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nit-2, the Major Nitrogen Regulatory Gene of *Neurospora crassa*, Encodes a Protein with a Putative Zinc Finger DNA-Binding Domain

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The nitrogen regulatory circuit of *Neurospora crassa* consists of a set of unlinked structural genes which specify various nitrogen catabolic enzymes plus control genes and metabolic effectors which regulate their expression. The positive-acting *nit-2* regulatory gene is required to turn on the expression of the nitrogen catabolic enzymes during conditions of nitrogen limitation. The complete nucleotide sequence of the *nit-2* gene was determined. The *nit-2* mRNA is 4.3 kilobases long and has a long nontranslated sequence at both its 5′ and 3′ ends. The *nit-2* gene nucleotide sequence can be translated to yield a protein containing 1,036 amino acid residues with a molecular weight of approximately 110,000. Deletion analyses demonstrated that approximately 21% of the NIT2 protein at its carboxy terminus can be removed without loss of function. The *nit-2* protein contains a single putative Cys_2/Cys_2 zinc finger domain which appears to function in DNA binding and which has striking homology to a mammalian *trans*-acting factor, GF-1.

In *Neurospora crassa*, limitation of preferred nitrogen sources, e.g., ammonia or glutamine, leads to the increased synthesis of various enzymes which are required for the use of a variety of secondary nitrogen sources, including nitrate, nitrite, purines, amino acids, and proteins (22). This response is governed by the nitrogen control circuit, one of several global metabolic regulatory circuits of *N. crassa*. The nitrogen regulatory circuit has been studied extensively and includes a number of unlinked structural genes which are controlled in parallel by both major and minor regulatory genes as well as by metabolic inducers and nitrogen catabolite repression (12, 17, 24). The *nit-3* gene, which encodes nitrate reductase, is a well-characterized structural gene of the nitrogen circuit, and expression of *nit-3* has been shown to be highly regulated by nitrogen repression, nitrate induction, and the activity of three separate control genes, *nit-2*, *nit-4*, and *nmr* (12).

Synthesis of enzymes of particular pathways within the nitrogen circuit requires specific induction of substrates or intermediates, mediated by minor control genes. For example, in the presence of the inducer nitrate, a pathway-specific control gene, *nit-4*, turns on the expression of the nitrate and nitrite reductase structural genes, *nit-3* and *nit-6*, respectively (11, 22).

The major positive-acting regulatory gene *nit-2* plays a central role in the nitrogen circuit and turns on the expression of the structural genes during conditions of nitrogen limitation (13, 22). In contrast to *nit-2*, a distinct and unlinked regulatory gene, *nmr* (for nitrogen metabolic regulation), acts in a negative manner (10, 15, 27). In *nmr* mutants, various nitrogen-related enzymes are expressed constitutively, even in the presence of sufficient levels of preferred nitrogen sources to fully repress enzyme synthesis in nmr^+ strains. It appears that the *nit-2* gene and the *nmr* gene are both directly involved in nitrogen catabolite repression in *N. crassa*, but the manner in which they may interact is not yet understood.

It was demonstrated that the *nit-2* gene product encoded in one nucleus is freely diffusible throughout the cell and can turn on the expression of structural genes not only in the same nucleus but also in other nuclei of a heterokaryon (5), suggesting that the *nit-2* product is a protein. The isolation of a suppressible amber nonsense *nit-2* mutant implies that the *nit-2* gene encodes a protein (26). Several different hypotheses have been proposed concerning the expression of the *nit-2* gene itself and the mechanism of operation of the nitrogen circuit (8, 9, 16). In each case, a functional *nit-2* product was postulated to turn on nitrogen structural gene expression. These hypotheses include (i) constitutive expression of the *nit-2* gene, with the activity of its protein product being sensitive to glutamine (16); (ii) negative control of *nit-2* expression by the *nmr* gene, whose product was envisaged to be a repressor that becomes active upon binding glutamine (8); and (iii) the enzyme glutamine synthetase, postulated to also act as a repressor which prevents *nit-2* expression (9). The recent molecular cloning of the *nit-2* gene has allowed us to examine directly this nitrogen regulatory gene (12, 31). The *nit-2* gene is transcribed to give a single 4.3-kilobase (kb) poly(A)$^+$ RNA whose content was shown to increase three- to fourfold during nitrogen limitation, suggesting that expression of the *nit-2* control gene is itself subject to regulation (12). However, the results obtained did not support the possibility that *nit-2* was controlled either by *nmr* or by glutamine synthetase. It appears obvious that an understanding of the operation of the nitrogen control circuit will be facilitated by a complete characterization of each of the regulatory genes. Here we report the complete nucleotide sequence of the *nit-2* gene and show that it contains two small introns near the 5′ end of the gene. The *nit-2* gene appears to encode a protein composed of 1,036 amino acids with a molecular weight of approximately 110,000. The translated *nit-2* protein appears to possess a single zinc finger domain that is essential for function and that probably plays a direct role in DNA binding. The single zinc finger of the NIT2 protein is remarkably similar to the two zinc finger domains found in a mammalian *trans*-acting factor, GF1.

