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nit-2, the major positive-acting nitrogen regulatory gene of *Neurospora crassa*, encodes a sequence-specific DNA-binding protein

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ABSTRACT **The *nit-2* major nitrogen regulatory gene of *Neurospora crassa* turns on the expression of various unlinked structural genes that specify nitrogen-catabolic enzymes under nitrogen-limitation conditions. The *nit-2* gene encodes a protein of 1036 amino acid residues with a single zinc finger and a downstream basic region that may make up a DNA-binding domain. The zinc-finger domain of the NIT2 protein was synthesized in two ways to examine its DNA-binding activity with gel-band-mobility shift and DNA-footprint experiments. The NIT2 protein binds to specific DNA recognition elements that are located upstream of nitrogen-regulated structural genes. Each recognition element contains at least two copies of a core sequence whose consensus is TATCTA.**

In the fungus *Neurospora crassa*, the expression of enzymes within various global areas of metabolism is highly regulated. *Neurospora* has been utilized as a model lower eukaryote to investigate complex regulatory circuits (1–3). The nitrogen regulatory circuit of *Neurospora* contains a substantial number of unlinked structural genes that encode enzymes required for utilization of various secondary nitrogen sources, when primary nitrogen sources (e.g., ammonia or glutamine) are not available (1, 4, 5). The product of *nit-2*, the major positive-acting regulatory gene of the nitrogen circuit, is necessary for the expression of the structural genes of the circuit during conditions of nitrogen limitation (6–9). A distinct unlinked regulatory gene, *nmr* (for "nitrogen metabolic regulation") acts in a negative manner (10–13). The *nit-2* gene and the *nmr* gene of *N. crassa* both appear to be directly involved in nitrogen catabolite repression, but the mechanism of their interaction is not yet understood. The *nit-3* gene, which encodes nitrate reductase, is a well-characterized structural gene of the nitrogen circuit (14). The expression of *nit-3* is highly regulated at the level of mRNA content by the positive-acting *nit-2* gene and by the metabolic signals, nitrogen derepression, and nitrate induction (14–16).

Several models have been proposed concerning the mechanism of operation of the nitrogen circuit (17–19). In each case the *nit-2* gene was postulated to specify a trans-acting regulatory protein that turns on the expression of the various nitrogen structural genes. The complete nucleotide sequence of the *nit-2* gene has been determined and can be translated to give a protein comprised of 1036 amino acids with a molecular weight of approximately 116,000 (20). The NIT2 protein appears to contain a single Cys_2/Cys_2-type zinc-finger element with a central loop comprised of 17 amino acid residues. A region of the NIT2 protein immediately on the carboxyl side of the zinc-finger motif is highly basic and may comprise part of a specific DNA-binding domain, as has been found in the yeast LAC9, GAL4, and PPR1 proteins (21, 22). The related filamentous fungus, *Aspergillus nidulans*, has a regulatory gene, designated *areA*, which is homologous to the *N. crassa nit-2* gene (23). Both *nit-2* and *areA* encode proteins that appear to contain a $Cys\text{-}Xaa_2\text{-}Cys\text{-}Xaa_{17}\text{-}Cys\text{-}Xaa_2\text{-}Cys$ zinc-finger domain. The *Neurospora nit-2* gene has been shown to substitute for *areA* function in *Aspergillus* and turn on the expression of various nitrogen structural genes (24). This result strongly suggests that the activation function and DNA sequence specificity of the NIT2 and the AREA proteins may be similar.

Here we present the results of gel-band-mobility-shift experiments and DNA-footprint studies that demonstrate that the NIT2 protein binds in a sequence-specific manner to three distinct sites in the 5′ promoter region of the *Neurospora* nitrate reductase gene. The NIT2 protein also binds to several closely related sites in the promoter region between the divergently transcribed *Aspergillus* nitrate and nitrite reductase genes.

MATERIALS AND METHODS

Strains. The *N. crassa* wild-type strain 74OR231A and *nit-2* mutant strains were obtained from the Fungal Genetics Stock Center (University of Kansas Medical Center). Cultures were grown in Vogels liquid medium supplemented as indicated for each experiment with shaking at 30°C as described (10–14).

