Analysis of a Distal Cluster of Binding Elements and Other Unusual Features of the Promoter of the Highly Regulated *nit-3* Gene of *Neurospora crassa*†

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ABSTRACT: In *Neurospora crassa*, the expression of the *nit-3* gene (nitrate reductase) is dependent upon nitrogen derepression and nitrate induction and is regulated by two positive-acting transcription factors, NIT2 and NIT4, and a negative regulator, NMR. The presence of a tightly linked cluster of NIT2 and NIT4 binding sites suggested that their close spacing might be required for a synergistic interaction of the NIT2 and NIT4 proteins. We show here that the NIT2 and NIT4 binding sites can be separated without affecting either the expression level or the precise regulation of the *nit-3* gene. Studies conducted on the NIT2 site II, which contains only a single GATA element and yet plays a major role in *nit-3* gene expression, showed that nucleotides both 5′ and 3′ of the GATA sequence were important for strong DNA binding in vitro and its activation function in vivo. The *nit-3* promoter contains two long AT-rich sequences, one of which is located just upstream of the transcription start sites and is required for optimal promoter function. The *nit-3* transcript contains eight TACC repeats in its 5′ noncoding region which appear to be involved in mRNA instability. Deletion of these TACC repeats led to a significant increase in the stability of *nit-3* mRNA.

In *Neurospora crassa*, *nit-3* (nitrate reductase) is one of the well-characterized genes in the nitrogen regulation circuit. The expression of nitrate reductase is precisely regulated by the availability of inducer nitrate and the absence of primary nitrogen sources such as glutamine, glutamate, and ammonia (*1*, *2*). When a primary nitrogen source is abundant, the expression of *nit-3* is fully repressed. Limitation of the favored nitrogenous compounds represents the signal for derepression of many structural genes in the nitrogen regulation circuit via a global transcription factor, NIT2. However, in order to utilize the secondary nitrogen sources, such as purines, amides, nitrate, nitrite, most amino acids, and proteins, a second pathway-specific positive regulator is usually also required for the full-scale derepression of the genes that encode the catabolic enzymes of a particular pathway. One well-documented example of a pathway-specific regulator is the NIT4 protein. In the presence of nitrate, NIT4 acts in conjunction with NIT2 to turn on the expression of *nit-3* and *nit-6* (nitrite reductase) genes (*3*).

Since the *nit-2* gene was identified (*4*), its regulation and function have been subject to extensive study. *nit-2* is constitutively expressed in *Neurospora*; however, the *nit-2* mRNA level increases 3−4-fold during nitrogen limitation (*5*). The *nit-2* gene encodes a 116 kDa protein with a single Cys-X_2-Cys-X_{17}-Cys-X_2-Cys zinc finger motif and a basic region that serves as the DNA binding domain (*6*). The *Aspergillus nidulans* homolog AREA protein and NIT2 share a high degree of homology with the carboxyl finger of the vertebrate GATA transcription factor family (*7*−*9*). In common with other members of this transcription factor family, NIT2 shows high binding affinity to the consensus DNA sequence T/A(GATA)A/G (*6*, *10*). NIT2 binding sites have been found in the promoters of *nit-3*, *nit-6*, and several other structural genes within the nitrogen regulation circuit, e.g., *alc* (allantoicase) and *lao* (L-amino acid oxidase), and even in the *nit-2* gene, suggesting a possible autogenuous regulation (*11*).

The pathway-specific transcription factor NIT4 is a protein with an amino-terminal $Zn(II)_2Cys_6$ type of zinc finger that constitutes its DNA binding domain (*12*). Northern analysis showed that the *nit-4* gene is constitutively expressed at an extremely low level (*13*). Mobility-shift and DNA-footprinting experiments showed that a symmetrical sequence, TCCGCGGA, and a closely related sequence are recognized by NIT4. Binding sites for NIT4 were found in the promoter of the *nit-3* gene (*14*), and potential NIT4 sites can be identified in the *nit-6* gene via sequence analysis.

Besides the two positive regulators, a negative-acting regulatory gene, *nmr* (nitrogen metabolic regulation), is also involved in the regulation of nitrogen circuit. Nitrate reductase and other nitrogen-related enzymes become constitutively expressed in *nmr* mutants (*15*, *16*). The NMR protein does not have DNA binding activity but functions as a negative regulator through a protein−protein interaction with NIT2, interfering with NIT2−DNA binding (*17*, *18*).

