Recognition of specific nucleotide bases and cooperative DNA binding by the *trans*-acting nitrogen regulatory protein NIT2 of *Neurospora crassa*

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ABSTRACT

The NIT2 nitrogen regulatory protein of *Neurospora* is a DNA binding protein which contains a single Cys₂/Cys₂ type finger motif followed immediately by a highly basic region. Several different approaches were employed to identify nucleotides which appear to be in contact with NIT2 in the DNA–protein complex. Methylation interference and missing contact analyses with the promoter DNA fragment of the L-amino acid oxidase gene showed that all three purines in both of two GATA core sequences and the single adenine residue in each of the complementary TATC sequences were in intimate contact with NIT2. Modification or loss of the three purine residues located between the two GATA core sequences also significantly reduced NIT2 binding, whereas alteration of purines which flank the binding element showed only minor effects. Chemical modification of all six thymine bases in the two GATA and TATC complement core sequences also strongly affected NIT2 binding. High affinity NIT2 binding sites appear to contain at least two GATA core sequences, with single GATA sequences acting only as weak binding sites. Mobility shift experiments with the DNA fragment upstream of *nit-3*, the structural gene for nitrate reductase, revealed two DNA–NIT2 protein complexes. In complex I, which is formed first, NIT2 was bound to a pair of GATA sites located at –180. In complex II, the paired GATA sites at –180 plus a single GATA site at –290 were all occupied by NIT2. A DNA fragment containing only the single –290 GATA element bound NIT2 very weakly. The affinity of this single GATA for NIT2 was ten to twenty times greater when it was situated on the same DNA fragment with the distant paired GATA elements than when alone.

INTRODUCTION

Regulation of gene expression is mediated by *trans*-acting regulatory proteins which recognize and bind at sequence-specific DNA elements in the promoters and enhancers that serve to

regulate one or more adjacent structural genes. Although both negative and positive-acting regulatory proteins control gene expression in eukaryotic organisms, those which positively turn on, i.e., activate transcription seem to predominate. A common feature of many such positive-acting regulatory proteins is that DNA binding and activation functions reside in different domains or regions of the protein (1,2). Activation is commonly believed to occur by the interaction of one or more activation domains, which may be rich in glutamine, proline or in acidic residues, with proteins of the basic transcription machinery, e.g. TFIID or TFIIB (3,4). In addition, distinct regions of *trans*-acting factors may serve as allosteric sites for the binding of ligands such as hormones, metal ions, or metabolic effectors, whereas still other regions function in protein–protein interactions.

A number of distinct types of DNA-binding motifs have been identified, which include helix–turn–helix, Cys₂/His₂ and Cys₂/Cys₂ zinc fingers, Cys₆/Zn₂ binuclear fingers, bZip motifs, and basic helix–loop–helix structures. A special type of DNA binding finger structure, which has a highly conserved motif characterized by Cys₂-17 amino acids–Cys₂, followed by a region rich in basic amino acids, has been identified in very diverse eukaryotic organisms, including yeast, filamentous fungi, plants, chickens, mouse, and human beings (5–12). Regulatory proteins which possess only a single finger structure of this type are found in fungi, including yeast, *Aspergillus*, and *Neurospora*, in which they play a fundamental role in control of nitrogen metabolism (5,11,13). In higher eukaryotes a family of such proteins, known as GATA-1, -2, -3, and -4 appear to act as tissue-specific *trans*-acting factors (14–16). GATA-1, whose characteristics are best understood, functions in the differentiation and function of the erythropoietic cell lineage, and binding sites for GATA-1 are found in the promoters and enhancers of all globin genes (6,7). Each of these mammalian GATA factors have two tandem repeated finger motifs, but only the carboxyl terminal finger, which is most similar to the single finger found in the fungal proteins, is required for specific DNA binding. Recently it has been demonstrated that binding of a metal ion is required for an *in vitro* synthesized single GATA finger peptide to adopt a helical conformation and to function in DNA binding (17).