Expression of the *nit-2* Zinc Finger Domain in *Escherichia coli*. The 0.65-kilobase (kb) *Bam*HI–*Eco*RI DNA fragment from *nit-2* deletion clone 22 (7) was cloned into the expression vector pET3b to obtain pN2ET, which was used to express the NIT2 zinc-finger domain protein in *E. coli* as described (25). The NIT2 finger protein was partially purified by precipitation with 50% ammonium sulfate saturation (4°C) before it was used in DNA-mobility-shift experiments.

Expression and Purification of a NIT2–β-Galactosidase (β-Gal) Fusion Protein. To construct an expression vector to express a NIT2–β-Gal fusion protein, the *Bam*HI–*Eco*RI fragment of *nit-2* deletion clone 22 was cloned into the corresponding sites of pSKS106 (26) to obtain pN2SKS. The *Bam*HI site was filled-in and the *Eco*RI site was made blunt-ended such that an in-frame fusion protein construction was achieved. *E. coli* strain JM103 transformed with pN2SKS was induced with 0.5 mM isopropyl β-D-thiogalactoside (when the bacterial culture reached an absorbance of 0.5 at 600 nm), and grown for an additional 2 hr. After harvesting by centrifugation, the bacterial pellet was resuspended in 20 mM Tris·HCl/10 mM $MgCl_2$, pH 7.4/1 mM phenylmethylsulfonyl fluoride and sonicated. After centrifugation, the supernatant was subjected to aminobenzyl-1-thio-β-D-galactopyranoside (ABTG) affinity chromatography (Sigma) as described by Ullmann (27).

Gel-Band-Mobility-Shift Experiments. DNA fragments used for gel-band-mobility-shift experiments were radioactively labeled with ^{32}P by filling-in with the Klenow fragment of DNA polymerase I. Mobility-shift experiments were carried out as described (28) except that the gels were electro-

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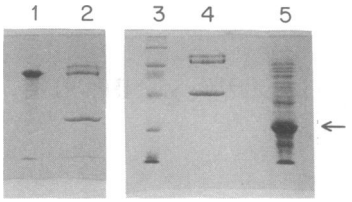

FIG. 1. Expression of NIT2 proteins in *E. coli*. Lanes: 1, β-Gal; 2, NIT2–β-Gal fusion protein; 3, molecular weight markers; 4, NIT2–β-Gal fusion protein; 5, NIT2 zinc-finger region protein (identified with an arrow), comprised of 217 amino acids from NIT2 plus 12 residues from the vector. The NIT2–β-Gal fusion protein (lanes 2 and 4) is larger than β-Gal and moves more slowly in the gel. Lanes 1 and 2 are from a different gel than lanes 3–5. The proteins were electrophoresed in a 7.5% polyacrylamide gel containing SDS and stained with Coomassie blue.

phoresed in 0.25× TBE buffer (1× TBE = 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3) without recirculation. The expressed protein (1 μg) and a ^{32}P-labeled DNA fragment (10,000 cpm) were incubated at room temperature for 25 min in a buffer containing 12 mM Hepes (pH 7.9), 2 mM dithiothreitol, 3.5 mM $MgCl_2$, 50 mM KCl, 15% (vol/vol) glycerol, and 2 μg of poly(dI)·poly(dC) before being loaded onto the gel.

DNase I Footprint Analysis. DNA-footprint experiments were carried out with a modification of the procedure described by Hope and Struhl (29). ^{32}P-labeled DNA fragments were incubated with the NIT2–β-Gal fusion protein in 25 μl of 20 mM Tris, pH 7.4/50 mM KCl/3 mM $MgCl_2$/1 mM EDTA/gelatin (100 μg/ml). After equilibration for 25 min at room temperature, 1 μl of DNase I (30 ng) was added and the mixture was incubated an additional 5 min. The DNase I digestion was then terminated by adding 25 μl of stop buffer [0.5% SDS/10 mM EDTA/yeast tRNA (0.5 mg/ml)]. The DNA was extracted with phenol and chloroform, precipitated with ethanol, resuspended in DNA-sequencing loading buffer, and electrophoresed on a sequencing gel.