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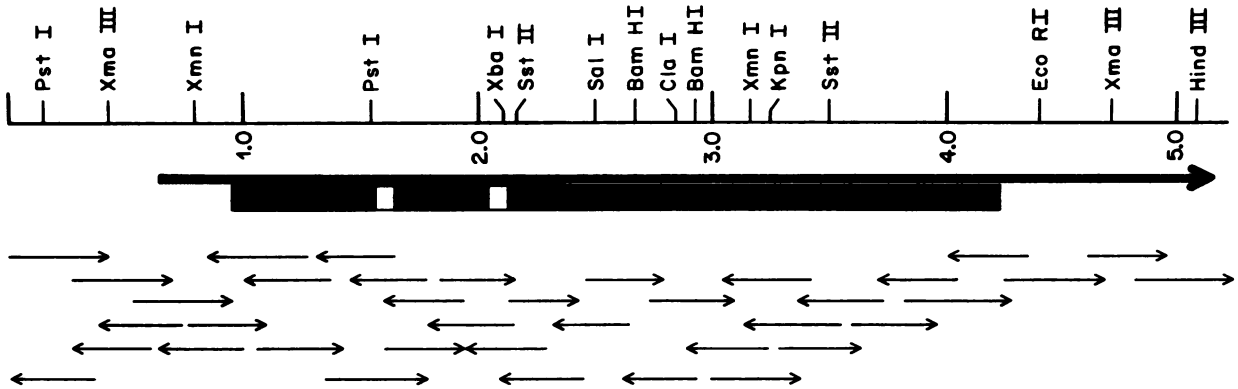

FIG. 1. *nit-2* gene structure and sequencing strategy. The direction of transcription is indicated with the bold horizontal arrow which represents the 4.5-kb transcript. The shaded box shows the predicted *nit-2* protein composed of 1,036 amino acid residues. The horizontal arrows show the sequencing strategy that was achieved with deletion clones and with oligonucleotide primers. Restriction sites and length of the genomic segment sequenced (in kilobases) are shown.

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 previously (12, 14).

DNA sequencing and S1 nuclease mapping. DNA sequencing was accomplished by the dideoxy-chain termination method (30) with [α-^{32}P]dATP and a modified T7 bacteriophage DNA polymerase, Sequenase (United States Biochemical Corp., Cleveland, Ohio). dITP was successfully used in place of dGTP to sequence through compression regions; some regions were sequenced at 70°C with the heat-resistant DNA polymerase of *Thermus aquaticus* (Promega Biotec, Madison, Wis.). Plasmid DNA templates were prepared as minipreparations (3). Oligonucleotide primers for DNA sequencing and site-directed mutagenesis were synthesized on an Applied Biosystems model 380B DNA synthesizer by the Ohio State University Biochemical Instrument Center. S1 mapping experiments were conducted by the method of Berk and Sharp (2). Primer extension experiments were accomplished by mixing a 5′-end-labeled 17-mer oligonucleotide primer that hybridizes with poly(A)$^+$ RNA as a template for Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.).

Site-directed mutagenesis. The cloned *nit-2* gene and the flanking region of 4.7 kb proved to be too large for efficient mutagenesis. Accordingly, a restriction fragment from the *nit-2* gene of approximately 250 base pairs (bp) that included a potential zinc finger domain was subcloned into the Bluescript vector, and after mutagenesis and confirmation by sequencing, it was moved back into an otherwise wild-type *nit-2* gene. Site-directed mutagenesis was performed as described by Kunkel (19).

Isolation of RNA and cDNA clones. *N. crassa* total RNA was prepared by the method of Reinert et al. (28). The poly(A)$^+$ RNA fraction was isolated by oligo(dT)-cellulose chromatography (1) for use in S1 mapping and primer extension studies and for cDNA synthesis. A cDNA library was constructed in λgt10 with *Eco*RI adaptors to eliminate the need for methylation of the double-stranded cDNA and *Eco*RI digestion before ligation into the vector arms, followed by packaging with the use of Packagene (Promega). Four rounds of plaque hybridization with pNit2 as a probe were required to isolate several *nit-2* cDNA clones. Lambda DNA was purified and digested with *Eco*RI, and the insert cDNA was subcloned into the Bluescript plasmid vector and sequenced as described above. In some cases, sequencing was accomplished directly with the recombinant lambda bacteriophage DNA. Plasmid DNAs for use as probes were labeled with [^{32}P]dCTP by nick translation (29).

***nit-2* deletions and transformation assays.** Deletion clones of pNit2 were constructed by the use of DNA polymerase I with the Cyclone system (International Biotechnologies, Inc., New Haven, Conn.) as described previously (13). Mutated or partially deleted *nit-2* genes were assayed for function by transformation into *nit-2* mutant protoplasts, with appropriate positive and negative controls (12, 13). Functional *nit-2* genes gave a high transformation rate, similar to that obtained with the wild-type *nit-2* gene, whereas nonfunctional genes did not transform at all.

Computer methods. The handling of sequences, their analysis and translation, and hydropathy and codon bias analyses were accomplished with Pustell software (International Biotechnologies). Protein homology searches were conducted with GenBank, the Protein Identification Resource (National Biomedical Research Foundation), which contains 3,800 different protein sequences.

RESULTS

***nit-2* nucleotide sequence.** A restriction map of the *nit-2* gene and the strategy used to sequence it are shown in Fig. 1. The dideoxy sequencing method was used to sequence the entire 5.2-kb region which encompasses the *nit-2* gene and flanking DNA. Both DNA strands were sequenced except for part of the 3′ noncoding region of the gene; overlapping clones and synthetic primers were employed to confirm the entire sequence. The nucleotide sequence of the *nit-2* gene and its flanking regions is presented in Fig. 2.