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Omichinski *et al.* (17) raised the interesting possibility that the ferrous ion, rather than zinc, might be involved in the finger motif of the GATA proteins.

NIT2 is a well characterized positive-acting control protein of *Neurospora crassa*; it is a global nitrogen regulatory protein which is responsible for turning on the expression of many different structural genes which specify nitrogen catabolic enzymes (18). Mobility shift and DNA footprinting analyses have identified binding sites for the NIT2 protein in the promoter regions of several structural genes, including *nit-3*, *alc*, and *lao*, which encode nitrate reductase, allantoinase, and L-amino acid oxidase, respectively (19). Exley *et al.* (20) have recently identified putative NIT2 binding sites in the 5' promoter region of *nit-6*, which specifies nitrite reductase. Much sequence variation is detected in the NIT2 binding sites, but the presence of at least two GATA core sequences appears to be a common feature of all NIT2 sites. Here we report studies designed to identify individual nucleotide bases within a recognition element which may be in direct contact with the NIT2 regulatory protein. We demonstrate with the *lao* promoter that all four bases of the GATA core sequence, as well as at least three bases of the complementary sequence, TATC, on the opposite strand, are essential for NIT2 DNA binding. We also report similar studies with the promoter region of the nitrate reductase structural gene, *nit-3*, which imply that cooperative NIT2 binding occurs

MATERIALS AND METHODS

Plasmids and DNA fragments and NIT2 protein expression

A 430 bp XhoI–Xba DNA fragment of the *nit-3* gene promoter region was isolated from plasmid pNIT3 (5). To obtain a DNA fragment containing a single high affinity NIT-2 recognition element, an 80 bp L-amino acid oxidase (LAO) gene promoter TaqI restriction fragment (21) was filled in with T4 DNA polymerase (22) and subcloned into the SmaI site of bluescript vector. Cloned DNA fragments were isolated by cutting with appropriate restriction enzymes and end-labeled at a single site by DNA polymerase Klenow with [α - 32 P]dATP (22). Expression and purification of a NIT2- β Gal fusion protein, in which 217 amino acid residues of the NIT2 zinc-finger domain are fused to the amino terminus of the entire β -Gal protein was described previously (19). The NIT2- β GAL fusion protein copurifies with native β -galactosidase and a degradation fragment of the enzyme and represents approximately 5% of the protein.

Chemical modification of DNA

Methylation. Partial methylation of end-labelled DNA fragments was performed as described by Siebenlist and Gilbert (23). DNA in 100 μ l of 50 mM sodium cacodylate buffer (pH 8.0) was mixed with 1 μ l of dimethyl sulfate for 1 minute (for the 430 bp fragment) or 5 minutes (for the 80 bp fragment) at room temperature, when the reaction was terminated by the addition of 50 μ l of 'DMS stop solution' (1 M 2-mercaptoethanol, 1.5 M sodium acetate, 10 mg/ml tRNA). After two rounds of precipitation with 3 volumes of ethanol, the DNA was vacuum-dried and dissolved in TE buffer at 20,000 cpm/ μ l.

Depurination. The end-labelled *lao* DNA fragment contained in 30 μ l of TE buffer containing 2 μ g poly(dI-dT) was mixed with 3 μ l of 1 M formate. After 8 minutes of incubation at 37°C, 3 μ l of 1 M sodium acetate (pH 7.0) was added. The DNA was ethanol-precipitated twice, and dissolved in TE buffer at 20,000 cpm/ μ l (24).

Thymine-specific modification. Modification of thymine residues in the end-labelled DNA fragment with KMnO₄ was performed as described (25). DNA in 10 μ l of 30 mM Tris–HCl (pH 7.5) was denatured at 95°C for 4 minutes and quickly cooled on ice, when 20 μ l of 0.25 mM KMnO₄ solution was added. After 20 minutes, the reaction was terminated by the addition of 50 μ l DMS stop solution. The DNA was ethanol-precipitated, dissolved in 30 mM Tris–HCl pH 7.5, 50 mM NaCl, 2 mM EDTA pH 8.0, heated to 95°C for 4 minutes and renatured by slowly cooling to 25°C.