Three NIT2 and two NIT4 binding sites have been identified in the *nit-3* promoter (*5*, *14*). Removal either of

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all the NIT2 sites or of the two NIT4 sites resulted in complete elimination of *nit-3* promoter activity, indicating that both NIT2 and NIT4 are required for expression (*19*). Inspection of the *nit-3* promoter revealed that it possesses some intriguing features, including the fact that a cluster of NIT2 and NIT4 binding sites is located at a considerable distance, approximately 1 kb, upstream of the gene; the promoter also contains two long AT-rich regions. Moreover, eight repeats of the sequence TACC occur in the transcribed region of the gene but prior to the initiation codon for synthesis of the nitrate reductase protein. In this paper, we report a detailed study to examine the functional significance, if any, of these unusual features of the *nit-3* promoter.

MATERIALS AND METHODS

N. crassa and E. coli Strains. The *N. crassa* wild-type strain 74-OR23-1A was obtained from the Fungal Genetics Stock Center, University of Kansas Medical Center, Kansas City, MO. The RIP15[1] (*nit-3 his3*) mutant has been described (*19*). Mycelia were cultured in Vogel's liquid medium (*20*) with shaking. The amount of glutamine, KNO_3, and uric acid supplement is indicated for each experiment. *E. coli* strain DH5α was used for plasmid propagation, and CJ236 for single-strand DNA template preparation.

Site-Directed Mutagenesis. Site-directed mutagenesis was used to generate all the *nit-3* mutant constructs. DNA oligos containing the desired mutation were synthesized and annealed to the single-strand *nit-3* DNA template which was generated using *E. coli* strain CJ236. The daughter strand was synthesized *in vitro*, and the double-strand product was used to transform *E. coli*. Each mutant was selected and confirmed by DNA sequencing. All the mutants were then constructed into vector pDE1 (FGSC).

Mobility Shift Assay. DNA mobility shifts were carried out as described before with minor modifications (*6*). *E. coli*-expressed NIT2 proteins (0.1−1.0 μg) were used in the mobility shift assays. A 34 bp DNA fragment containing the NIT2 binding site II was used as probe. The mixtures of proteins and DNA were incubated at room temperature for 20 min in a total volume of 25 μL in binding buffer (12 mM HEPES, 4 mM Tris-HCl, pH 7.9, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 0.3 mg/mL BSA, 10% glycerol) with 3 μg of poly(dI-dC) as nonspecific competitor. Samples were separated on 4% polyacrylamide gels (acrylamide:bisacrylamide = 19:1) in 1/4× TBE buffer.

RNA Preparation and Northern Analysis. Total RNA from *N. crassa* was isolated as described by Weaver et al. (*21*) with modifications. Mycelia were ground to a fine powder with a mortar and pestle in liquid nitrogen and suspended in lysis buffer (50 mM NaOAc, pH 5.3, 10 mM EDTA, 1% SDS) at a 1 g/5 mL ratio. An equal volume of acidic phenol/chloroform (equilibrated with the lysis buffer without SDS, pH 5.3, prewarmed at 65 °C) was added to the lysate, and the reaction mixture was incubated at 65 °C for 30 min with shaking. The aqueous phase was recovered by centrifugation, and this process was repeated several times until no protein interface could be seen. Total RNA was precipitated with 0.6 volume of isopropyl alcohol followed by centrifugation. The RNA samples were fractionated in agarose gels, and transferred to nitrocellulose filters.

nit-3 cDNA and *β-tubulin* cDNA were labeled with random primers using [^{32}P]dATP for use as probes for Northern analysis (GIBCO-BRL). The hybridization was carried out at 65 °C in 1× hybridization buffer (0.5 M NaCl, 0.1 M $NaPO_4$, pH 7.0, 6 mM EDTA, 1% SDS). The membranes were washed at 65 °C for 60 min with 1/4× hybridization buffer. The *nit-3* and *β-tubulin* mRNAs were identified by their sizes.