Intervening sequences in the *nit-2* gene. We determined the nucleotide sequence of two overlapping *nit-2* cDNA clones, which together compose a full-length cDNA copy of the *nit-2* transcript. The cDNA sequence agreed completely with the genomic sequence, except for the presence of introns, whose locations were determined by comparing the cDNA sequence with the genomic sequence (Fig. 2). The *nit-2* gene is interrupted by two introns of 98 and 78 bp which occur in the protein-coding region near the 5′ end of the gene. Both

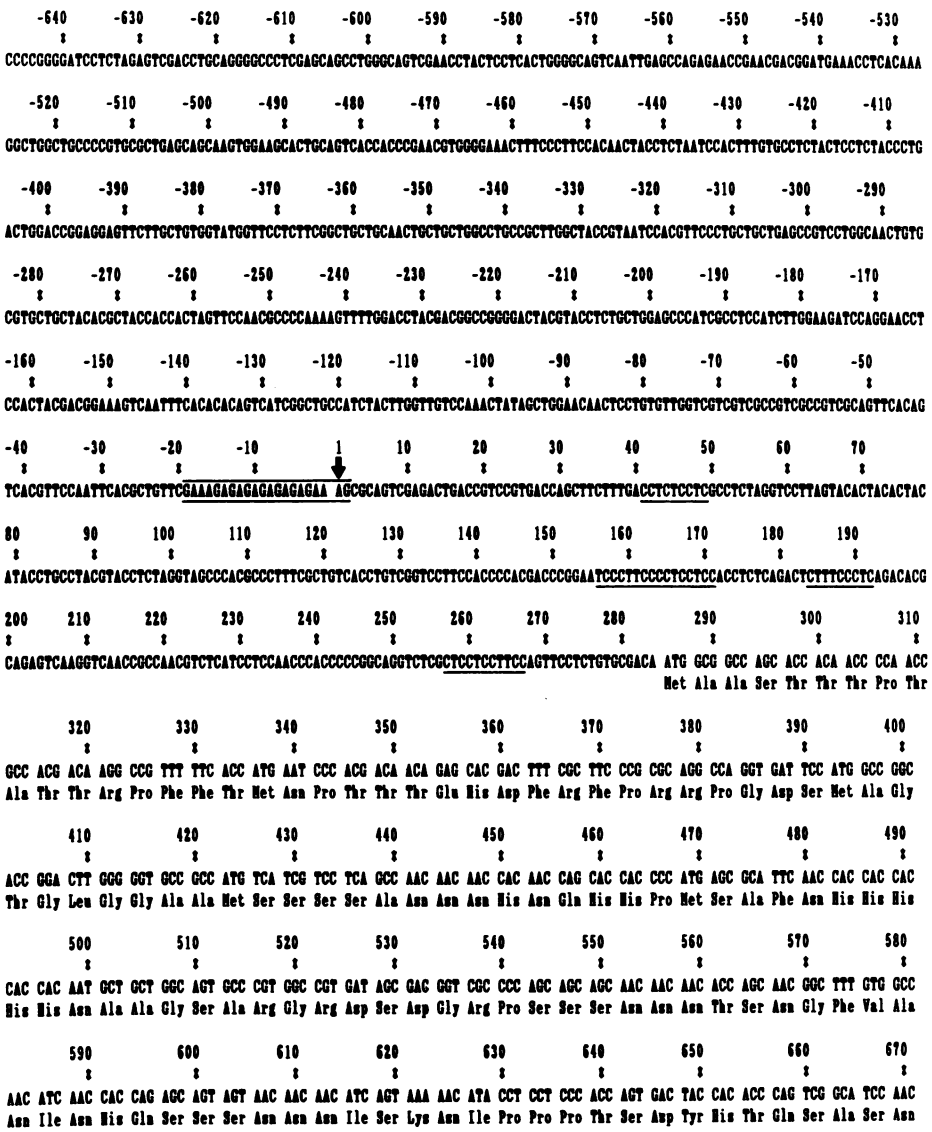

FIG. 2. Nucleotide sequence of *nit-2* and its flanking regions. The adenine base which composes the single transcription start site is numbered +1. Vertical arrows indicate 5′ and 3′ termini of the *nit-2* transcript. The 5′ terminus was determined by primer extension analysis, and the 3′ end was identified as being adjacent to the poly(A) tract of a cDNA clone. Transcription initiation begins at the 3′ end of a 21-bp repeated sequence composed entirely of purine bases in the coding strand (underlined and overlined). Pyrimidine-rich and thymidine-rich tracts within the 5′ and 3′ noncoding regions of the *nit-2* mRNA are underlined. The translated amino acid sequence which encodes a protein of 1,036 amino acids is shown beneath the DNA sequence. The putative Cys_2/Cys_2 zinc finger domain is underlined. Intron 1 (98 bp) and intron 2 (78 bp) both have the consensus 5′ GT and 3′ AG splice site sequence.

introns have good consensus 5′ and 3′ splice site sequences. Removal of the two introns from the primary transcript yields an mRNA of 4.3 kb which contains a protein-coding region of 3.1 kb and long 5′ and 3′ nontranslated segments.

Organization of *nit-2* gene. The initiation site for transcription of the *nit-2* gene was determined by primer extension analysis. The results indicated that transcription initiation occurs at a single site located 284 bp upstream of the first AUG codon which begins a long open reading frame (Fig. 2). No consensus TATA box sequence is found within 150 bp upstream of the initiation site. Initiation occurs with an adenine residue located at the 3′ end of an unusual, highly symmetrical 21-bp sequence which consists entirely of purine bases on the coding strand (Fig. 2). The 5′ nontranslated region of the transcript is very rich in pyrimidine bases and contains four stretches (of 9, 11, 11, and 16 bp) composed entirely of C and T residues (Fig. 2). The first AUG codon is closely followed by four additional in-frame AUG codons. It is unknown which of these five in-frame AUG codons actually represents the initiation codon; however, the sequence surrounding the second AUG codon has the best match to the *N. crassa* consensus translational start sequence. Translating from the initial AUG codon to the UAA stop codon yields a protein of 1,036 amino acids, with a molecular weight of approximately 110,000. Comparison of the sequences of genomic and cDNA clones revealed that polyadenylation of the *nit-2* transcript occurs 933 bp downstream of the UAA stop codon. Therefore, the *nit-2* transcript has long nontranslated regions at both its 5′ and 3′ ends. The very long 3′ nontranslated region of the *nit-2*