Cleavage of DMS-methylated DNA was as described previously (26). Depurinated DNA was cleaved by dissolving it in 2 mM phosphate buffer (pH 7.0), adding 1 μ l of 1 M NaOH, and heating the sample for 15 minutes at 90°C (26). Piperidine cleavage was used for thymine-specific modified DNA (27). After cleaving, the samples were resolved by electrophoresis in sequencing gels and autoradiographed. The relative intensity of relevant bands was determined with a BioRad model GS-300 densitometer. The extent of interference for protein–DNA binding for molecules represented by a particular band was determined by the ratio of optical density of the same band in both bound and unbound DNAs. The interference values were normalized by use of the ratios observed with bands for the same nucleotide bases but for positions which occur outside of the NIT2 recognition element.

Nick interference with binding of NIT2

The 80 bp *lao* promoter DNA fragment was first uniquely end-labelled with 32 P by the Klenow fill-in reaction on either strand. The DNA was lightly digested in 50 μ l of 1 $\times$ binding buffer containing 2 μ g of poly(dI-dC), 2 μ l of 100 mM MgCl₂ and 5 μ l of 1 U/ μ l DNaseI. The reaction was carried out for 5 minutes at room temperature and stopped by adding 50 μ l of 0.5% SDS, 10 mM EDTA, and 0.5 mg/ml tRNA. After two rounds of phenol extraction, the DNA was ethanol precipitated, dried, and dissolved in TE buffer at 20,000 rpm/ μ l.

Mobility shift assays

NIT2 binding reactions and gel band mobility shifts were carried out as described before (19) with minor modifications. Typically, binding was accomplished in 20 μ l of 12 mM HEPES–NaOH pH 7.9, 4 mM Tris–HCl pH 7.5, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 2 μ g poly (dI-dC) and 10% glycerol, using a P³²-end-labelled DNA fragment (10,000 to 20,000 cpm) plus affinity-purified NIT2- β Gal fusion protein. The reaction mixture was equilibrated at room temperature for 20 minutes, and then loaded onto 4% polyacrylamide gels in 0.25 $\times$ TBE. After electrophoresis, the gels were autoradiographed or analyzed via a β -scanner to determine the radioactivity present in individual bands. The free DNA band and DNA–protein complex band(s) were excised from the gel, electroeluted onto a DEAE membrane (NA45; Schleicher & Schuell, Inc.) and eluted according to the manufacturer's instructions. Hydroxyl radical footprinting was performed as described (28).

RESULTS

Methylation and depurination interference of DNA binding by NIT2

In order to determine whether specific guanine or adenine residues were important for DNA binding by the NIT2 protein, both methylation interference and missing contact (depurination) experiments were carried out. The 80 bp LAO (L-amino acid

Figure 1. Interference of NIT2 binding by methylation or depurination of the 80 bp *lao* DNA fragment. The DNA fragment, labeled at its 3' end with ^{32}P on the transcribed strand (a) or the nontranscribed strand (b), was partially methylated or depurinated and subjected to interference analysis. U, the transcribed strand; L, the nontranscribed strand; M, methylation interference; D, depurination interference; C, bound probe; P, free probe; IN, input probe; T, G and G+A show the T, G and G+A ladders, respectively, generated by chemical cleavage at these specific bases (see materials and methods) of the same fragment. Bases whose modification interfered with NIT2 binding are shown by closed circles (strong effects) and open circles (weak effects). The results of A methylation interference are difficult to discern but were readily visible in the autoradiographs. Panels c and d summarize the results of the methylation (c) and depurination (d) interference experiments. The magnitude of the effects on binding is shown by the height of the bar above or below a base. The full height of the scale represents 100% interference and bases at the binding site are numbered.

oxidase) gene promoter fragment containing a 11 bp palindromic sequence, 5'-GATACTGTATC, was employed. This 11 bp palindrome, which contains two GATA core elements facing toward each other, has previously been shown to display high binding affinity for the NIT2 protein in both mobility shift and DNaseI footprinting experiments (29). After partial methylation or depurination of this DNA fragment, it was incubated with the NIT2 protein and the NIT2-DNA complex and the unbound probe were separated by the gel mobility shift technique. A single DNA-protein complex was present in the mobility shift assay (results not shown).