Nitrate Reductase Assay. Mycelia were grown at 30 °C in liquid Vogel's minimum medium containing 20 mM glutamine as the only nitrogen source for 12−16 h, and then transferred to medium containing 40 mM KNO_3 for 3−4 h to allow expression of the *nit-3* gene. Mycelia were harvested by filtering through mirocloth and were lysed in lysate buffer (0.1 M KPO_4, pH 6.8, 1 mM β-mercaptoethanol, 0.5 mM EDTA, 1% NaCl) using a Mini-beadbeater BX-4 (Biospec Products). Protein concentrations were determined with the Bio-Rad protein assay. Nitrate reductase enzyme assays were performed as described before with a few modifications (*22*). Cell lysate (100 μL) was mixed with 400 μL of buffer (22 mM KPO_4, pH 7.75, 22 mM $NaNO_3$, 5.5 mM Na_2SO_3, 0.11 mM FAD) and incubated at 30 °C for 30 min. Fifty microliters of NADPH (0.2 mM) was added to initiate the reaction, and the mixture was incubated at 30 °C for 15−30 min depending on the amount of activity; 0.1 mL of stop solution (25% barium acetate) was added, and the precipitate was removed by centrifuging in a microfuge at full speed for 1 min. For the zero-time control, the stop solution was added to the mixture without incubation. Then 0.5 mL of the supernatant of each reaction mixture was mixed with 2.5 mL of 0.2% sulfanilamide (diluted from 1% stock) in 20% v/v HCl, and 0.026% w/v naphthylenediamine dihydrochloride. The absorbance of these samples was measured at 540 nm with a spectrophotometer.

RESULTS

Potential Communication between NIT2 and NIT4 Sites. Of the binding sites found in the *nit-3* promoter, two NIT2 and two NIT4 binding sites are clustered together about 1 kb upstream of the transcription start sites (Figure 1). A third NIT2 site is located very close to the transcription start sites. Site-directed mutagenesis was used to study the biological function of the individual NIT2 and NIT 4 sites. Surprisingly, the proximal NIT2 site III which consists of four GATA elements and has strong in vitro binding affinity for the NIT2 protein is the least important one for *nit-3* promoter activity. Loss of the most upstream NIT2 site I, which also contains multiple GATA elements, reduced promoter activity by 50%. Mutation of NIT2 site II with a single GATA element showed the most dramatic effect, with a 90% loss of promoter activity (*19*; Tao and Marzluf, unpublished data). The two NIT4 sites showed an equal contribution to promoter activity, and mutation of either one resulted in a 50% reduction and loss of both leads to complete loss of *nit-3* expression (*19*; Tao and Marzluf, unpublished data).

[1] Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetracetic acid disodium salt; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; RIP, repeat induced point mutation; SDS, sodium dodecyl sulfate; TBE, Tris−boric acid−EDTA.

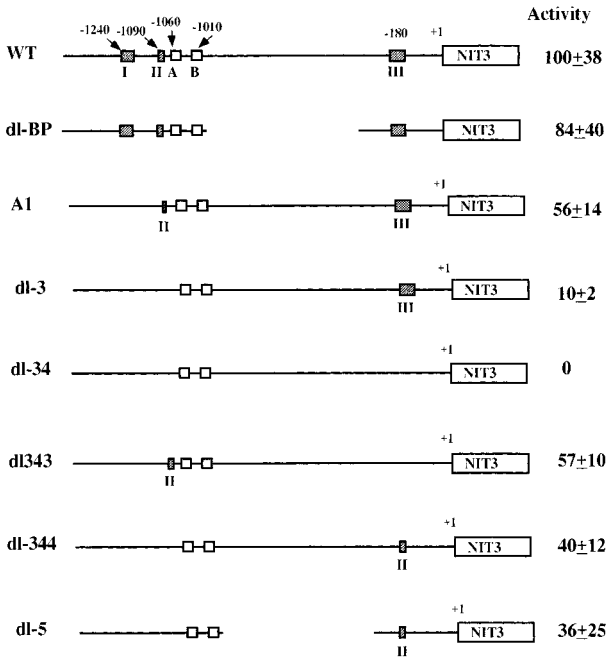

FIGURE 1: In vivo *nit-3* promoter analysis. Diagram of *nit-3* promoters showing combinations of deletions and insertions of NIT2 binding sites. All constructs were built in pDE1 and targeted to the *his3* locus in RIP15A, and transformants were assayed for nitrate reductase activity. Relative activities are indicated with activity from the wild-type construct set equal to 100. I, II, III, NIT2 sites; A and B, NIT4 sites.