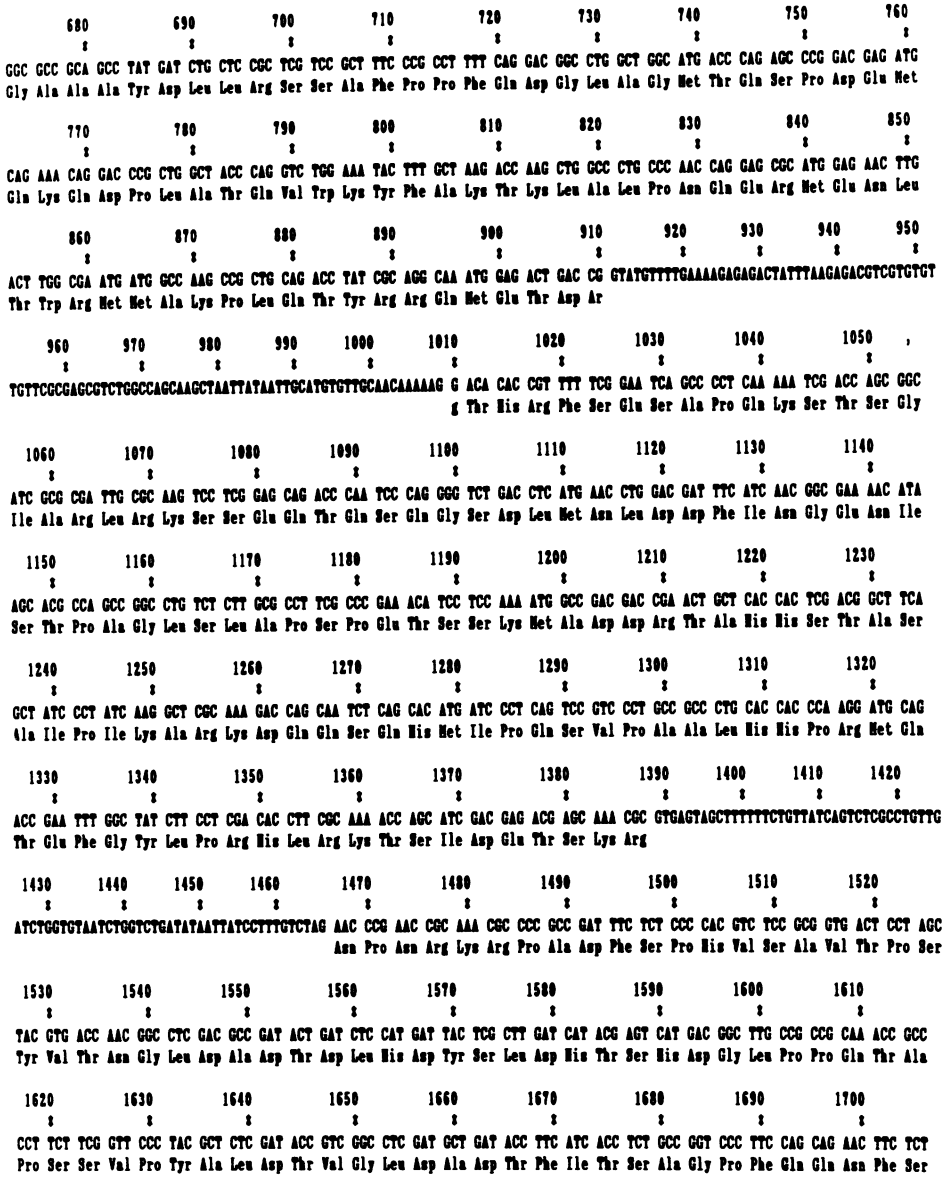

FIG. 2—*Continued.*

mRNA also contains multiple pyrimidine-rich, especially thymidine-rich, stretches (Fig. 2).

Analysis of 3′ deletion clones of *nit-2*. Deletion clones which truncated the 3′ end of the *nit-2* gene were constructed as described in Materials and Methods (Fig. 3). Each of these deletion clones was transformed into the *nit-2* mutant strain to test its functional activity. The subclone which contains the entire 4.3-kb *Eco*RI fragment and the deletion clones 41 and 22 all complemented the *nit-2* mutant, indicating that each was expressed to give a functional *nit-2* gene product. However, deletion clones 76 and 19 failed to transform the *nit-2* mutant. These results suggest that amino acid residues in the region between the sites defined by deletions 22 and 76 are important for function of the *nit-2* protein. However, the manner in which the *nit-2* deletions were made resulted in fusion proteins in which each truncated *nit-2* protein was fused to amino acids specified by plasmid sequences (deletions 19, 22, 41, and 76 have, respectively, 51, 11, 12, and 50 extra amino acids fused to the truncated *nit-2* protein). It is not clear whether these additional amino acids at the carboxy terminus affected the activity of the various proteins. However, it should be emphasized that the truncated *nit-2* protein encoded by deletion 22, which lacks the carboxy-terminal 214 amino acids, was functional, thus demonstrating that these amino acids residues are dispensable for activity.

The *nit-2* protein deduced from the nucleotide sequence is composed of 1,036 amino acids and contains only four cysteine residues, all of which are located in the region near the endpoint of deletion 76. The cysteine residues form a consensus zinc finger motif CYS-Xaa$_2$-CYS-Xaa$_{17}$-Cys-Xaa$_2$-Cys, in which the cysteine residues are postulated to serve as zinc-binding ligands (Fig. 4). Deletion 76 occurs in the 17-amino-acid loop of the potential zinc finger domain; thus, the lack of function of the deletion 76 protein is consistent with the suggestion that the *nit-2* protein is a DNA-binding protein with an essential zinc finger domain.