DNA molecules, in which bases important for NIT2 binding were methylated or missing, would be significantly reduced or missing entirely in the DNA-NIT2 protein complex band, and would accordingly be enriched in the free probe band. The protein-bound and free DNA probe bands were isolated, cleaved, and analyzed by electrophoresis in a sequencing gel as described in Materials and Methods. Guanine and adenine residues important for NIT2 binding were revealed as bands with reduced intensities in the lane corresponding to bound DNA in comparison with the same bands in the lane with the free probe DNA.

Figure 1 shows the results of both the methylation interference and the missing contact experiments. Methylation of the guanines at positions L5 and R1 on the transcribed DNA strand and at L1 and R5 on nontranscribed strand drastically reduced NIT2 binding affinity (Fig. 1 a,b and c). Adenine residues at L2, L4, and R3 on the transcribed strand and at L3, 0, R2, and R4 on the nontranscribed strand, when methylated, similarly strongly reduced NIT2 binding. Only minor interference with NIT2 binding was observed for DNA molecules with methylated

purines at positions L6, L7, L8, R6, R7, R8, R9 and R10 on either strand. In the depurination or missing contact analysis, the affect of loss of individual adenine or guanine bases on DNA binding by NIT2 was examined. The results generated by missing purine contact assay generally agree with those of methylation interference but appeared even more striking (Fig 1d). Depurination at positions L2A, R1G or R3A on the transcribed strand, for instance, reduced the binding affinity for NIT2 to about 5% of unmodified DNA molecules, while DNA molecules methylated at these same positions retained almost 30% binding affinity (Figure 1c and d).

These results indicate that interaction of the NIT2 protein with its DNA binding site is fulfilled through close contacts of the DNA binding domain of NIT2 with A and G bases within the GATA core sequence element. However, purines that were required for NIT2 interaction were not entirely confined to the GATA core sequences, and other purines, particularly bases between the two core elements, were also important (see Figure 1c and d).

Thymine-specific modification interference

Seven thymidine bases lie within the *lao* 11 bp palindromic NIT2 binding element. To investigate any possible interactions between thymine residues and the zinc finger region which constitutes the NIT2 protein DNA binding domain, the 80 bp DNA fragment which contains the 11 bp NIT2 binding element was treated with KMnO_4 using conditions which specifically modify thymine residues (30). Thymine residues important for DNA binding by NIT2 were identified by an interference assay, as described above for methylation interference. Chemical modification of thymidine

Figure 2. Interference of NIT2 binding by thymine modification. The 80 bp *lao* promoter DNA fragment labelled at the 3' end on the transcribed strand (a) or the nontranscribed strand (b) was treated with KMnO_4 and subjected to interference assay. In each gel, chemically generated G ladders of the same DNA strand was run along with the samples. U, transcribed strand; L, nontranscribed strand; G, G ladders; C, bound probe; P, free probe. The bands of corresponding to thymines whose modification interfered with NIT2 binding are indicated by closed circles (strong effects) or open circles (weak effects). (c) The magnitude of thymine modification on NIT2 binding is shown as described in the legend for Figure 1.

residues at positions L3, R2, R4, and R6 on the upper DNA strand and at L4 and R3 on the lower strand strongly interfered with DNA binding by the NIT2 protein (Fig. 2). In contrast, modification of thymidines at position O on the upper strand and at L2 on the lower strand had only minor effects upon NIT2 DNA binding. These results, together with those of methylation and depurination interference, provide strong evidence for intimate contacts between the NIT2 protein and bases within the GATA core sequences.