RESULTS

Expression of NIT2 Zinc-Finger Domain in *E. coli*. The NIT2 protein is comprised of 1036 amino acids with a molecular weight of approximately 116,000. We were unable to express the entire NIT2 protein in *E. coli*, presumably because of its very large size. Thus, a *nit-2* gene restriction fragment was cloned into the pET3b vector so that 217 amino acids of NIT2, which included the zinc-finger domain, were fused to 12 amino acids specified by the vector. Addition of the inducer isopropyl β-D-thiogalactoside to the *E. coli* host cells results in the synthesis of T7 RNA polymerase that then leads to strong expression of a gene cloned adjacent to the T7-specific promoter in the pET vector. SDS/PAGE analysis revealed that upon induction the cloned *nit-2* gene segment was expressed at a high level to give a protein of the predicted size (Fig. 1). *E. coli* cells that contained the expression vector lacking the *nit-2* DNA fragment did not produce any of this protein (data not shown).

Synthesis of a NIT2–β-Gel Fusion Protein. A NIT2 zinc-finger–β-Gal fusion protein was expressed using pSKS, an expression vector that permits the fusion of a foreign protein to the amino terminus of *E. coli* β-Gal (26). The NIT2–β-Gal fusion protein construct was designed such that 217 amino acids of the NIT2 zinc-finger domain were fused to a short amino-terminal sequence (13 residues) coded for by the vector, and at its carboxyl terminus the finger domain was fused to the entire β-Gal protein. The fusion protein was soluble and was extracted from the bacterial cells and purified by affinity chromatography on aminobenzyl-1-thio-β-D-galactopyranoside (ABTG)-agarose. An aliquot of the purified fraction was analyzed by SDS/PAGE as shown in Fig. 1. Except for a full-length β-Gal (116 kDa) band and a shorter β-Gal fragment (46 kDa), the fusion protein (140 kDa) was the only visible band on the gel.

Mobility-Shift Experiments. The *nit-2* gene of *Neurospora* encodes a major positive-acting regulatory protein that controls the expression of various nitrogen structural genes, including the nitrate reductase gene, *nit-3*. Since the NIT2 protein contains a putative zinc-finger-DNA-binding domain, we anticipated that the promoter regions of various structural genes of the nitrogen circuit might have one or more specific DNA recognition sites for NIT2. Gel-band-mobility-shift analyses were utilized to investigate possible DNA binding by NIT2. The results presented in Figs. 2 and 3 demonstrate that NIT2 indeed binds to specific DNA fragments upstream of the *nit-3* structural gene. The NIT2 zinc-finger domain protein and the NIT2–β-Gal fusion protein both appear to be functional in DNA binding. Several lines of evidence demonstrated that the binding is specific. Certain DNA fragments in the *nit-3* promoter region display obvious mobility shifts, whereas other fragments do not show any detectable binding

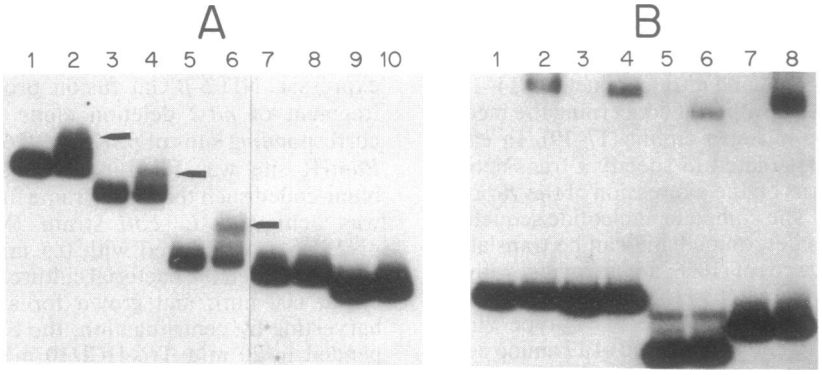

FIG. 2. Gel-band-mobility-shift experiments with 5′ flanking DNA fragments of the *Neurospora nit-3* and the *Aspergillus niaD* and *niiA* genes. DNA fragments were incubated with NIT2 proteins and subjected to polyacrylamide gel electrophoresis. (*A*) Analysis of the NIT2 zinc-finger-protein domain expressed in *E. coli*. Protein extracts were incubated with a 5′ flanking DNA fragment of *niiA* (lanes 1 and 2), *niaD* (lanes 3 and 4), *nit-3* (lanes 5 and 6), *cys-3* (lanes 7 and 8), and *cys-14* (lanes 9 and 10). Protein (0.3 μg) expressed in bacterial cells with vector only was used in lanes 1, 3, 5, 7, and 9. Expressed NIT2 protein extracts (0.3 μg) were used in lanes 2, 4, 6, 8, and 10. An arrowhead identifies each shifted band. No shift occurred with *cys-3* or *cys-14* DNA; the large *niiA* and *niaD* fragments used in this experiment display only a small mobility shift. (*B*) Analysis with the NIT2–β-Gal fusion protein. Protein was incubated with a 5′ flanking DNA fragment of *nit-3* (lanes 1 and 2), a *Bam*HI–*Pst* I *niaD* fragment (lanes 3 and 4), a 5′ *niiA* fragment (lanes 5 and 6), and a *Dde* I–*Pst*I *niaD* fragment (lanes 7 and 8). *E. coli* β-Gal (1 μg) was used in lanes 1, 3, 5, and 7; 1 μg of the NIT2–β-Gal fusion protein preparation was used in lanes 2, 4, 6, and 8.