Mutagenesis of potential *nit-2* zinc finger motif. To investi-

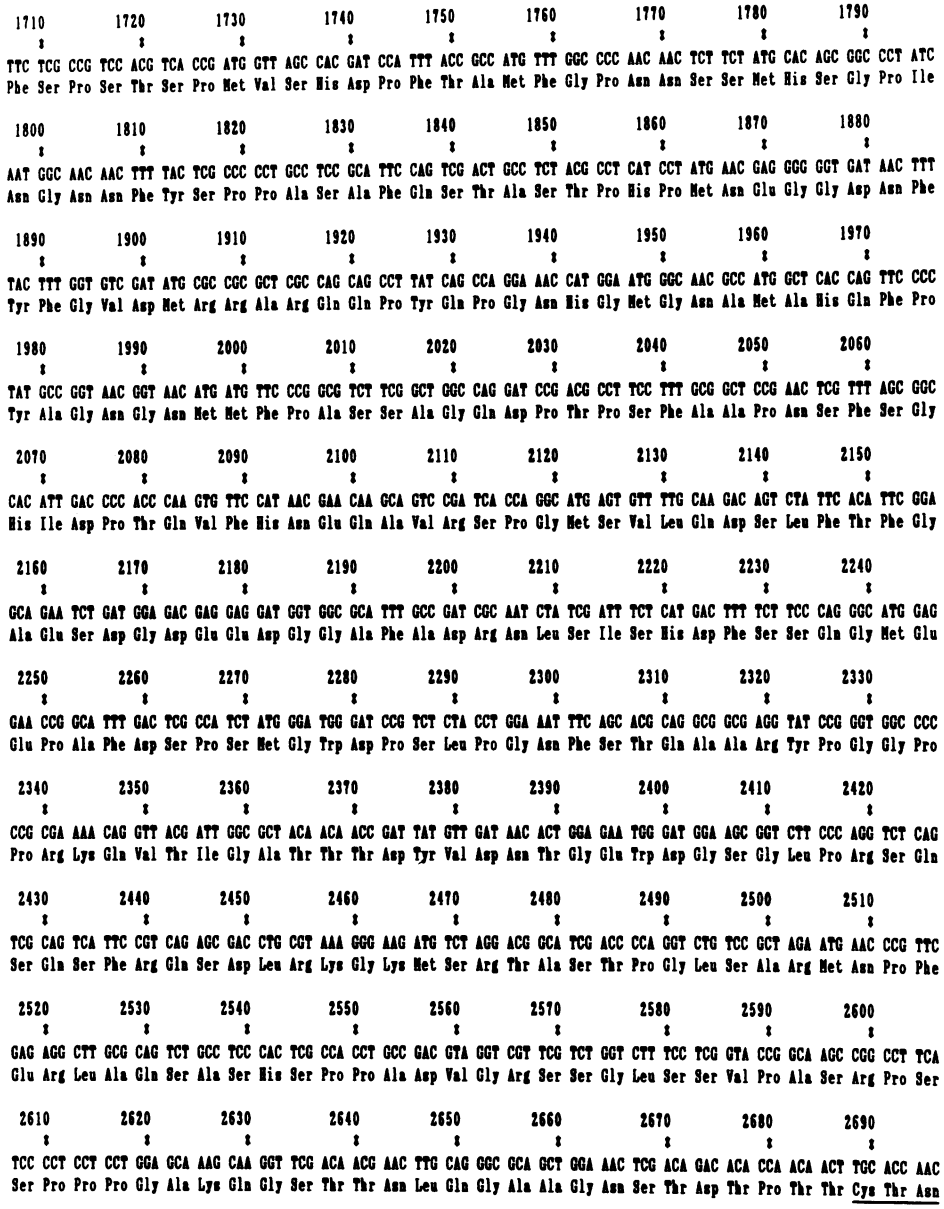

FIG. 2—*Continued.*

gate whether integrity of the potential zinc finger domain was necessary for function of the *nit-2* protein, we changed two of the four cysteine residues simultaneously by site-directed mutagenesis; cysteine residues 743 and 746 were changed to serine and glycine, respectively. The mutated gene segment was sequenced to confirm that the desired changes had been obtained, and then a restriction fragment containing the altered sequence was inserted into the corresponding position of an otherwise wild-type *nit-2* gene. The mutant gene thus constructed was found to be nonfunctional, i.e., incapable of transforming the *nit-2* mutant strain, demonstrating that the cysteine residues are essential, consistent with their potential role in formation of a zinc finger DNA-binding motif.

DISCUSSION

The *nit-2* gene is the major positive-acting nitrogen control gene of *N. crassa*. During conditions of nitrogen limitation, the *nit-2* gene product is required to turn on the expression of an entire series of unlinked structural genes which specify nitrogen catabolic enzymes. Here we report the complete nucleotide sequence of the *nit-2* gene and its flanking regions. The 4.3-kb *nit-2* mRNA has a long nontranslated 5′ leader (284 bp) and an unusually long 3′ nontranslated sequence (933 bp), both of which contain stretches that are pyrimidine rich. Although the significance of these extensive nontranslated regions of the *nit-2* mRNA is unknown, they represent potential areas for control of mRNA translation or stability. The presence of the five closely spaced in-frame AUG codons at the beginning of the protein-coding region also suggests a potential for translational control of *nit-2*, perhaps similar to that suggested for the *N. crassa cpc-1* and the yeast *GCN-4* proteins (25, 32). No other potential start sites or open reading frames occur in the 284-bp 5′ leader sequence which precedes the first of the five closely spaced AUG codons.

