 3′ end, was constructed as described in Materials and Methods and verified by Southern blot analysis (not shown). As expected, this double mutant required histidine, could not utilize nitrate, and completely lacked nitrate reductase activity.

In vivo analysis of 5′ deletion constructs of the *nit-3* promoter. NIT2 binding sites and NIT4 binding sites in the *nit-3* promoter region have been identified in vitro via EMSA and footprinting experiments (4, 7). However, it was unknown whether these binding sites have any physiological significance in controlling either the level or regulation of expression of the *nit-3* gene. Several NIT2 and NIT4 binding sites are located far upstream (−1000 to −1300) of the transcriptional start site. A series of 5′ deletions was constructed to determine whether these distal binding sites are required for *nit-3* gene expression (Fig. 3).

These constructs, with various 5′ deletions of the *nit-3* promoter, were inserted into the pDE vector containing a truncated *his-3* gene and transformed into the *N. crassa nit-3 his-3* double-mutant strain as described in Materials and Methods. Transformants were selected for the ability to grow without

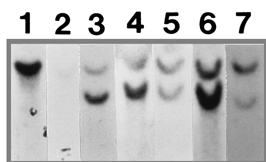

FIG. 5. Southern analysis of the *nit-3* RIP15A mutant transformed with constructs containing a *nit-3*$^+$ gene with various 5′ deletions of the promoter. Genomic DNA was isolated from various strains, digested with *Eco*RV, and electrophoresed, and Southern blots were hybridized with a *nit-3* gene probe. Lanes: 1, wild-type 74A; 2, the *nit-3* RIP15A mutant; 3, RIP15A transformed with pDEn3DD; 4, pDEn3SK transformant; 5, pDEn3KKw transformant; 6, pDEn3KKM transformant; 7, pDEn3PK transformant. The upper band in each case represents the genomic copy of *nit-3*; the lower bands identify the single copy of the transformed *nit-3* promoter deletion constructs, which had integrated at the *his-3* locus.

histidine, which required that the plasmid had integrated at the *his-3* locus by homologous recombination. Southern blots of the transformants demonstrated insertion of a single copy of each construct at the *his-3* genomic location (Fig. 5). Each transformant was assayed for nitrate reductase activity under both nitrogen repression and derepression-nitrate induction conditions. The nitrate reductase specific activity observed in transformants for each construct was standardized by comparison with the nitrate reductase activity of transformants pDEn3DD, with the entire 5′ promoter region of *nit-3* serving as a wild-type control (Fig. 6).

The most distal NIT2 binding site (site A) was deleted in pDEn3SK, transformants of which contain only 15% of the nitrate reductase activity found in the wild-type control, although it was regulated in a normal fashion by induction and nitrogen repression. This result demonstrated that NIT2 binding site A, located at approximately −1200 bp, is important to fully turn on the expression of *nit-3* expression, but its loss had no effect on control since normal nitrogen repression and induction were still fully operational. pDEn3KKW possesses approximately 1 kb of the *nit-3* 5′ promoter region including the intact NIT2 site E but lacks the far-upstream NIT2 and NIT4 binding sites; transformants of pDEn3KKW failed to express any detectable nitrate reductase under either repression or derepression-nitrate induction conditions, demonstrating that the distal control elements located at least 1 kb upstream are required for activation of the *nit-3* gene. Transformants of pDEn3KKM and pDEn3PK similarly lacked any nitrate reductase activity (Fig. 6).

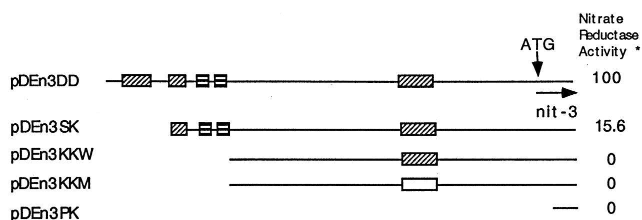

FIG. 6. In vivo analysis of 5′ deletions of the *nit-3* promoter region. Schematic diagrams of constructs carrying 5′ deletions in the *nit-3* promoter are shown. ▨, NIT2 binding site; ⊟, NIT4 binding site; □, mutated binding site. pDEn3DD, pDEn3SK, pDEn3KKW, pDEn3KKM, and pDEn3PK were constructed by cloning the *nit-3* full-length protein-coding region with 3′ flanking sequences and the indicated deleted 5′ promoter sequence into the pDE vector carrying the 5′-truncated *his-3* gene. Transformants with pDEn3DD served as a wild-type control. Specific nitrate reductase activity under derepressed and nitrate-induced conditions shown for each transformant of a *nit-3 his-3 N. crassa* strain containing each construct was standardized by comparison with the specific activity of pDEn3DD transformants. Under nitrogen repression conditions, the transformed *nit-3* constructs were controlled normally and possessed either no nitrate reductase activity or only low basal levels of the enzyme. Transformants of all constructs lacking the distal NIT2 and NIT4 binding sites were incapable of growing on nitrate and lacked any detectable nitrate reductase activity.