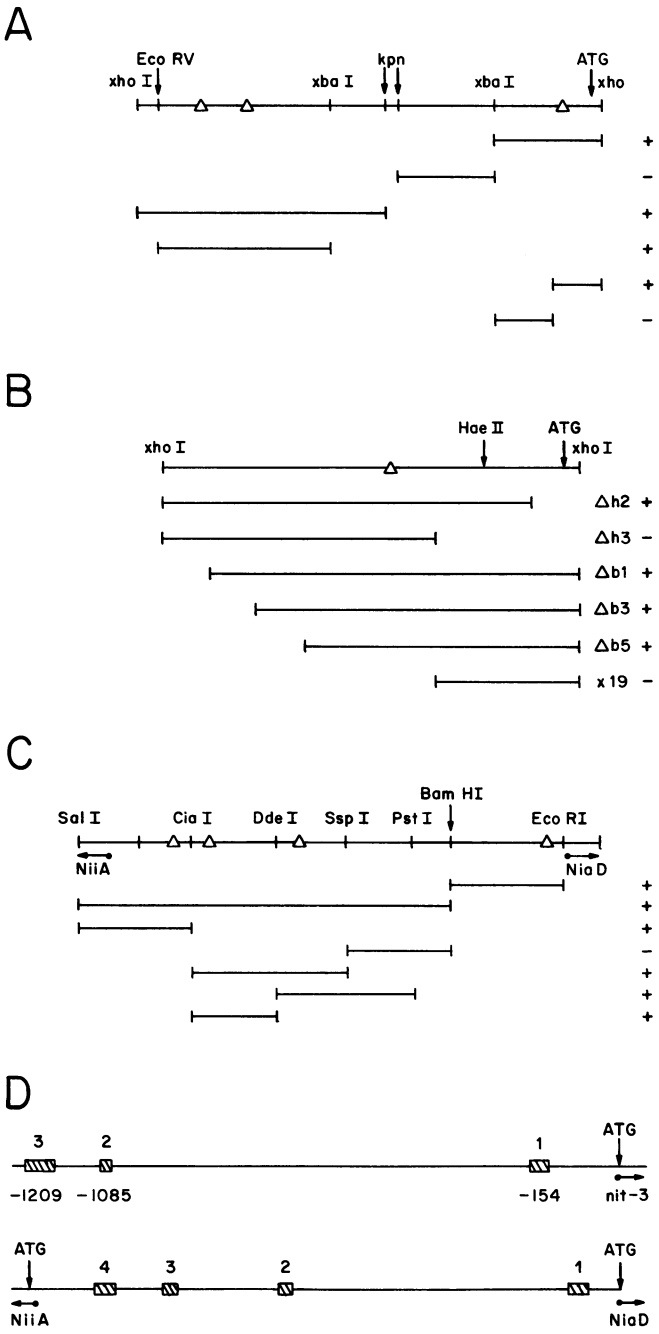

FIG. 3. Summary of gel-band-mobility-shift experiments. Mobility-shift experiments were carried out as described in Fig. 2 with the fragments identified as solid lines. +, Fragments shifted with NIT2 protein; −, fragments not shifted. (*A*) Restriction map and results of mobility-shift experiments with the 5′ flanking DNA fragments of *nit-3*. (*B*) Detailed analysis of the immediate 5′ upstream DNA (440-bp *Xho* I/*Xba* I fragment) of *nit-3*. Mobility-shift experiments were carried out with DNA fragments from various deletion clones in this region. (*C*) The results of mobility-shift experiments with DNA fragments located between *niaD* and *niiA*. (*D*) Location of binding sites for the NIT2 protein upstream of the *nit-3* gene (three sites) and between the *niaD* and *niiA* (four sites).

by NIT2 (Fig. 3). In addition, no binding of any DNA fragments was observed when protein extracts obtained from bacterial hosts containing the expression vector but lacking the cloned *nit-2* segment were used (Fig. 2). Moreover, both of the NIT2 proteins failed to bind to DNA fragments from the promoter regions of the *cys-3* and *cys-14* genes, neither of which is regulated by the *nit-2* gene (Fig. 2).