Interference of backbone nicks on NIT2 DNA binding

Introduction of nicks into the deoxyribose-phosphate backbone of the DNA double helix by DNaseI can be expected to induce subtle change in DNA double helix geometry. A nick interference assay may reveal groups, e.g. phosphates, which are closely contacted by a protein or phosphodiester bonds which are important for maintaining a native DNA conformation that is essential for recognition by a DNA-binding protein. In this approach, the end-labelled *lao* promoter DNA fragment was treated with DNaseI in the presence of Mg^{++} to produce random nicks in a limited fashion so that each modified DNA fragment was nicked at approximately only one position. The population of randomly nicked DNA fragments was incubated with the NIT2- β Gal fusion protein and analyzed as described above for methylation interference assays. DNA molecules with nicks that interfered with NIT2 binding were missing in the DNA-protein complex and correspondingly enriched in the free probe band in mobility shift gels. Thus, in sequencing gels, one observes DNase I generated ladders, with certain bands having reduced intensities in the lanes corresponding to the DNA-protein complex relative to that of the free probe and input DNA.

This technique allowed us to identify a variety of phosphodiester bonds in the NIT2 binding site which, when broken, significantly reduced NIT2 binding. As showing in Figure 3a and 3b, nicks in the region between L2A and R7C on the transcribed strand and between R3T and L7G on the nontranscribed strand severely interfered with NIT2 binding. The interference pattern appeared to be in a quasisymmetric fashion. Positions which can when nicked interfere with DNA binding by NIT2 were also found outside the binding site. For instance, some nicks even 10 to 12 bases away from the NIT2 binding site still showed some interference with NIT2 binding (Figure 3b). These results indicate that the particular local topography around the 11 bp binding element may play role in its serving as a high affinity NIT2 binding site. A major effect of such nicks, however, may be to allow the DNA to become partially single stranded, thus disrupting the native double stranded form which may be required for a DNA binding site.

Hydroxyl radical footprinting

The possibility that the NIT2 protein makes tight contacts with deoxyribose moieties in the DNA backbone was examined by Fe(II)EDTA -generated hydroxyl radical footprinting. The 80 bp LAO DNA fragment was end-labelled, equilibrated with NIT2 protein, treated with hydroxyl radicals, and the DNA-protein complex and unbound probe were separated by the gel shift technique and then analyzed with a sequencing gel. A diagram which represents the densitometric results is shown in Figure 4a. A stretch of 19 contiguous nucleosides on the transcribed strand and 15 contiguous nucleosides on the nontranscribed strand showed apparent protection by NIT2. In addition, some enhanced hydroxyl radical attacks were found on both strands of sequences flanking the 11 bp recognition element, which suggests that

Figure 3. DNaseI-generated nick interference of NIT2 binding. (a) The 80 bp *lao* DNA fragment ^{32}P -labelled at 3' end on the transcribed strand (U) or the nontranscribed strand (L) was treated with DNaseI to generate approximately one nick per DNA molecule. The nicked DNA fragments were then subjected to NIT2 binding. The bound and unbound DNA fractions were separated on a 4% polyacrylamide gel in $0.25\times\text{TBE}$ and analyzed with a 6% sequencing gel. The vertical box along either side of the diagram shows the region spanning NIT2 binding site. C, bound probe; P, free probe; G, G DNA sequence ladders of the same fragment. The horizontal arrows indicate the center of dyad symmetry of the binding site. (b) The magnitude of nick interference with binding of NIT2 is indicated by the length of arrows which point to the position of the nicks.

protein-induced DNA bending might occur in the DNA – NIT2 complex (31).