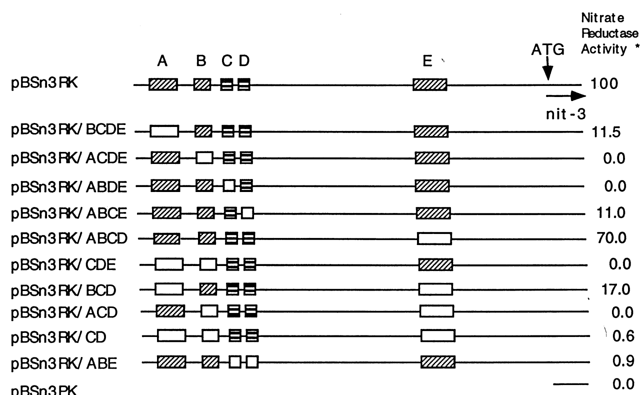

FIG. 7. In vivo analysis of NIT2 and NIT4 binding sites of the *nit-3* promoter region. Schematic diagram of constructs carrying NIT2 or NIT4 binding-site mutations or deletions are shown. ▨, NIT2 binding site; ⊟, NIT4 binding site; □, mutated or deleted binding site. pBSn3RK through pBSn3PK were constructed by cloning the *nit-3* full-length protein-coding region with 3′ flanking sequences and each 5′ mutated promoter into a pBluescript vector together with a 5′-truncated *his-3* gene. All constructs were transformed into RIP15A (*nit-3 his-3*) and selected for *his-3*$^+$ wild-type phenotype. Specific nitrate reductase activity under derepressed and nitrate-induced conditions of each of the transformants of RIP15A containing each construct was standardized by comparison with the specific activity of construct pBSn3RK transformants. Values are averages of assays with at least three independent transformants for each construct, which yielded consistent results. Under nitrogen repression conditions, all transformants with these constructs were regulated normally and expressed either no nitrate reductase activity or only basal levels of this enzyme. Transformants which had less than 1% of the control level of nitrate reductase (which is within the limit of sensitivity of the assay) were not able to grow with nitrate as the nitrogen source.

In vivo analysis of individual NIT2 and NIT4 binding sites. To precisely define the functional role played by each of the multiple NIT2 and NIT4 binding sites, we designed a number of constructs which had mutated NIT2 sites or small deletions that had no NIT4 binding sites but otherwise contained the entire upstream *nit-3* promoter region (Fig. 7). Each of these constructs was inserted into the pBluescript vector together with a truncated *his-3* gene and transformed into an *N. crassa nit-3 his-3* double-mutant strain. Transformants were selected for growth on minimal medium and shown to have integrated a single copy of the transforming vector at the *his-3* locus by Southern blot analysis (results not shown). At least three independent transformants for each construct were assayed for nitrate reductase activity under both nitrogen catabolite repression and nitrogen derepression-nitrate induction conditions. The specific activity of nitrate reductase in each case was standardized by comparison with the activity of transformants that received the wild-type promoter sequence pBSn3RK, with its nitrate reductase activity set at 100% (Fig. 7).

Transformants with construct pBSn3RK/BCDE, which has a mutated NIT2 site A, has considerably reduced nitrate reductase activity under derepression and nitrate induction conditions, approximately 12% of the wild-type control level, although the strain was still sensitive to nitrogen catabolite repression. This result was in excellent agreement with the analysis with deletion construct pDEn3SK (Fig. 6), confirming that the most-upstream NIT2 site A was important for *nit-3* expression, but its loss had no effect on nitrogen repression or nitrate induction regulation. Mutation of NIT2 site B in pBSn3RK/ACDE resulted in the total loss of nitrate reductase activity under both repressed and induced conditions, which

was surprising considering that there was only one base change in the entire *nit-3* promoter. Transformants of construct pBSn3RK/ABDE, which had a small deletion that removed NIT4 site C, located 32 bp downstream of NIT2 site B (Fig. 1), also failed to express any nitrate reductase during either nitrogen repression or derepression and induction. Deletion of NIT4 site D in construct pBSn3RK/ABCE, which is an imperfect palindrome and a relatively weaker NIT4 binding site (4), resulted in the loss of 89% of the wild-type level of nitrate reductase activity. Loss of NIT2 site E, which is the element closest to the translational start site (Fig. 1), only reduced nitrate reductase activity to 70% of that found with the wild-type promoter; moreover, transformants of this construct (pBSn3RK/ABCD) were subject to proper repression and induction regulation. Transformants of constructs pBSn3RK/CDE, pBSn3RK/ACD, and pBSn3RK/CD, all of which lacked a functional NIT2 site B and were also missing either NIT2 site A or E or both, failed to express any nitrate reductase; this result was consistent with the finding that transformants of construct pBSn3RK/ACDE, which was missing only NIT2 site B, lacked any detectable nitrate reductase activity. Transformants of construct pBSn3RK/BCD had induced nitrate reductase activity reduced to 17% of the wild-type control level, similar to that found with construct pBSn3RK/BCDE. Finally, transformants of construct pBSn3RK/ABE, which was missing both NIT4 sites, failed to express any nitrate reductase, as expected.

