ASD4, a New GATA Factor of *Neurospora crassa*, Displays Sequence-Specific DNA Binding and Functions in Ascus and Ascospore Development†

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ABSTRACT: A new gene encoding a novel GATA factor, ASD4, of *Neurospora crassa* was isolated and demonstrated to possess one intron and to specify an open reading frame encoding a protein with 427 amino acid residues. The ASD4 protein contains a single GATA-type zinc finger and a putative coiled-coil domain. Unlike related proteins, DAL80 in yeast and NREB in *Penicillium*, ASD4 does not appear to be involved in regulation of nitrogen metabolism. An Asd-4 null mutant obtained by the rip procedure did not show any effect upon nitrogen control, but instead resulted in severe defects in ascus and ascospore genesis. The Asd-4 rip mutant is dominant to Asd-4+. A cross of the Asd-4 mutant with wild-type resulted in fruiting bodies that appeared to be normal macroscopically but which were complete devoid of asci and ascospores. Introduction of the Asd-4+ gene into the Asd-4 rip mutant corrected the defect in ascus and ascospore development in crosses with wild-type. Mobility shift assays demonstrated that ASD4 acts as a sequence-specific DNA binding protein and recognizes DNA fragments that contain GATA core elements. Gel filtration and cross-linking experiments revealed that the ASD4 protein exists as a tetramer in solution. These results suggest that the ASD4 protein functions positively as a transcriptional regulator of sexual development in *Neurospora*.

GATA factors, regulatory DNA binding proteins which possess one or two Cys2/Cys2-type zinc fingers, are widely distributed in eukaryotic organisms from yeast and worms to plants and mammals. The functions performed by GATA factors are very diverse, ranging from controlling transcription of a variety of downstream genes involved in metabolic circuits (*1*) and cellular activities, to that of cell differentiation and development (*2*, *3*).

Mammals, such as humans and mice, possess at least six different GATA factors that show tissue- and cell-specific patterns of expression and which control different types of differentiation and development. GATA-1, the best-studied member of this family, controls the erythropoetic cell lineage specification (*4*). Other members, such as GATA-2 and GATA-3, act in early development of the central nervous system (*6*), while members GATA-4/5/6 are implicated in the gastrulation step of early embryonic development (*6*) and cardiac-specific gene expression (*7*, *8*). Two tandem Cys2/Cys2-type zinc fingers are present in all the GATA factors discovered in higher organisms. The C-terminal finger is required for specific DNA binding, while the N-terminal finger is not essential for DNA binding but required for interacting with cofactors such FOG-1 (*9*, *10*) and FOG-2 (*11*). In addition, GATA factors have been implicated as agents in chromatin remodeling which accompanies gene activation (*12*). Although most GATA factors act in a positive fashion to turn on gene expression, some of them, e.g., the yeast DAL80 protein (*13*) and *Penicillium* NREB, act in a negative way to regulate downstream genes (*14*).

In lower eukaryotes such as yeast and filamentous fungi, most GATA factors found thus far are involved in the control of transcription of metabolic genes. A total of five GATA factors have been identified in yeast. GLN3 (*15*) and NIL1/GAT1 (*16*, *17*) are positive regulators involved in nitrogen metabolic control, while DAL80 (*13*) and NIL2 (*18*) regulate nitrogen metabolism in a negative fashion. Ash1, a far relative of the GATA factor family, is a daughter cell specific protein required for the inhibition of daughter cell mating-type switch in yeast (*19*).

NIT2, the first GATA factor identified in *Neurospora crassa*, is a global regulator of nitrogen metabolism and is required for the expression of genes which encode catabolic enzymes for different pathways of nitrogen source utilization (*1*). Two additional GATA factors, WC1 (*20*) and WC2 (*21*), were subsequently identified and shown to control blue light responses and are involved in light-induced resetting of the circadian rhythm. WC2 also serves as an essential component of the clock machinery (*22*). GATA factors present within the same cells would display overlapping DNA binding specificities and might be expected to interfere with each other's ability to control a specific set of downstream genes. It is thus interesting to consider whether lower eukaryotes with relatively simple cell differentiation, such as the filamentous fungus *Neurospora crassa*, could also possess multiple GATA factors. A PCR approach, using degenerate primers that correspond to the zinc finger DNA binding

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domain of GATA proteins, was employed to determine if any additional GATA factors could be detected. This approach allowed the identification of two new GATA factors from *Neurospora crassa* which possess the characteristic Cys2/Cys2-type zinc finger motif. One of the newly identified genes, sre, encodes a *Neurospora* GATA factor with two zinc fingers and acts negatively to prevent the expression of siderophore biosynthetic genes during conditions of iron sufficiency (*23*, *24*).