In an attempt to further dissect the *nit-3* promoter, we wanted to know whether there were additional unknown elements located distal to the transcription start sites. A deletion mutant, dl-BP, was generated by removing 600 bp of *nit-3* promoter between the NIT2−NIT4 cluster and the proximal NIT2 binding site. This construct was targeted to the *his-3* locus of a *nit-3* RIP mutant host (RIP15). Analysis of multiple transformants showed that the mutant promoter gave approximately the same activity as the wild-type promoter (Figure 1). This result suggested that there is no obvious functional reason for the large distance between the cluster of NIT2−NIT4 elements and the transcription start sites, and also showed that the intervening 600 bp of the promoter region does not appear to contain any important regulatory element(s).

The next question addressed was whether it is necessary that the NIT2 and NIT4 sites of the upstream cluster be close together to maintain proper function, possibly to allow a direct protein−protein interaction between NIT2 and NIT4 factors. In order to answer the question, a series of mutants was generated. First a unique restriction enzyme site (*Sma*I) was engineered between the NIT2 and NIT4 sites, and then DNA fragments ranging from 22 to 200 bp were inserted at this *Sma*I site. The constructs [*Sma*I(−8), 22, 34, 56, 68, 124, 200] were named after the length of DNA fragment inserted. These insertion mutant constructs as well as one carrying the wild-type promoter were targeted to the *his-3* locus, and multiple transformants of each were examined. The promoter activity showed a slight decrease for constructs *Sma*I(−8), 22, and 34 and was at the wild type or a somewhat greater level for constructs 56, 68, 124, and 200 (data not shown). Nitrogen regulation was normal in the transformants carrying each of these mutant constructs. The presence of

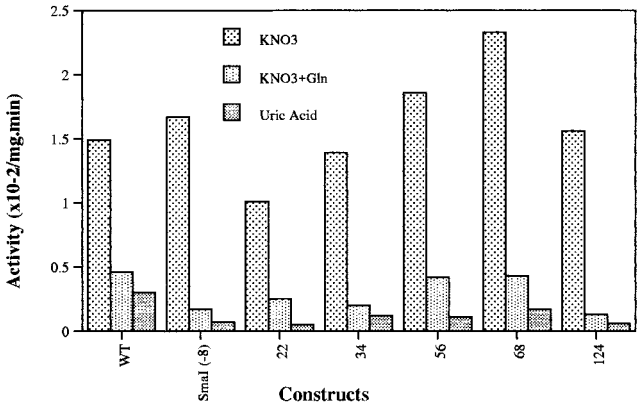

FIGURE 2: In vivo analysis of nitrogen regulation of the *nit-3* promoter mutants. Mycelia were initially grown in Vogel's minimum medium with 20 mM glutamine as the nitrogen source for 12−16 h, then transferred to media containing 40 mM KNO_3, 40 mM KNO_3, and 20 mM glutamine or 20 mM uric acid as nitrogen sources, and grown for 3 h. Nitrate reductase was analyzed, and the activity [$OD_{540} \times 10^{-2}$/(mg·min)] of each sample is shown.

glutamine fully repressed the expression of the *nit-3* gene, while nitrate was required for induction. The presence of both glutamine and nitrate led to a low level of expression (approximately 10% of fully derepressed value), while other poor nitrogen sources like allantoin or uric acid did not induce *nit-3* at all (less than 5% of induced level) (Figure 2). This study suggested that it is not critical that NIT2 and NIT4 elements of the cluster be close to one another for proper promoter function and for normal nitrogen regulation.

Anatomy of a Single GATA Site. Nearly all strong NIT2 binding sites have two or more closely spaced GATA elements, with the exception of site II in the *nit-3* promoter. In vitro mobility shift studies demonstrated that this site, which contains only a single GATA element, strongly binds the NIT2 protein. Deletion of this site results in up to 90% loss of the in vivo activity of the promoter. Deletion of all three NIT2 sites (in construct dl-34) resulted in essentially a complete loss of *nit-3* expression, and dl-34 transformants could not grow in medium using nitrate as the only nitrogen source. In order to further analyze the function of the single GATA site, a series of mutants were generated. By using dl-34 as the recipient, a short double-stranded fragment containing the GATA core element and its flanking sequence (12 bp on each side) was inserted, either at the original location (named dl-343) or at a different location about 180 bp upstream of the transcription start sites (named dl-344). Analysis of transformants that received these constructs showed that returning the GATA element back to its original location restored about 60% of the wild-type promoter activity, suggesting that this single GATA has a unique feature in regulating the promoter. Transformants with the single GATA element inserted just before the transcription start sites had about 40% of the wild-type promoter activity. By comparison to construct dl-3 in which the NIT2 cluster (sites I and II) was removed, this single GATA element is stronger at activation (3-fold greater) than the NIT2 site III which contains multiple GATA copies (Figure 1).