The *nit-2* gene of *Neurospora* can substitute for the corresponding gene (*areA*) in *Aspergillus* to turn on the expression of various nitrogen structural genes in this related filamentous fungus (24). Both the NIT2 protein and the AREA protein appear to contain a single Cys_2/Cys_2-type zinc-finger-DNA-binding domain (20, 30). Therefore, it seemed possible that the *Neurospora* NIT2 protein would recognize specific elements in the promoter region of *Aspergillus* structural genes. The results displayed in Figs. 2 and 3 demonstrate that the NIT2 protein binds to specific DNA fragments that lie between the divergently transcribed nitrate and nitrite reductase genes, *niaD* and *niiA*, of *Aspergillus*. Specificity of the binding is indicated by the fact that certain DNA fragments between *niaD* and *niiA* genes are strongly bound by NIT2 whereas other fragments from this same region are not bound. The regions depicted as DNA binding elements in Fig. 3*D* were defined by DNA footprinting experiments presented below (Fig. 4).

DNA Footprints with the NIT2–β-Gal Fusion Protein. DNA footprint experiments were employed to determine the identity, number, and location of DNA binding sequences for the NIT2 protein in the promoter regions of the *nit-3*, *niaD*, and *niiA* structural genes. The footprint experiments were carried out with the NIT2–β-Gal fusion protein and various 5′ flanking DNA fragments of the *nit-3*, *niaD*, and *niiA* genes that tested positive in the mobility-shift experiments. Fig. 4 presents the results of these DNA footprint experiments that revealed that the NIT2 protein indeed binds to several specific sites upstream of each of these three genes. In the *Neurospora nit-3* promoter region, three NIT2 binding sites were found at −0.19, −1.1, and −1.2 kb (measured from the start codon for translation). A single NIT2 binding site was located relatively close to the start codon of both the *Aspergillus niaD* gene and the *niiA* gene, located at −0.1 kb and −0.13 kb, respectively. Two additional NIT2 sites were identified in the intercistronic DNA that lies between the *niaD* and *niiA* genes (Fig. 4). All of these binding sites show some similarity and possess an identical core sequence, TATCT, as found in GF1 binding sites (see *Discussion*).

DISCUSSION

The *nit-2* gene of *Neurospora* encodes the major positive-acting regulatory protein that turns on the expression of various nitrogen structural genes during conditions of nitrogen limitation. The NIT2 protein consists of 1036 amino acids and appears to contain a single Cys_2/Cys_2-type zinc-finger motif and an adjacent positively charged region, which together may comprise a DNA-binding domain (20). We were not able to express the complete NIT2 protein in bacterial cells, presumably because of its very large size. However, for some regulatory proteins (e.g., the yeast GCN4 and GAL4 proteins), DNA binding is an attribute of a particular domain that represents only a small portion of the entire protein (22). Thus, a region of the NIT2 protein that contains the zinc finger was expressed alone and as a NIT2–β-Gal fusion protein. These two proteins were both functional in specific DNA binding as demonstrated by gel-mobility-shift experiments.

The expression of the nitrate reductase structural gene *nit-3* has been shown to be highly regulated at the transcriptional level and to require a functional *nit-2* gene for its expression (14). Results presented in this paper demonstrate that the NIT2 protein binds to three distinct recognition elements situated at approximately −0.19, −1.1, and −1.2 kb in the 5′ DNA upstream of the *nit-3* gene. The *N. crassa nit-2* gene can substitute for the corresponding *A. nidulans* major nitrogen regulatory gene *areA*, and the NIT2 protein and the AREA protein have a very similar single zinc-finger domain. We demonstrate here that NIT2 binds to four recognition