Neurospora crassa has two life cycles, a vegetative cycle and a sexual cycle. Complex morphological changes occur during the sexual cycle that result in developmental of specialized female structures, the protoperithecia (*25*−*27*). Fertilization occurs when conidia or hyphae of the opposite mating-type are encountered. After fusion of the gamete cells but before nuclear fusion, they undergo about 10 rounds of cell division inside the fruiting body. Upon nuclear fusion, the diploid cell immediately undergoes the two meiotic divisions and one mitotic division to produce a total of eight haploid cells. Meanwhile, cell specialization occurs, resulting in the formation of a tube-like structure called an ascus, within which haploid cells mature to form eight ascospores (*26*, *27*). Although the morphological features of the sexual cycle have been extensively described, the molecular details governing the developmental process are largely unknown. Here, we report the identification and characterization of a fifth *Neurospora* GATA factor, ASD-4, and describe results which indicate that ASD4 serves as a major transcription regulator in specifying the lineage of asci and ascospores during *Neurospora* sexual development.

MATERIALS AND METHODS

Strains and Transformation. E. coli XL-1 blue was used for most cloning purposes. BL21 lys(−) was used for expressing GST fusion and his6-tagged proteins. 2×YT (yeast extract−tryptone medium) was used for growing *E. coli*. The *Neurospora crassa* wild-type strains 740R23 A and a were used for genetic crossing and other procedures. *Neurospora* his-3(−) strain Y234M723 was used as host for targeted transformation of the pDE (*28*) based constructs. *Neurospora* was grown on Vogel's minimal medium (*14*) plus proper supplements. The protoplast protocol was used for *Neurospora* transformation (*29*, *30*). For nitrate reductase assay, conidia of wild-type and Asd-4Rip were grown overnight in Vogel's-N plus 20 mM glutamine at 30 °C. The mycelia were harvested and split into four equal parts and inoculated into four flasks containing different nitrogen sources, namely, 20 mM glutamine, 20 mM $NaNO_3$, 20 mM glutamine + 20 mM $NaNO_3$, or 4 mM hypoxanthine. Cell-free extracts were prepared using a Mini-bead beater. Nitrate reductase assay was done as described (*31*).

DNA and RNA Procedures. General techniques for DNA manipulation, such as restriction digestions, subcloning, Southern blot analysis, and DNA sequencing, were according to (*32*). The degenerate primers used for PCR-based homologous cloning of Asd-4 were 5′-TGYCAPAAYTGYGGNAC-3′ and 5′-TTCTTPATNACPTCNGTYTT-3′, where Y = T, C; P = A, G; N = A, T, C, G. The RACE protocol (*33*) was used for PCR. The PCR products were subcloned into pGEN-T vector (Promega) and sequenced with the chain-termination method. Screening of *Neurospora crassa* genomic and cDNA libraries was done as described (*34*). Both strands of the 5.6 kb *Eco*RI DNA fragment containing the Asd-4 gene were sequenced using a ^{33}P-labeled dideoxide nucleotide sequencing kit (Amersham). The sequencing data were analyzed with Wisconsin package (GCG). Isolation of *Neurospora* genomic DNA was done by the LETS buffer method described (*35*).

To isolate *Neurospora* total RNA, an overnight culture in Vogel's-N plus 20 mM glutamine was harvested by filtration and split into three flasks containing Vogel's-N plus 20 mM glutamine, 20 mM $NaNO_3$, or 4 mM hypoxanthine, respectively. Growth was continued at 30 °C for 2.5 h. RNA preparation and subsequent Northern analysis were done as described in (*34*).

Mapping of the Asd-4 Gene. The RFLP method was used to map the Asd-4 gene. The result using *Kpn*I digestion revealed a RFLP pattern of "$O_1M_2M_3M_4M_5M_6M_7M_8O_9M_{10}$-$O_{11}O_{12}O_{13}O_{14}O_{15}O_{16}O_{17}M_{18}O_{19}O_{20}$".