Interactions of NIT2 with NIT3 proximal promoter elements

The XhoI/XbaI fragment of the proximal promoter region of the *nit-3* gene, a highly regulated gene (19,32), contains several NIT2-binding GATA core sequences (Figure 5c). To further understand the interaction of NIT2 with the *nit-3* promoter, we first titrated this promoter fragment with increasing amounts of the NIT2 protein. Two distinct DNA – protein complexes were observed in mobility shift gels (Fig. 5a). As the amount of NIT2 protein was increased, the amount of complex I (CI) decreased in parallel with an increase in the slower-migrating complex II (CII), implying that CII was formed from CI by binding an additional NIT2 molecule(s) to the same DNA fragment.

To investigate the nature of these two complexes formed via the NIT2 protein – *nit-3* promoter interaction, a methylation interference analysis was performed. Complex I and complex II and the unbound probe were each isolated from a mobility shift gel and examined with a sequencing gel (Fig. 5b). Complex I results from NIT2 protein binding at a site centered at –180 in the *nit-3* promoter, which contains three closely-spaced GATA core sequences, and was previously identified by DNA footprinting as the most proximal NIT2 binding site (33). Within this site, NIT2 binding seems to occur primarily at doublet GATA elements which are separated by 16 bp (Fig. 5c). Surprisingly, complex II results from NIT2 occupation the paired GATA elements plus that of a single GATA sequence which is 110 bp upstream of the –180 binding site. Thus, methylation of G and A residues in either of the doublet GATA core elements at –180 interferes with formation of both complex I and II (Fig. 5b). In contrast, methylation of G and A residues in the single upstream

Figure 4. Hydroxyl radical NIT2 footprint of the *lao* promoter DNA fragment. The 80 bp *lao* promoter DNA fragment was end labelled with ^{32}P (20,000 counts/min), incubated with $1\ \mu\text{l}$ of the NIT2- βGal fusion protein in binding buffer, and briefly treated with hydroxyl radical generating reagents. The DNA – NIT2 complexes were separated from free probe with a 2.5% agarose gel in $0.5\times\text{TBE}$ buffer and analyzed on a 6% sequencing gel. The vertical boxes indicate the NIT2 binding site. L, untranscribed strand; P, free probe; C, bound probe; G, G DNA sequence ladders of the fragment.

GATA sequence interferes only with complex II formation (Fig. 5b). These results suggest that NIT2 first binds at the GATA elements at –180 in the *nit-3* promoter to form complex I, and that subsequent binding of NIT2 to the single upstream GATA element results in the formation of complex II. No NIT2 protein binding to the single GATA site without occupation of the GATA doublet could be detected. This result led us to speculate that the single upstream GATA sequence was itself a weak NIT2 binding site, but that it might become a higher affinity site when the –180 site on the same DNA fragment had been occupied by NIT2. This prediction was examined and confirmed by the following experiment. The NIT3 promoter DNA fragment (F1)