In an attempt to determine why the NIT2 site II when inserted near the transcription start site was somewhat less effective in restoring the promoter activity than when located far upstream, the deletion mutant dl-5 which reduced the

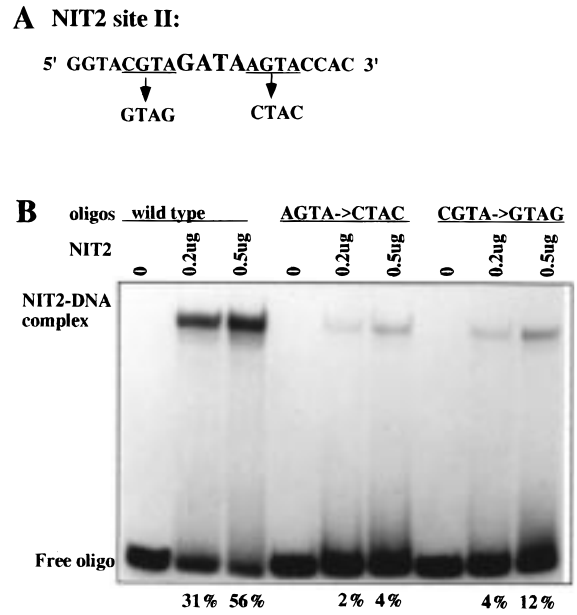

FIGURE 3: In vitro mobility shift assay using a synthetic double-stranded oligonucleotide with NIT2 site II (5′ AATTCTATGG-TACGTAGATAAGTACCACCCGTG 3′) and related mutant DNAs and *E. coli* expressed NIT2 protein (DNA binding region). (A) Sequence of NIT2 site II and the two mutants with altered 5′ and 3′ flanking regions. (B) DNA mobility shift experiment. 34 bp DNA oligos labeled with ^{32}P were quantified, and equal amounts of each were tested for NIT2 binding as described under Materials and Methods. The NIT2−DNA complex and free oligo are indicated. The percentage shift of the probe in each reaction as determined by a phosphoimager is indicated at the bottom of each lane.

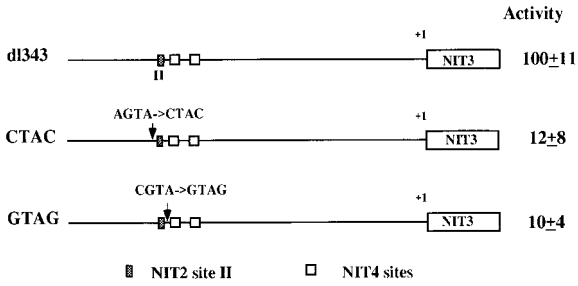

FIGURE 4: In vivo *nit-3* promoter analysis. dl-343 contains only the NIT2 site II while CTAC and GTAG contain NIT2 site II with altered flanking sequences. Transformants of each were assayed for nitrate reductase activity, with that for dl-343 set at 100. Open boxes represent NIT4 binding sites; the filled box represents NIT2 site II.

distance between NIT4 and NIT2 sites from 600 bp to about 100 bp was constructed from dl-344. In dl-5, the two NIT4 sites are located about 100 bp upstream of the inserted NIT2 site II. The dl-5 promoter functioned at the same level as dl-344. This result suggested that the relative orientation of NIT4 and NIT2 sites may have some effect on the promoter activity (Figure 1).

Previous results had suggested that substitution of single nucleotides 5′ or 3′ of a GATA element had marginal effects upon NIT2 binding affinity (*11*). To further analyze why the single GATA element of site II was so highly active, site-directed mutagenesis was used to change its flanking sequences. The 5′ flanking sequence was changed from AGTA to CTAC; the 3′ flanking sequence was changed from CGTA to GTAG (Figure 3A). The effect, if any, of these changes was analyzed both in vivo and in vitro. The in vitro