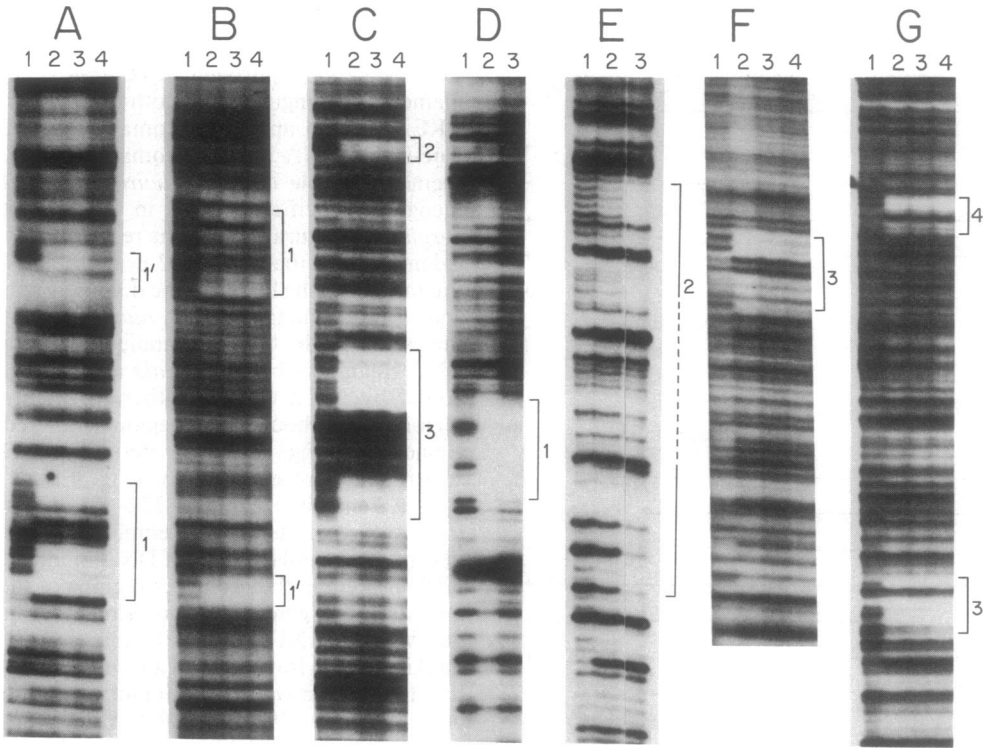

FIG. 4. Representative DNA DNase I footprints. (*A*) *nit-3* site 1 in an *Xba* I–*Xho* I fragment end-labeled at the *Xho* I site. A short secondary binding site (labeled 1′) that contains a single TATCT sequence is also visible. (*B*) *nit-3* site 1 (opposite strand), the *Xba* I–*Xho* I fragment was labeled at the *Xba* I site. (*C*) *nit-3* sites 2 and 3; a subcloned *Eco*RV–*Kpn* I *nit-3* promoter fragment was end-labeled at an *Eco*RI site present in the polylinker next to the *Eco*RV site. Site 2 is at a considerable distance from the labeled end and thus is compressed. (*D*) *Aspergillus niaD–niiA* site 1 in a *Bam*HI–*Eco*RI fragment end-labeled at the *Eco*RI site. (*E*) *niaD–niiA* site 2; a *Dde* I–*Pst* I fragment that contains the long site 2 footprint with a central unprotected region was labeled at its *Dde* I site. (*F*) *niaD–niiA* site 3 in an *Sty* I–*Sst* II fragment labeled at the *Sty* I site. (*G*) *niaD–niiA* sites 3 and 4. An *Sst* II–*Bam*HI fragment was labeled at the *Bam*HI site. Samples for all lanes contained the respective end-labeled DNA fragment that was incubated in the presence or absence of NIT2-β-Gal fusion protein before treatment with DNase I. Lanes: 1, DNA only; 2, + 0.5 μg of protein; 3, + 1.0 μg of protein; 4, + 1.5 μg of protein. The number by each footprint identifies the corresponding binding site indicated in Fig. 3.