Figure 5. Methylation interference and titration of the *nit-3* promoter fragment with NIT2 protein. (a) Titration of NIT3 promoter fragment with NIT2 protein. Identical amounts (10 ng) of the 437 bp 32 P-end labelled NIT3 XhoI/XbaI fragment (10,000 cpm) were incubated with increasing amounts of the NIT2- β Gal protein. The results of the gel mobility shift were analyzed with a β -scanner. The amount of NIT2 protein used in the binding assay in Lanes: 1, 5 μ g; 2, 2.5 μ g; 3, 1.25 μ g; 4, 0.6 μ g; 5, 0.3 μ g; 6, 0.15 μ g; 7, 0.08 μ g; 8, 0 μ g. The arrows indicate the position of free probe (P), fast moving complex (CI) and slow moving complex (CII). (b) Methylation interference of NIT2 binding to the *nit-3* promoter fragment. The 437 bp NIT3 XhoI/XbaI fragment 32 P-labelled at the XbaI end by filling-in was treated with DMS, incubated with the NIT2- β Gal protein, and analyzed by a mobility shift gel. The free probe and both retarded complexes I and II were isolated, cleaved, and examined with a 6% sequencing gel. The bands corresponding to the GATA sequences are identified; bases which, when methylated, affect complex I or II formation are circled. P, free probe; IN, input probe; CI, fast moving complex; CII, slow moving complex. (c) Diagram of proximal region of the *nit-3* promoter fragment. The three GATA elements involved in NIT2 binding are indicated. (d) Titration of different binding sites in the NIT3 promoter fragment with NIT2 protein. The 437 bp NIT3 promoter fragment (F1) was cut by AluI restriction enzyme. The fragment containing the single 'GATA' (F2) and the fragment containing the GATA doublet (F3) were isolated. A fixed amount of each of these three DNA fragments (F1, F2 and F3) were incubated with different amounts of the NIT2 protein in 20 μ l binding buffer. The results of the mobility shift experiments were quantitated by β -scanning. Symbols: CII, F1 DNA-NIT2 fast moving complex; CII, F1 DNA-NIT2 slow moving complex; C2, F2 DNA-NIT2 complex; C3, F3 DNA-NIT2 complex. P1, P2, and P3 indicate the position of F1, F2, and F3 free probes. The amount of NIT2- β Gal protein used in lanes 0, 1 and 2 (or 2') were 0, 0.5 μ g and 2.5 μ g, respectively. (e) NIT2-DNA complex(es) for different binding sites on the *nit-3* promoter. The values above each bar shows the percentage of binding sites occupied by NIT2 in the titrations (see 5d). F1, F2, F3 and 1, 2 are defined above (5c). The results with the single 'GATA' site and the 'GATA' doublet site are indicated.

was cut by AluI restriction enzyme which separated the single GATA sequence from the GATA doublet onto two fragments, F2 and F3 (Fig. 5c). The F1, F2 and F3 DNA fragments were each titrated with NIT2 protein in quantitative mobility shifts. The results are shown in Figure 5d and summarized in Figure 5e. Both the GATA doublet and the single GATA site showed higher NIT2 binding affinities when together on the same DNA fragment than when on separate fragments. The increase in the binding affinity of the single GATA site was particularly striking, presumably because it possesses only a weak binding ability when alone on F2 (Figure 5e). In fact, very little if any binding of NIT2 to DNA fragment F2, with the single GATA element, was observed, whether or not a separate fragment containing the strong binding site (DNA fragment F3) was included in the binding reaction (Figure 5d, lanes F3 and F2+F3). The single GATA site showed a very substantial increase (10 to 20-fold) in binding affinity when situated on a DNA fragment that also included the stronger -180 binding site (Figure 5e). These results suggest a cooperative binding of the NIT2 protein to the duplex GATA site and the single upstream GATA site.

Cooperative binding could possibly occur by a signal conveyed along the DNA molecule such that protein binding at one site would alter a second site and thus change its affinity for the same or a different protein. An alternative, and more likely, possibility is that protein-protein interaction would be responsible for cooperative DNA binding. In order to examine the manner in which NIT2 binds at these two separated sites in an apparent cooperative manner, we introduced nicks in the DNA backbone and also used a missing nucleoside interference analysis with the *nit-3* promoter fragment. Neither nicks nor gaps introduced between the GATA doublet and the single GATA prevented the formation of complex II formation (data not shown). These results indicate that the cooperative binding was not accomplished by communication between the two sites via the DNA double helix itself. Rather, these results suggest that DNA looping and direct protein-protein interactions are responsible for the cooperative effects.