Genetic Crosses. The 1× synthetic crossing medium containing 0.5% sucrose and 2% agar noble was used for all crosses (*25*). The female parental strain was applied onto the medium and grown in the dark at 25 °C for 5 days. The male parental strain was then applied as a conidia suspension. Fruiting body formation was followed by periodical checking. Fruiting bodies were collected from day 2 to day 20 after the cross, and fixed either in 5% paraformalin at RT or as described (*26*). Ascus and ascospore formation were examined under light microscopy by squeezing out the content of the microscopically dissected fruiting body. Pictures were taken using a microscope-attatched camera.

Rip Protocol. A 5.6 kb *Eco*RI fragment containing the Asd-4 gene was subcloned into the pDE vector by replacing the *Eco*RI fragment. The resulting pDE-ngf5.6 was used to transform *Neurospora* his3(−) strain Y234M723 to obtain a strain with two copies of the Asd-4 gene. The successful targeting of the Asd-4 gene to the his-3 locus was confirmed by a Southern blot. The strain bearing two copies of the Asd-4 gene was crossed with wild-type strain to create Asd-4 Rip mutants. Twenty days after the cross, ascospores were collected and germinated. Forty progeny strains were screened by Southern blot for the Asd-4 Rip mutant using the 1.2 kb *Pst*I fragment from the Asd-4 gene. A 2.0 kb nit-3 DNA fragment was also used for Southern blot as a control probe.

Mobility Shift Assays. GST-ASD(1−102) was expressed in BL21 and purified using glutathione−agarose resin (Sigma) as described (*36*). The double-stranded DNA probes for mobility shift assays were annealed from synthetic oligos. The sequences are given in the Figure 1 legend. The probes were end-labeled with ^{32}P and incubated with GST-ASD-(1−102) in binding buffer [20 mM Hepes, pH 7.9, 50 mM KCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 20 μM $ZnCl_2$, 1 μg/25 μL poly(dI-dC)] at 25 °C for 20 min. The samples were resolved on a 5% polyacrylamide gel in 0.25× TBE (Tris−boric acid−EDTA). The gel was then dried and autoradiographed (*16*).

Chemical Cross-Linking Assay. ASD4(103−319)-his6-tagged protein was expressed in BL21 lys(−) strain and purified using NTA−agarose resin according to the manufacturer's instructions (Invitrogen). Imidazole (100 mM) was used to elute the protein. The reaction for the cross-linking assay contained 1−2 μg of protein and 0.05% freshly diluted glutaraldehyde in either PBS buffer (50 mM phosphate, pH

7.0, 150 mM KCl) or imidazole buffer (60 mM imidazole, pH 8.0, 150 mM KCl). The reaction was allowed to proceed at RT for 10 min, and was terminated by adding an equal volume of SDS-gel loading buffer. The samples were resolved on SDS−PAGE and stained with Coomassie blue R-250 (*37*).

RESULTS

Isolation of Asd-4 Which Encodes a Novel GATA Factor. GATA-type zinc finger proteins perform important regulatory functions in many different organisms. To identify possible new GATA factors in *Neurospora crassa*, a PCR-based approach was employed using degenerate primers corresponding to the conserved regions of GATA-type zinc fingers. A novel sequence that appeared to encode a GATA-type zinc finger was identified by sequencing the PCR products. This sequence was used to probe a *Neurospora* cosmid library, which yielded a genomic DNA clone of the corresponding gene, which subsequently was named Asd-4 (Ascus Development-4).

ASD4 Contains a Cys2/Cys2-Type Zinc Finger and a Coiled-Coil Domain. Sequencing the Asd-4 genomic DNA revealed an open reading frame encoding 424 amino acid residues (Figure 1 A). A 71 nucleotide intron was identified between the 78th and 79th codon, and its presence was confirmed by comparing the genomic and cDNA sequences. The amino acid sequence of the ASD4 protein revealed interesting features. It contains a single GATA-type zinc finger domain located near its N-terminus which is homologous to that found in other members of the GATA protein family (Figure 1B), with the greatest homology to *Penicillium* NREB (*22*), *S. cerevisiae* DAL80 (*13*), and *Neurospora* NIT2 (*38*) and SRE (*23*) zinc fingers. A putative coiled-coil domain was identified in the middle of the protein, and it shows significant homology to the coiled-coil domain of NREB and DAL80 and also of motor proteins such as myosin (*39*), paramyosin (*40*), and kinesin (*41*). The C-terminal region of ASD4, which consists of 120 residues, is rich in acidic residues and bears a negative charge of −24. In addition, the amino acid sequence 'PAPAPE' is repeated 4 times within the C-terminal region. Eight 5′-GATA-3′ core DNA elements were identified in the upstream promoter region of the Asd-4 gene.