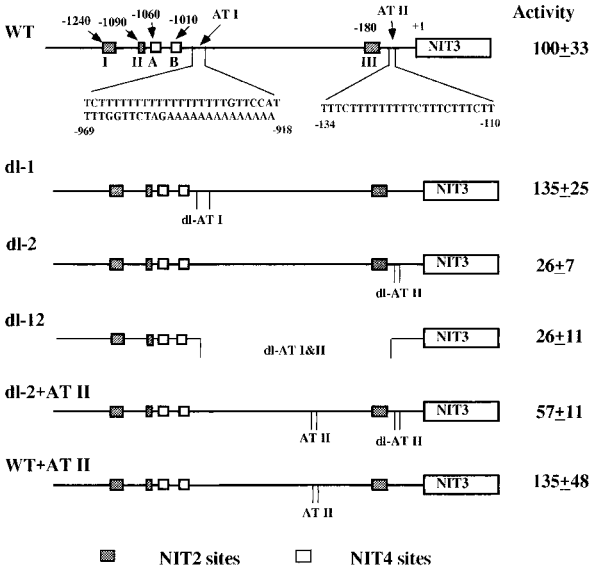

FIGURE 5: In vivo analysis of manipulated *nit-3* promoters. The two AT-rich regions are indicated in the diagram. Schematic drawings show the deletion and insertion of AT-rich regions in the *nit-3* promoter in different constructs. Each construct was transformed into RIP15A and assayed for nitrate reductases activity. The activity for the transformants with wild-type construct was set at 100. dl = deleted segment.

mobility shift analysis with a synthetic oligo containing this single GATA with its wild-type flanking sequences showed very good NIT2 protein binding. In contrast, identical oligonucleotides with base substitutions on the 5′ or on the 3′ side of the GATA core element showed a significantly reduced binding affinity for NIT2, 15-fold and 7-fold lower, respectively (Figure 3B). The results of in vivo expression analysis with these altered promoters correlated very well with the mobility shift analysis. The engineered *nit-3* promoters containing either the wild-type copy of NIT2 site II or the mutant forms were transformed into the *Neurospora* RIP15 mutant. Transformants carrying the two mutant promoters with altered flanking sequences showed a dramatic reduction in promoter activity compared to the wild-type copy (Figure 4). This result indicated that both the 5′ and 3′ flanking nucleotides and the GATA core sequence contribute to the NIT2 protein−DNA interaction in vivo, as reflected in *nit-3* gene expression.

AT-Rich Sequences. Besides the major transcription factor binding sites, the *nit-3* promoter has several other interesting features. Two AT-rich sequence are found in the *nit-3* promoter: one is located about 1 kb upstream of the transcription start sites (AT I), and the other is located just in front of the transcription start sites (AT II). Deletion of AT I (construct dl-1) increased *nit-3* expression slightly, while deletion of AT II (construct dl-2) resulted in more than 70% loss of *nit-3* gene expression (Figure 5). Deletion of both AT sequences and the 1 kb promoter region between them (construct dl-12) resulted in the same loss of promoter activity as did deletion of just the AT II sequence. Insertion of the AT II sequence 200 bp upstream of the transcription start sites into construct dl-2 (yielding dl-2+ATII) partially restored activity (60% of the wild-type promoter). Insertion of an additional copy of the AT II sequence to the wild-type promoter (construct WT+ATII) resulted in only a slight increase of the promoter activity. These results suggested

that the proximal AT-rich sequence II is essential for full-level transcription of the *nit-3* gene.

Study of TACC Repeated Sequence. Eight repeats of the sequence TACC are situated about 30 bp downstream of the transcription start sites and thus will be present in the *nit-3* mRNA. Deletion of this repeated sequence resulted in an increase of nitrate reductase activity. As shown in Figure 6A, the TACC deletion mutant had about twice the activity as did transformants carrying the wild-type construct. It was suspected that this increase in enzyme activity was due to a change in the stability of the *nit-3* transcript. In order to test this hypothesis, the *nit-3* mRNA half-life was determined at the induction−repression transition. Mycelia were initially grown in medium with 40 mM KNO_3 as the sole nitrogen source, and then transferred to medium with 20 mM glutamine. This change in the nitrogen source prevented any further synthesis of *nit-3* mRNA, thus allowing us to analyze the turnover rate of the preexisting *nit-3* transcripts. Cells were harvested at 0, 5, 10, and 20 min after the shift, and total RNA was prepared. Northern analysis revealed that the mRNA stability of the TACC-deletion mutant is increased, with an estimated half-life of 10 min (Figure 6B) in comparison to the wild-type mRNA which has a half-life of 5 min (*23*). This result suggested that an increase in the half-life of *nit-3* transcript is responsible for the increase in enzyme activity in transformants containing the *nit-3* mutant lacking the TACC repeated sequences.