DISCUSSION

The NIT2 global-acting nitrogen regulatory protein of *Neurospora* is a member of a special class of DNA binding proteins which contact DNA with a domain that contains a finger motif with a characteristic structure Cys-X₂-Cys-X₁₇-Cys-X₂-Cys followed immediately by a highly basic region. In the work reported here, several different approaches were employed to identify nucleotides which appear to be in intimate contact with NIT2 in the DNA-protein complex. Close interactions of NIT2 with purine bases were examined by methylation interference and by missing contact analyses of the promoter of the *lao* gene, which contains two closely spaced GATA core sequences in a palindromic arrangement. All three purines in both of the two GATA core sequences and the single adenine residue in each of the complementary TATC sequences were identified as major NIT2 contact sites. In addition, modification or loss of the three purine residues located between the two GATA core sequences also significantly reduced NIT2 binding, whereas purines which lie outside of this element showed only minor effects. The results of thymine modification upon NIT2 binding were completely consistent with the above findings. Chemical modification of all six of the thymine bases in both of the GATA and TATC complement core sequences, plus the one thymine residue which lies between them, strongly reduced NIT2 binding. Our findings

with NIT2 agreement well with methylation interference and in vivo dimethyl sulfate footprinting analyses conducted with GATA-1 (7,34), a mammalian trans-acting protein which has a homologous DNA binding finger motif.

It appears that high affinity NIT2 binding sites contain two (or more) GATA core sequences and that single GATA sequences serve only as weak binding sites. Surprisingly, it appears that the paired GATA elements of a strong binding site can be oriented in the same direction or facing one another and, furthermore, their spacing can vary, in the range of 3 to 30 bp (Chiang and Marzluf, unpublished results). When two GATA core sequences are separated by greater than approximately 30 bp, NIT2 binding is very weak, similar to that found with single GATA sites. The results presented here support this model for NIT2 binding. Only a single DNA-protein complex, in which both of the two GATA sequences were simultaneously occupied by NIT2, could be observed with the *lao* promoter. The NIT2 protein may bind as a monomer to a single GATA sequence with low affinity; however, when two such GATA elements are in close proximity, two NIT2 monomers may interact and stabilize their joint binding. Such a model implies that NIT2 binding is cooperative.

The results obtained with the DNA fragment upstream of *nit-3*, the structural gene for nitrate reductase, provide additional support for the concept that a single GATA element serves only as a weak NIT2 site, whereas two or more closely spaced GATA sequences act as a high affinity NIT2 binding site. Our experiments with the *nit-3* promoter fragment revealed two DNA-NIT2 protein complexes that were resolved by the mobility shift technique. In Complex I, which appeared first with increasing protein concentrations, NIT2 was bound to a pair of GATA sites separated by 16 bp. In complex II, which seemed to be formed at the expense of complex I, the paired GATA sites (at -180) plus a single GATA site at -290 were all occupied by NIT2. Moreover, a DNA fragment containing only the single -290 GATA site did not bind NIT2 to a detectable level, i.e. the affinity of this single GATA element for NIT2 was ten to twenty times greater when it was situated on the same DNA fragment with the distant paired GATA elements than when alone. These results further suggest that NIT2 binds as a monomeric species, and indicate that a looping out of DNA allows protein-protein interaction to stabilize binding of NIT2 to a single GATA site greater than 100 bp from a high affinity site. The possibility that a signal is conveyed along the rigid double helix seems unlikely, particularly since nicks in the backbone and even missing bases did not prevent complex II formation. Omichinski *et al.* (17) reported that the avian GATA-1 protein binds as a monomer, and several reports have suggested that avian or mammalian GATA factors can bind strongly to a single GATA sequence (12,15-17). In a very recent study, Ko and Engel (15) found that the highest-affinity sites detected for avian GATA-1, -2, and -3 proteins were double sites in inverted orientation. Although all of the results which we have obtained to date suggest that high affinity NIT2 binding requires the presence of two or more closely spaced GATA core sequences, we cannot yet exclude the possibility that certain single GATA elements with special features might serve as strong NIT2 binding sites; however, we have found that substitution of nucleotides 5' or 3' to the GATA core elements have only modest effects, two to three-fold, upon NIT2 binding (Chiang and Marzluf, unpublished results). An important unresolved question is to identify the amino acid residues within the DNA-binding domain of NIT2 and other members of the GATA binding protein family which directly contact individual bases of the GATA core elements.

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