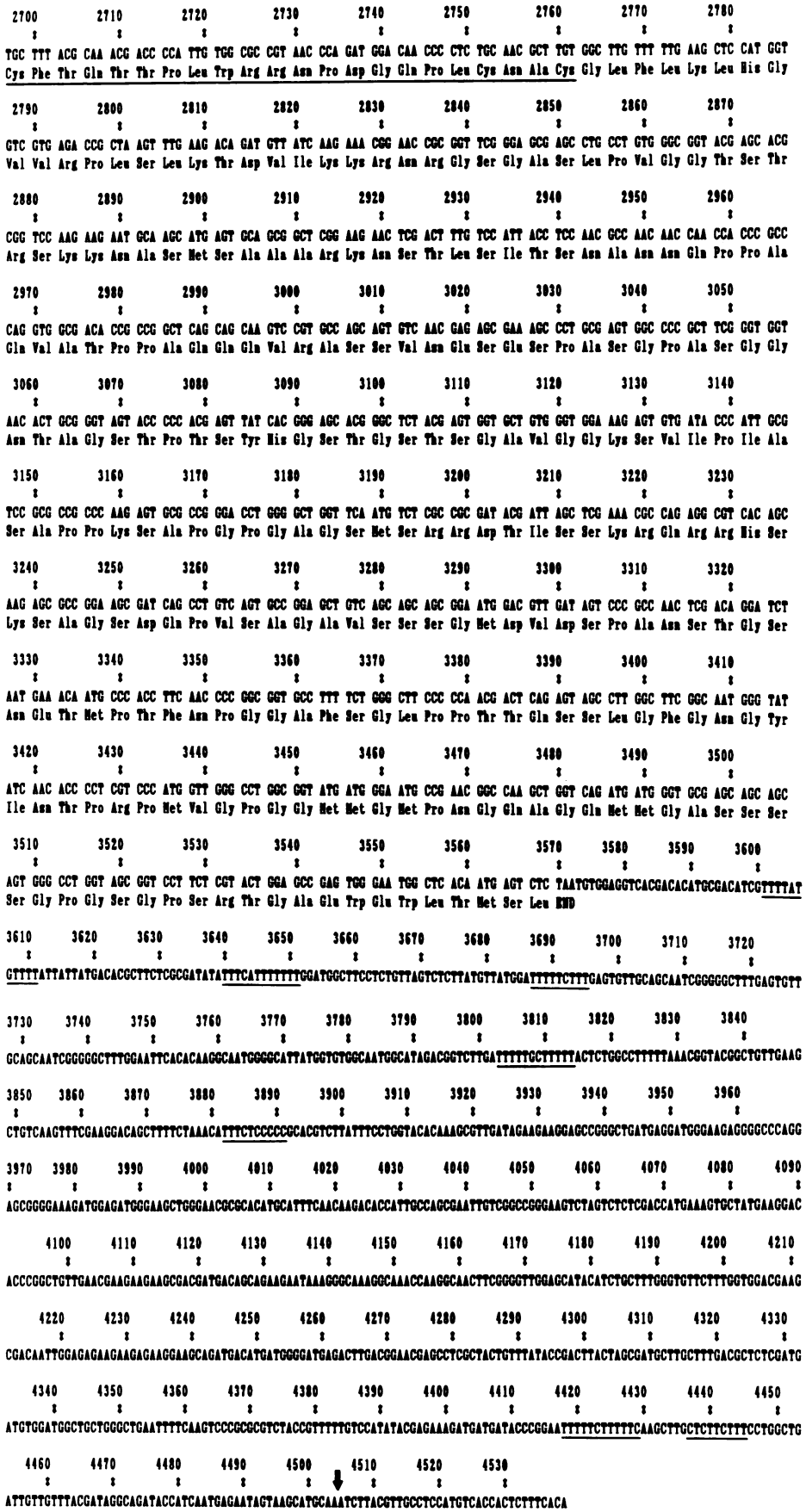

FIG. 2—*Continued.*

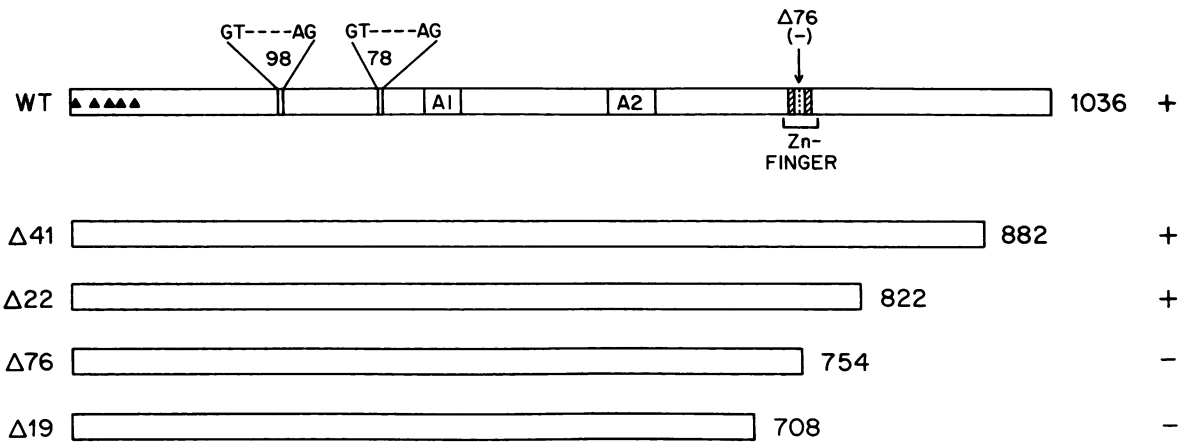

FIG. 3. *nit-2* gene structure. The *nit-2* gene has five in-frame potential AUG initiation codons which occur in the 5′ end of the mRNA at positions corresponding to amino acid residues 1, 8, 12, 51, 62, and 68 (displayed as solid triangles). The *nit-2* sequence can be translated to yield a protein of 1,036 amino acids, ending with a UAA termination codon. Two short introns of 98 and 78 bp occur near the 5′ end of the gene within the protein-coding region. A single putative zinc finger element is composed of four cysteine residues at positions 743, 746, 764, and 767. Two acidic regions of the translated *nit-2* protein begin at residue 362 (A1, net charge, −6) and at residue 559 (A2, net charge, −11). Basic regions begin at residues 330 (B1) and 912 (B2) and immediately downstream of the putative zinc finger element (not shown). Four different 3′ deletions are shown. Deletions 22 and 41 result in a functional *nit-2* protein as assayed by transformation, whereas deletions 19 and 76 are inactive. WT, Wild type.