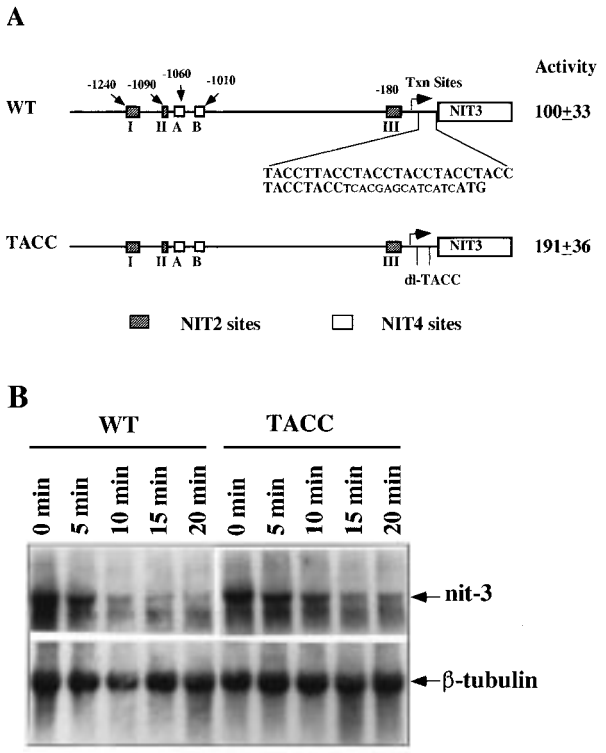

FIGURE 6: Functional analysis of TACC repeats. (A) In vivo analysis of TACC repeated sequence. The deletion mutant lacking the TACC repeats was generated, and nitrate reductase of multiple transformants was examined. The relative enzyme activity was calculated using that for a wild-type construct as 100. (B) RNA blot analysis of *nit-3* transcript (3.1 kb) (wild type and TACC mutant) and β-tubulin (2.0 kb) control was carried out as described under Materials and Methods.

DISCUSSION

In *Neurospora crassa*, environmental signals determine the expression pattern of nitrate and nitrite reductase. Limitation for a primary nitrogen source and the presence of inducer nitrate, metabolic signals integrated via NIT2 and NIT4 proteins, respectively, are both required for the expression of these enzymes. In the 5′ promoter region of the *nit-3* gene, multiple NIT2 and NIT4 binding sites have been identified. The arrangement of these binding sites is rather intriguing, with only a single NIT2 site located at −180 bp, and a cluster of two NIT2 and two NIT4 sites situated at about 1 kb upstream of the transcription start sites. In our mutagenesis studies, we found that it is the far upstream cluster that has the major effect for the *nit-3* promoter activity, while the proximal NIT2 plays only a limited role despite its having multiple GATA core elements and showing strong in vitro binding to NIT2 protein. Removal of up to 600 bp of the promoter sequence between the cluster of NIT2 and NIT4 elements and the proximal NIT2 site showed no detectable effect on the promoter activity, implying that there are no important elements in this entire stretch of 600 bp that function to modulate the level of promoter activity or to regulate its function. This result also indicates that there is no obvious requirement that the cluster of regulatory elements be so far (at least 1 kb) upstream of the transcription start sites. A similar phenomenon has been observed in *Aspergillus nidulans* as well (*24*). The *niaD* and *niiA* genes, which specify nitrate and nitrite reductase, respectively, are transcribed divergently from a common intergenic control region, within which multiple NIRA (NIT4 homolog) and AREA (NIT2 homolog) binding sites were identified scattered throughout the entire region. Mutagenesis results showed that of the 10 AREA binding sites, only 4 located in the central region appear to be biologically important for nitrogen repression/derepression. Loss of those AREA sites located near the transcription start sites of *niaD* and *niiA* showed no detectable effect.

Like many *Neurospora* genes, *nit-3* lacks a TATAA box, and deletion of a TATA-like sequence located near the start sites showed no demonstrable effect (Tao and Marzluf, unpublished result). Another unusual feature of *nit-3* promoter is the presence of two long AT-rich sequences, one located far upstream, the other immediately before the transcription start sites. The upstream AT-rich sequence is immediately downstream of the cluster of NIT2 and NIT4 binding elements, which suggested the possibility that it might function to keep these sites in an open conformation for interaction with the regulatory factors, for example, by excluding nucleosomes. However, our results reveal that this AT-rich sequence does not appear to play a visible role in modulating either the regulation or the expression level of the *nit-3* gene. In contrast, the AT-rich sequence just upstream of the transcription start sites is required for the optimal expression of *nit-3*, and its loss led to a significant decline (about 70% loss) of promoter activity. Studies in yeast, plants, and mammals have suggested that AT-rich sequences in promoter regions may have very divergent effects on transcription. DNA binding proteins have been identified in these diverse organisms which can bind specifically to AT-rich sequences and act either as an activator or as a repressor (*25*−*27*).