Northern Blot Analysis. The first 238 residues of ASD4 exhibits extensive homology to yeast DAL80 and *Penicillium* NREB, both of which appear to be negative-acting nitrogen regulatory proteins. The expression of both DAL80 and NREB is controlled by nitrogen repression (*13, 14*). This prompted us to examine the effects of nitrogen repression/derepression on the expression of Asd-4. As revealed by a Northern blot, two Asd-4 messenger RNAs are detected, the larger, slower migrating mRNA being predominant (Figure 2). The level of Asd-4 mRNA was largely unaffected by nitrogen sources which represent nitrogen repression (glutamine) and derepression (nitrate or hypoxanthine) conditions (Figure 2). The expression level of Asd-4 was similar in both wild-type and nit-2 null mutant strains, indicating that the major positive nitrogen regulator NIT2 does not play a role in Asd-4 expression. These results demonstrate that, unlike nreb and dal80, the expression of the Asd-4 gene is not subject to nitrogen control and suggested that Asd-4 might not serve a nitrogen regulatory function.

Disruption of Asd-4 Does Not Affect Nitrogen Regulation but Prevents Ascus Development. To establish the functional role, if any, played by the ASD4 protein, the Rip mutagenic protocol (*42*) was used to disrupt the Asd-4 gene. The cloned Asd-4 gene was transformed into wild-type *Neurospora*, yielding a transformant with two copies of this gene, which was then crossed with wild-type. Progeny from this cross bearing the disrupted Asd-4 gene were identified by Southern blot analysis, with the nit-3 gene employed as an internal control (Figure 2A). The Asd-4 gene in one of the progeny was so severely damaged by the Rip process that it could not be detected on the Southern blot. This strain with the ripped Asd-4 gene was used for further analysis. It is noteworthy that the Asd-4 rip mutants were rare, and, in fact, only 1 confirmed rip mutant was found of 40 progeny examined. Furthermore, only about half of the ascospores from the original cross to obtain rip mutants were viable.

To examine the possible role of Asd-4 in nitrogen control, the expression of the nitrate reductase gene, a highly regulated gene in the nitrogen regulatory circuit, was examined in the Asd-4 Rip mutant strain under nitrogen repression, repression/induction, derepression/noninduction, and depression/induction conditions. The levels of expression and regulation of nitrate reductase in response to these different conditions were completely normal, as compared with wild-type (data not shown). The growth rate of the Asd-4 Rip strain on a variety of nitrogen sources was also normal. These results strongly suggest that, unlike its homologues in yeast and *Penicillium*, Asd-4 is not involved in nitrogen regulation but instead functions in another aspect of cell regulation.

Repeat sequence-induced mutation, or rip, occurs in the premeiotic stage. The unusual low frequency of Asd-4 Rip mutants and the very poor viability of ascospores from the cross suggested the possibility that Asd-4 may play a role in controlling sexual development of *Neurospora*, a process for which molecular details are poorly understood. *Neurospora* exhibits two mating types, namely, A and a, either of which can serve as male or female parental strains during a cross. To examine whether the loss of the ASD4 protein results in any defects in sexual cycle development, the Asd-4 Rip strain was crossed with a standard wild-type strain. The development of fruiting bodies, asci and ascospores, was examined at different time points by microscopy (see Materials and Methods). When the Asd-4 strain served as either the female or the male parent, fruiting body formation appeared normal, as judged by comparing their number, color, and size with fruiting bodies resulting from crosses between two wild-type strains. However, ascus development was severely blocked by Asd-4 gene disruption. Asci with mature ascospores can be observed within 7 days after a cross in which both parents are wild-type (Figure 3B, left panel). In contrast, for crosses between wild-type strain and the Asd-4 Rip mutant, no asci or ascospores were observed inside the fruiting bodies even 20 days after the cross (Figure 3B, right panel). Only cellular masses with multiple polygonal cells were seen inside the fruiting bodies under light microscopy. These results demonstrate that Asd-4 plays an important role in the development of asci and ascospores in *Neurospora*. It is important to note that when crossed with wild-type, the strain with the disrupted Asd-4 gene exhibited a dominant detrimental effect on ascus development, which