The translated *nit-2* protein contains 11 hydroxyl amino acids within the first 23 amino-terminal residues, which could possibly be phosphorylation sites for posttranslational control of *nit-2* protein activity. An amino acid sequence which begins at residue 48, S-S-S-A-N-N-N, is repeated two more times starting at residue 87 and again at residue 105 (except that the later two repeats lack the central alanine), suggesting that duplication events have helped shape the *nit-2* gene and its protein product. The hydroxyl amino acids compose 22% of the residues of the *nit-2* protein, with serine being the most abundant individual amino acid (14%). Proline represents nearly 10% (99 residues) of the protein, whereas all the remaining nonpolar amino acids compose only 27% of the total residues; in contrast, 63% of the *nit-2* protein is composed of polar or charged amino acids. Surprisingly, a computer homology search revealed that the *nit-2* protein more closely resembles collagen than any other protein in the data base; collagen is known to be deficient in alpha-helical or beta-sheet structures. A hydropathy plot for the deduced *nit-2* protein showed that it is an extremely hydrophilic protein with only a few hydrophobic regions (data not shown). Moreover, the predicted secondary structure of the protein suggests only a small amount of alpha-helical and beta-sheet structures. The *nit-2* protein contains 40 more basic than acidic amino acids and has a predicted pI of 9.0. The translated *nit-2* protein has two acidic regions and three basic regions whose significance, if any, is unknown. Acidic regions have been shown to be responsible for transcriptional activation by the yeast GAL4 and GCN4 proteins (18, 21). A highly basic region, with a net charge of +12, occurs in the 50 amino acids immediately downstream of the putative zinc finger (see below).

The *nit-2* protein contains a single putative Cys_2/Cys_2-type zinc finger domain which has a loop composed of 17 amino acid residues. Preliminary results indicate that a domain of the *nit-2* protein containing this potential zinc finger functions in DNA binding (Y.-H. Fu and G. A. Marzluf, unpublished data). Almost no homology is apparent when the zinc finger domain of *nit-2* is compared with the single Cys_2/Cys_2-type zinc finger structures of five yeast regulatory proteins, GAL4, PPR1, ARGRII, and LEU3 of *Saccharomyces cerevisiae* (20, 23, 35) and LAC9 of *Kluyveromyces lactis* (34), each of which has 13 amino acids in the internal loop, except for LEU3, which has 16 residues (Fig. 4). A region of the NIT2 protein on the carboxy side of the zinc finger motif is highly basic (net charge, +12); this basic region may represent part of a DNA-binding domain (Fig. 4), perhaps acting as a specificity region, as has been found in the yeast LAC9, GAL4, and PPR1 proteins (6, 34). Each of the single zinc finger proteins has a basic region immediately downstream of the zinc finger structure. The *qa-1F* gene of *N. crassa* encodes an activator protein for quinic acid catabolic genes and appears to contain a single zinc finger that shows significant homology to the zinc finger region of GAL4 and other yeast regulatory proteins.

GF1, a *trans*-acting DNA-binding protein which appears to activate gene expression specifically in mammalian cells of the erythroid lineage, possesses two zinc finger domains, each with a central loop composed of 17 amino acids (33). The two zinc finger structures of GF1 and their immediate downstream basic regions are remarkably similar to the single zinc finger and basic region of NIT2 (Fig. 4). This segment of the NIT2 protein has 32 of 50 amino acids identical to those in the second GF1 zinc finger region, i.e., 64% homology in this region, and it also displays strong homology to the first GF1 zinc finger. In fact, the zinc finger of NIT2 is more homologous to each of the GF1 fingers than they are to each other. The GF1 protein is composed of 413 amino acid residues and is particularly rich in serine (12%), threonine (8%), and proline (9.4%), very similar to the NIT2 protein, which is also rich in serine (14%), threonine (8%), and proline (9.9%). However, despite this similarity in amino acid composition, only the zinc finger regions of GF1 and NIT2 display any homology in amino acid sequence. The GF1 protein recognizes a core consensus DNA sequence, TATCT, present in promoter and enhancer elements of alpha, beta, and gamma globin and related genes of chickens, mice, and humans (33). This sequence is also present at least once in the 5′ promoter region of structural genes controlled by the *nit-2* gene and thus may represent all or