Many organisms possess multiple GATA factors, each of which recognizes binding sites with the same core GATA

Table 1: Summary of Nucleotides That Occupy 5′ and 3′ Flanking Regions of the GATA Element of NIT2 Binding Sites

site I	G	A	T	T	**GATA**	A	G	G	T							strong site
	G	T	T	T	**GATA**	G	T	G	C							
	T	G	T	A	**GATA**	G	G	C	G							
site II	C	G	T	A	**GATA**	A	G	T	A							essential site
site III	A	G	T	A	**GATA**	C	A	C	C							weak site
	A	T	G	T	**GATA**	A	C	C	T							
	T	T	T	A	**GATA**	T	G	T	A							
	T	G	C	A	**GATA**	A	C	A	G							
	-4	-3	-2	-1		-1	-2	-3	-4							
A	2	1	0	5		4	1	1	2							
C	1	0	1	0		1	2	3	2							
G	2	4	1	0		2	4	2	2							
T	3	3	6	3		1	1	2	2							
CONSENSUS	N	G	T	A	**GATA**	A	G	N	N							

sequence. Mice and humans contain at least six different GATA factors, each of which activates a particular set of target genes. The expression of these GATA factors is developmentally regulated to give a distinctive but overlapping pattern of tissue-specific distribution, which is at least one major determinant for their specificity in controlling downstream genes (*10*, *28*).

Neurospora crassa contains at least five distinct GATA factors, NIT2, WC1, WC2, SRE, and NGF1, all of which appear to be constitutively expressed within the same vegetative cells despite their diverse functions (*29*; Zhou and Marzluf, unpublished; Bo and Marzluf, unpublished). The coexistence of these multiple GATA factors, with similar DNA binding specificity, raises a question as to how each controls its own unique set of target genes. The studies presented here highlight two features which may be central to the specificity exerted by NIT2 in controlling nitrogen-regulated structural genes. First, activation of the *nit-3* gene is completely dependent upon the presence of both NIT2 and NIT4 which bind at a cluster of elements in the promoter (*2*). Second, it was shown that both the 5′ and 3′ flanking nucleotides of the single GATA core element in NIT2 binding site II are of critical importance for its strong NIT2 binding affinity in vitro and its physiological function in vivo. The NIT2 binding sites I and III, each with multiple GATA sequences, bind the NIT2 protein in vitro with a greater affinity than does the single GATA element in site II. Yet site II was found to be the most important for in vivo function, revealing that DNA binding examined in vitro cannot be relied upon to indicate function in vivo.

Comparison of the flanking nucleotides in all eight of the GATA elements present in the *nit-3* promoter revealed an expanded consensus NIT2 binding site, 5′ GTA**GATA**AG (Table 1). The single GATA element in site II, the one most important for *nit-3* gene expression, is the only GATA sequence in the promoter with a perfect match to this expanded binding sequence. Moreover, changing the nucleotide sequence on either side of this special GATA element led to a drastic reduction in both the strength of NIT2 binding in vitro and *nit-3* gene activation in vivo. These findings show that the context of the single GATA element of site II is important and that a proper extended sequence is required for optimal function. This feature may help explain the weak level of derepression observed with two other genes of the nitrogen control circuit, *lao* and *alc*, which possess less perfect NIT2 binding sites.

In many cases, the regulation of gene expression is achieved at different levels. Within the *Neurospora* nitrate assimilation pathway, the expression of nitrate reductase appears to be regulated mainly at the transcriptional level by NIT2 and NIT4. However, our mutagenesis study suggested that the stability of *nit-3* messenger RNA might also affect *nit-3* gene expression. Removal of eight TACC repeats from the 5′ untranslated region doubles the half-life of *nit-3* mRNA as determined at the transition stage from nitrogen-derepression to nitrogen-repression conditions. Differential turnover of nitrate reductase mRNA has been observed in *Chlorella vulgaris* under nitrogen-repressed conditions (*30*).

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