DISCUSSION

Expression of *nit-3*, the structural gene which encodes nitrate reductase, is completely dependent on the presence of NIT2, the global-acting positive regulatory protein that mediates nitrogen catabolite derepression (5, 15). The two distal NIT2 binding sites 1.2 kb upstream of *nit-3* and the single proximal NIT2 site at 0.3 kb were each studied for their in vitro binding ability and in vivo functional significance. The proximal NIT2 binding site E exhibited the strongest binding ability in vitro yet showed the least functional role in vivo in controlling *nit-3* gene expression. The distal NIT2 binding sites A and B showed comparable in vitro binding activity, surprisingly since site B contains only a single GATA element. Transformants of constructs deleted for the most distal element, site A, expressed approximately 15% of the normal level of nitrate reductase, and its regulation via nitrogen repression and nitrate induction was intact. In contrast, the loss of NIT2 site B resulted in a complete loss of *nit-3* expression.

Expression of the *nit-3* gene is also totally dependent on NIT4, the pathway-specific DNA-binding regulatory protein believed to mediate nitrate induction. The two NIT4 binding sites are located far upstream of the *nit-3* structural gene and adjacent to each other (4). Deletion of the stronger NIT4 binding site C totally eliminated nitrate reductase expression in vivo, while constructs deleted for NIT4 site D expressed approximately 10% of the full activity.

These results indicate that each of the NIT2 and NIT4 binding sites contributes to the full-level expression of the *nit-3* gene, although two sites, NIT2 site B and NIT4 site C, seem of paramount importance for any expression. The finding that NIT2 site B is clearly of greater physiological importance in vivo than either site A or site E raises the question of whether its location close to the two NIT4 binding sites is of functional significance. The clustering of the far-upstream NIT2 and NIT4 binding elements suggests that their close spacing may be important in gene expression. Binding of the NIT2 protein to sites A and B and of the NIT4 protein to sites C and D might occur in a cooperative fashion; the binding of one protein might increase the affinity of nearby elements for the second protein. No interaction between NIT2 and NIT4 proteins free in solution could be detected (19). However, upon occupation of nearby sites, an interaction between NIT2 and NIT4 might stabilize the binding of each to their respective promoter elements.

An interesting feature of the regulation of the *nit-3* gene is that its expression requires the presence of both NIT2 and NIT4, and the lack of either of these regulatory proteins totally precludes any *nit-3* function. In addition to possible cooperative DNA binding, it is conceivable that the NIT2 and NIT4 proteins each contribute to an activation domain, such that the proteins must be close to one another in order to turn on the *nit-3* gene. Future experiments will be required to test the potential significance of the proximity of NIT2 and NIT4 sites.

Putative NIT2 and NIT4 binding sites were also found in the *N. crassa nit-6* gene promoter sequence (3), which is also under the control of both NIT2 and NIT4. The distribution of sites is quite distinct from that for *nit-3*, with three possible NIT4 binding sites at −695, −677, and −547 upstream of *nit-6*, one GATA element at −713, just 18 bp upstream of the NIT4 binding site cluster, and a cluster of seven GATA elements between −445 and −235. Determination of the functional importance of these putative NIT2 and NIT4 binding sites in the *nit-6* promoter will be of considerable interest in understanding this complex control network.

In *Aspergillus nidulans*, the *niaD* and *niiA* genes, coding for nitrate and nitrite reductase, are divergently transcribed from an intergenic region of 1.3 kb (11). Binding sites for both AREA and NIRA, the *A. nidulans* NIT2 and NIT4 homologs, respectively, have been identified in the intergenic region, and their physiological importance has been investigated (16a). A cluster of four GATA elements located in the middle of the intergenic region out of a total 10 identified AREA binding sites appear to be contribute to *niaD* and *niiA* expression in vivo. Three of the four NirA binding sites, whose consensus sequence is similar to that of NIT4, affect the bidirectional expression of *niaD* and *niiA*. One NIRA binding site close to the important AREA site cluster was of particular significance; its deletion resulted in a total loss of *niaD* and *niiA* expression, similar to our results with the *nit-3* promoter, with respect to which NIT4 site C had a comparable effect. The possibility exists for an interaction between the AREA and NIRA proteins, as speculated above for NIT2 and NIT4, as a result of the presence of clustered functional binding sites.

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