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-264                                                                CTGCAGCGCCCCAAACCTGGAAGCGGC

-237 CCCGTCAAAAAATTTCCTTATCATTTCCGTGCTTCGTTCTTATCGACATCCCTCCACGTTCTATCTCATTCACCAGCTT

-158 CCACTCCATTCCCGCATTTATTTATCAAGGTTGGCTTATCGCCAGCATCTAATCGGCCTTCTATCTCTCCCTCTCGATA

 -79 CCATTGAGGTTCCCATCTCTTATCCGACAGCGCACCTGCACCACCACCACCACCACCACCACCGCCAACAACAGCAAAC

   1 ATG GCG ACA ATG GCT AAC CAT GAT CGC GAA ACC ACA CAG CCC ACG TGC CAG AAC TGC GCG
   1  M   A   T   M   A   N   H   D   R   E   T   T   Q   P   T   C   Q   N   C   A
  61 ACC TCG ACA ACG CCG CTA TGG CGC CGC GAC GAG ATG GGC CAG GTC CTC TGC AAT GCC TGT
  21  T   S   T   T   P   L   W   R   R   D   E   M   G   Q   V   L   C   N   A   C
 121 GGT CTC TTC CTC AAG CTG CAT GGC CGT CCA CGC CCA ATC TCC CTA AAG ACC GAT GTC ATC
  41  G   L   F   L   K   L   H   G   R   P   R   P   I   S   L   K   T   D   V   I
 181 AAG AGT CGC AAC CGC GTC AAG ACC ATG CGC CCT GAC TTG GCC AAG CAG AAA AAGgcaagccg
  61  K   S   R   N   R   V   K   T   M   R   P   D   L   A   K   Q   K   K
 243 ttcatatccgtccgacctcggcaagcttcagtcagtaaactaactgtgtgtgcccagcaacag CAG CAA CAA CAA
                                                                     Q   Q   Q   Q
 319 CAG CAG CAG CAG CAG AAC CTA GCT GCC ACC GCC GAC ATG AAC GGA GGA GTA GGC ATG ATG
  84  Q   Q   Q   Q   Q   N   L   A   A   T   A   D   M   N   G   G   V   G   M   M
 379 GAT CCC AAC AAC CCT GCC GCT GCT GCC CGA AGA GCA TCC CAG AAG TCC ATC AAT GGC CAC
 104  D   P   N   N   P   A   A   A   A   R   R   A   S   Q   K   S   I   N   G   H
 439 CCC GTC GAC GAT AAC TCG CCC GTT TCG CGG ACC GGC ACC CCC AAC GTA TAC AAT CCG CAC
 124  P   V   D   D   N   S   P   V   S   R   T   G   T   P   N   V   Y   N   P   H
 499 ATT CCC ATT GAT CAC AGC CTA GAG TAC CAA TTT CAA GCC CAA CAG ATT CCC GGA TTT GGC
 144  I   P   I   D   H   S   L   E   Y   Q   F   Q   A   Q   Q   I   P   G   F   G
 559 GTC CCG ACT GCC TCT CCC GGC CGA GCC CCA TCG CCC ATG AAT GGC GAG CAC ATG CCA CAA
 164  V   P   T   A   S   P   G   R   A   P   S   P   M   N   G   E   H   M   P   Q
 619 ACC CAC GAG CAG CTC CTC GCT GCC AAT GCC AGC CTG AAG ACC CGA GTC AGC GAG TTG GAG