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NIT3 # 2  TATCATGTACGTAGATAAGT
          ATAGTACATGCATCTATTCA

NIT3 # 1  CAGGGGAGGTTATCACA-7-TATACATATCTAAAGTATATTTGAAGTAGATAC
          GTCCCCTCCAATAGTGT-7-ATATGTATAGATTTCATATAAACTTCATCTATG

NIT3 # 3  GCCGCCTATCTACACT-22-TGCACTATCAAACCTTATCAATC
          CGGCGGATAGATGTGA-22-ACGTGATAGTTTGGAATAGTTAG

NIAD # 1  CTATCTAATCGACCTTGATCTGGCATATCTACCAGTTCA
          GATAGATTAGCTGGAACTAGACCGTATAGATGGTCAAGT

NIAD # 2  TAGATAGAGATAGGGCTTGATTATCGTTGAT-19-TCGAATCTCTTATCTC
          ATCTATCTCTATCCCGAACTAATAGCAACTA-19-AGCTTAGAGAATAGAG

NIAD # 3  TCATGCTTATCGCCGGCTCATTATCGTCAAGC
          AGTACGAATAGCGGCCGAGTAATAGCAGTTCG

NIAD # 4  TTTAATTATCTCATGCTCCGATGCCACGGAGATACAA
          AAATTAATAGAGTACGAGGCTACGGTGCCTCTATGTT
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FIG. 5. Binding sites for NIT2 in the promoter region of the *Neurospora nit-3* gene and between the divergently transcribed *Aspergillus niiA* and *niaD* genes. The *Aspergillus* sites are numbered 1 to 4 from the *niaD* gene; thus site 4 is actually just upstream of the *niiA* gene (see Fig. 3). Site 2 of *nit3* is listed first only because it is shorter than the other sites. All sites contain at least two copies of a core sequence TATCTA (or a closely related sequence), which are underlined or overlined. Three of the sites are bipartite and show a long footprint with a central unprotected region; the number of base pairs within the unprotected region is indicated in each case.

sites in the intergenic DNA that lies between the divergently transcribed *niaD* and *niiA* genes. These results strongly suggest that the NIT2 and AREA proteins recognize similar DNA-binding sequences. However, it is possible that the *Neurospora* NIT2 protein binds to cryptic sites in the *Aspergillus niaD–niiA* promoter region that are distinct from the elements recognized by the AREA protein. An analysis of AREA binding sites will provide valuable insight about possible relationships between NIT2 and AREA.

The DNA sequences at the three binding sites upstream of the *Neurospora nit-3* gene and at the four binding sites between the *Aspergillus niaD* and *niiA* genes identified by the footprint experiments are presented in Fig. 5. It is intriguing that these seven sites display only a limited amount of sequence homology. However, all of the sites share one feature; namely, all seven of the NIT2 recognition elements identified in this study contain at least two copies of a core sequence whose consensus is TATCTA, with some sites having three or even four such core sequences (Fig. 5). The region of the NIT2 protein immediately on the carboxyl side of the zinc-finger motif is highly basic (net charge +12). The zinc-finger motif and the downstream basic region of NIT2 is remarkably similar to the two zinc fingers and basic regions of GF1, a trans-acting DNA-binding protein that functions in the erythroid lineage of mammalian and avian cells (31–33). This 50-amino acid segment of NIT2 has 64% amino acid identity with the second GF1 zinc finger. The mammalian GF1 protein also recognizes a core consensus DNA sequence, TATCT, present in promoter and enhancer elements of all members of the globin gene family (31). Moreover, beyond the core sequence, the various binding sites for GF1 appear to lack any sequence homology (31). It is well established that some regulatory proteins can recognize two or more distinct DNA sequences—e.g., the yeast HAP1 protein (34), the glucocorticoid receptor, CCAAT-binding protein/enhancer-binding protein (C/EBP), and the octamer binding protein OBP-100 (35).

A total of 18 core sequences were found at the seven binding sites examined in this work; many of these are present in a paired fashion, either tandemly repeated or inverted with dyad symmetry. These hexameric core sequences appear to be the primary specificity elements recognized by the NIT2 protein in the control regions of *Neurospora* and *Aspergillus* genes that we examined. We anticipate that other nitrogen catabolic genes of these two filamentous fungi will be found to have similar recognition elements. It is important to note that these recognition sequences have only been identified *in vitro* and studies are needed to determine whether any or all of these sites function *in vivo* in controlling gene expression. The length of the individual footprints shows some variation; furthermore, three of the NIT2 binding sites appear to be bipartite—i.e., the footprints show two regions with protected and enhanced cleavages, separated by 10–20 bp that show the usual DNase I sensitivity. These observations suggest the possibility that two or more molecules of the NIT2 protein can bind at some of these elements, perhaps even in a cooperative manner. Since NIT2 can turn on various structural genes to widely different extents, it would not be surprising if individual sites were to show significant differences in binding affinity for NIT2.

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