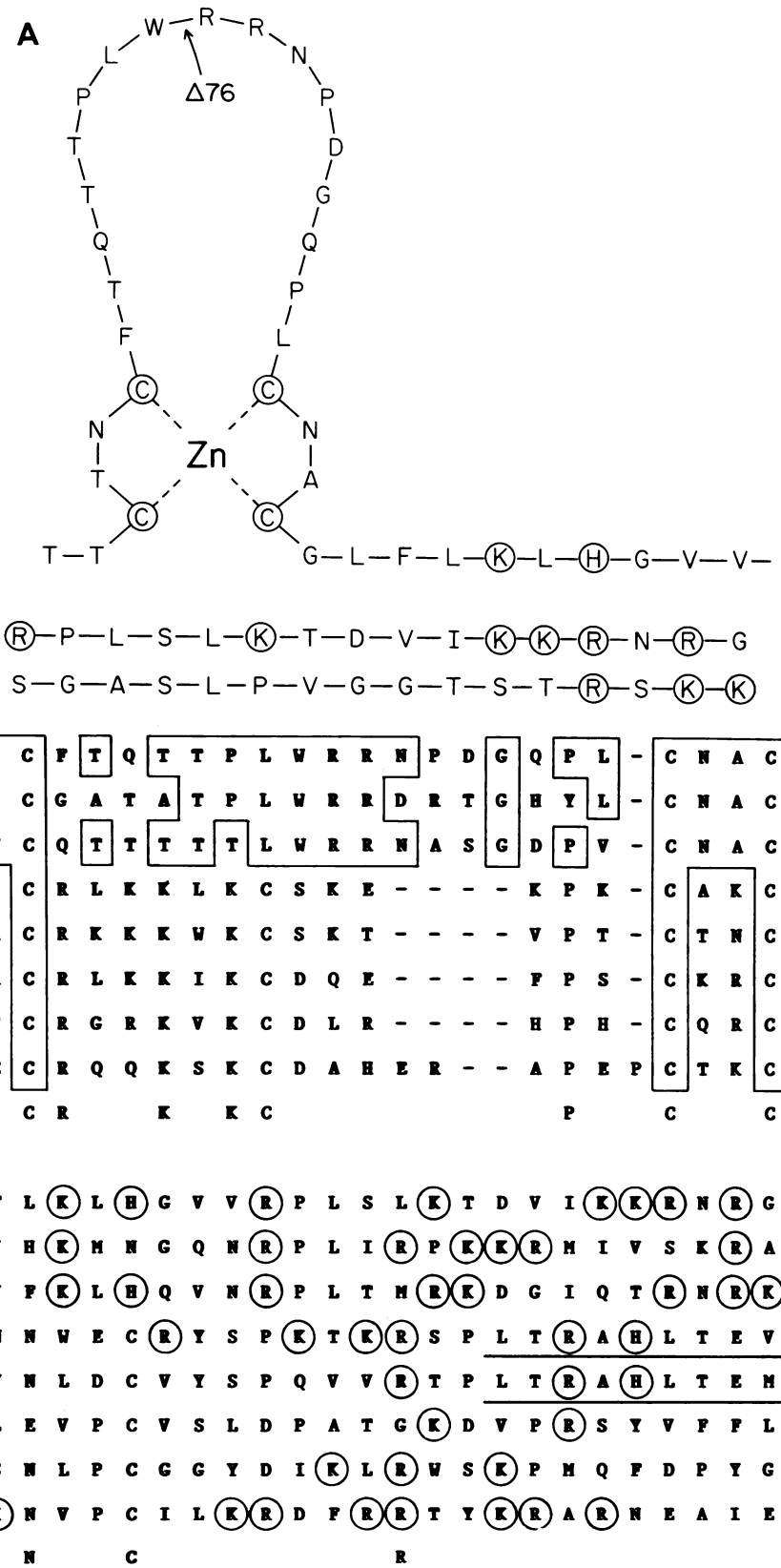

FIG. 4. (A) Putative single zinc finger element of the *nit-2* protein. The four cysteine residues are shown coordinating a zinc atom with a loop of 17 amino acid residues. The negatively charged region immediately downstream of the zinc finger is shown (basic amino acids are circled). (B) Comparison of *nit-2* zinc finger and downstream basic region with yeast single zinc finger proteins GAL4, LAC9, PPR1, ARGRII, and LEU3 and with the mammalian factor GF1, which has two zinc fingers (GF1a and GF1b). YCS, Yeast consensus residues. The cysteine residues which participate in zinc binding are boxed, as are identical amino acids of NIT2 and the two GF1 fingers; basic amino acids in the downstream 30 residues are circled. The region of high homology between GAL4 and the closely related LAC9 protein (12 amino acids) is underlined. In this region, replacement of amino acids in the LAC9 protein resulted in a deficiency in DNA binding (33).

part of the recognition site for the NIT2 protein. It is intriguing that a zinc finger DNA-binding motif of a protein responsible for regulation of genes of the nitrogen control circuit of the lower eucaryote *N. crassa* plays a major role in specific gene expression in the differentiated erythroid cell lineage of mammals.

The deletion analysis revealed that a *nit-2* protein lacking the carboxy-terminal 214 amino acids, approximately 21% of the protein, was still functional in activating gene expression. This seems to be a common feature of regulatory proteins, namely, that relatively large segments of control proteins such as the yeast GAL4 and GCN4 proteins can be deleted without noticeably altering their function (18, 21).

In the related filamentous fungus *Aspergillus nidulans*, a regulatory gene designated *areA* appears to be homologous to the *N. crassa nit-2* gene (22). In fact, the *nit-2* gene can complement an *areA* mutant of *A. nidulans* and turn on the expression of nitrate reductase, acetamidase, and related catabolic enzymes (7). This result implies that the activation function and DNA sequence specificity of the *nit-2* and *areA* proteins must be quite similar; moreover, the upstream recognition elements that serve the nitrogen structural genes in *A nidulans* and *N. crassa* also must be remarkably alike. The *areA* gene encodes a protein which also possesses a single zinc finger domain with a central loop of 17 amino acids (4). On the other hand, *A. nidulans* lacks any counterpart of the *N. crassa nmr* gene. Thus, the nitrogen repression mechanism for these two fungi may be different, although it could also occur in a similar manner, perhaps with the *areA* protein playing the regulatory role carried out by a multimeric protein composed of *nit-2* and *nmr* subunits in *N. crassa*. A detailed comparison of the structure and function of these regulatory proteins should be informative and provide new insight concerning nitrogen control in lower eucaryotes.

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