 184  T   H   E   Q   L   L   A   A   N   A   S   L   K   T   R   V   S   E   L   E
 679 GTC ATC CAA GAG CTC TAC CGC GGT CGC CTT CAC CAA CTC GAG ACG GAA GAG AAC ATT CGA
 204  V   I   Q   E   L   Y   R   G   R   L   H   Q   L   E   T   E   E   N   I   R
 739 CAA GCT TCA GAG CCC GGC AAG CTT GAA GCG CAG CTT CGC GCC CAG ATC GAT GCC ATG GGC
 224  L   Q   A   S   E   P   G   K   L   E   A   Q   R   A   Q   I   D   A   M   G
 799 GAA GCT CAT CAG CAG CTG CAA AAG GAA CTG GAG GAA AGC CAC AGG AGG GAG AAT ATG CTC
 244  E   A   H   Q   Q   L   Q   K   E   L   E   E   S   H   R   R   E   N   M   L
 859 AAG AGA CGC CTT GAT GAG CTC GAG GTT GAG CTC AAG GAC GTC AAG GAT GCT CTC GAA TCT
 264  K   R   R   L   D   E   L   E   V   E   L   K   D   V   K   D   A   L   E   S
 919 CAG GAT AAT GGA CGC CAC AAG AAG ATC CGC TTG GAC GAA AAC GTC AAG ACC GAG CCC TAT
 284  Q   D   N   G   R   H   K   K   I   R   L   D   E   N   V   K   T   E   P   Y
 979 GCA GAA GTT GTT GAG CCA CAG CAG CCG GAA CAG CAG CAG CCA GCA CCT GCA GAG CAG CCA
 304  A   E   V   V   E   P   Q   Q   P   E   Q   Q   Q   P   A   P   A   E   Q   P
1039 ATT CCC ACC CCT ATG GCC ATC GAC GAA GCC ACC CCT GCC CCT GCG CCA GCA CCC GAG GCA
 324  I   P   T   P   M   A   I   D   E   A   T   P   A   P   A   P   A   P   E   A
1099 GCA CCC GAG CAG GCT CCG GCA CCC GCG CCC GAA CCC GTC CAA GAA CAA GCC CAA GAG CCA
 344  A   P   E   Q   A   P   A   P   A   P   E   P   V   Q   E   Q   A   Q   E   P
1159 GAG CCT GCG CCC GTT AGC GAG CCC ACT GAG GCT TCA GCC CCG GCC CCG GCC CCG GAA GCC
 364  E   P   A   P   V   S   E   P   T   E   A   S   A   P   A   P   A   P   E   A
1219 GAT TCT GTT GTT CCC GAG CCG ACC CCC GCC GCG CCA GAG TCT GCT CCC ACC GAA GAG CCT
 384  D   S   V   V   P   E   P   T   P   A   A   P   E   S   A   P   T   E   E   P
1279 GCC GCA CCG GAG ACT GAG GCC TCT GAG CCT CCC ACC ACC GCC CCC GTG GAA GAA GCT CCC
 404  A   A   P   E   T   E   A   S   E   P   P   T   T   A   P   V   E   E   A   P
1339 AAG GCG GAG TCA TAG TGTCCCTGCCGCCAGTGCTTTGCTTCTTTTTCTGGTGGTCCAATGGATACTACTGCCTA
 424  K   A   E   S   *
1412 AGAGTCGTCTGCAACATACTTGGCTTTGGATGGATGGATGGATGGATGGACGGATGGCTGGATGGCTGGATGGAAACTG

1491 TCGCGTTAATGATTTTTATACTATCACCTGAGTCTTTGGCGTCGTCGACAATGACTGGATGGAACTATCAAGAGTTGAT
1570 GTGGTCAGCTAAAAAGAGGAATCGGGATGGTCATGCTCGGACAAAGATGGCTTGGTTGGGATTGCCTTGATTGAATGTT
1649 GATTACTCCAATGATCACCACTTGGTTTTGGCTGGTTAGTCCAATTCGGACGGGGTTATGGAGCTTCCGATCCCATTTT

1728 TCTTTTTTTCCCAATAATAAATGGGTTTACGGAAAATTCCCTTAATTTTAACATTTCCCCTTCAATTCCAAGTTTCCCC

1807 TTAAAACCCCCCTTCCTGGTCCCTTTCCGGTGGGAGGAAAAAGGTTCCCTTAACGGAATTTGAACATAAATGGTCCTTG

1886 GCGTGGGTTTAACCGGGGGTGGGCCTCCCAAAGGGGGTCGGAACAAAAAAAAAAAAATTTTTCCCC
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was observed when the Asd-4 Rip strain served either as the female or as the male parent.

To further test and confirm that the severe defect in ascus development observed during the cross of the rip mutant with wild-type is due to the disruption of the Asd-4 gene, we transformed the cloned Asd-4+ gene into the Asd-4 Rip strain to determine whether it corrects the defect. The vector alone, which confers benomyl resistance, was used as a negative control. Transformants of the Asd-4 rip mutant which received the Asd-4+ gene regained the ability to produce asci and ascospores in crosses with wild-type, although the number of the inviable, nonpigmented ascospores was somewhat higher (~30%) compared with a wild-type × wild-type cross (less than 5% inviable spores). Transformation of the Asd-4 rip mutant with the cosmid vector alone did not restore ascus development in crosses

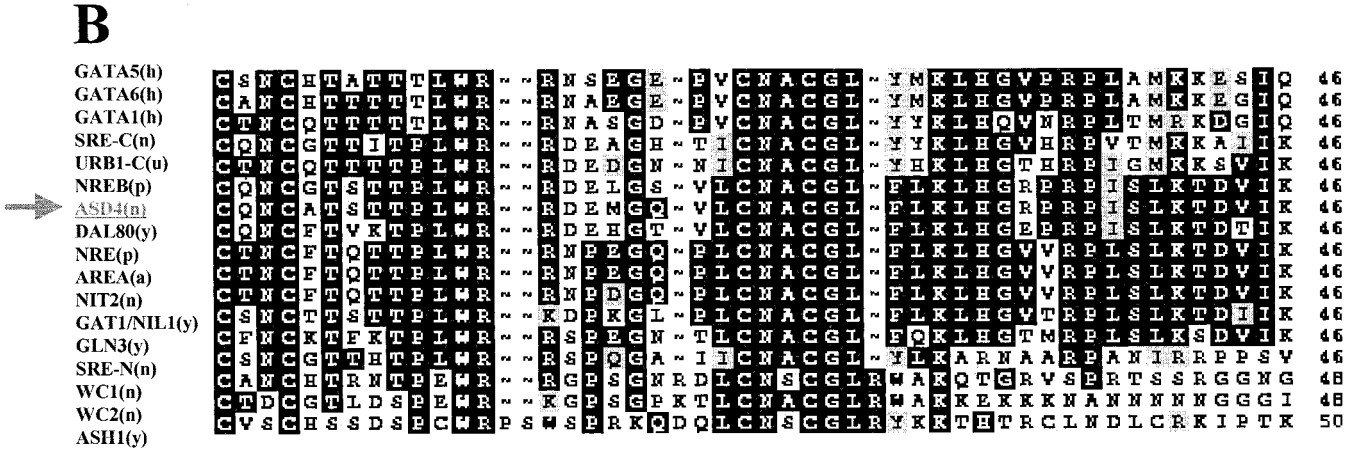

FIGURE 1: (A) Nucleotide and amino acid sequence of Asd-4. The zinc finger region is in larger font and underlined. The putative coiled-coil region is also underlined. 'GATA' elements upstream of the Asd-4 gene are double-underlined. Arrows indicate amino acid repeat sequences in the C-terminal region. The dashed lines indicate triplet and quadruplet repeats 5′ and 3′ of the Asd-4 gene, respectively. Putative transcription termination signals are boxed. (B) Sequence alignment of ASD4 zinc finger with other GATA-type zinc fingers. The alignment was done by Prettybox(GCG). n, *Neurospora*; h, human; y, yeast; a, *Aspergillus*; p, *Penicillium*; u, *Ustilago*.

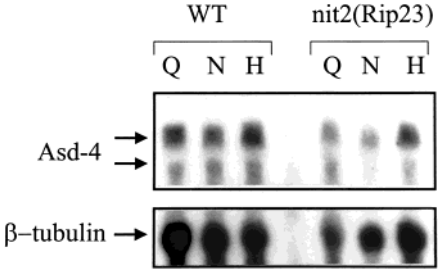

FIGURE 2: Northern blot analysis of Asd-4. The growth conditions used are indicated as Q (20 mM glutamine); N (20 mM sodium nitrate); or H (4 mM hypoxanthine). The 3.0 kb and 1.7 kb Asd-4 transcripts as well as the 1.5 kb tubulin mRNA are identified by arrows. Both the 1.2 kb *Pst*I and the 5.6 kb *Eco*RI fragment containing the Asd-4 gene were used as probes for Northern blots and gave identical results. WT, wild-type strain; nit2(Rip23), nit-2 Rip mutant strain. The relative level of Asd-4 mRNA was determined by Lab Works Analysis version 3.0.02.00. After being normalized by the level of β-tubulin mRNA, ASD-4 mRNA levels for lanes Q, N, and H of the wild-type were 1, 1.08, and 1.68, respectively; and for lanes Q, N, and H of the nit2 Rip mutant were 1.75, 0.93, and 1.30, respectively.

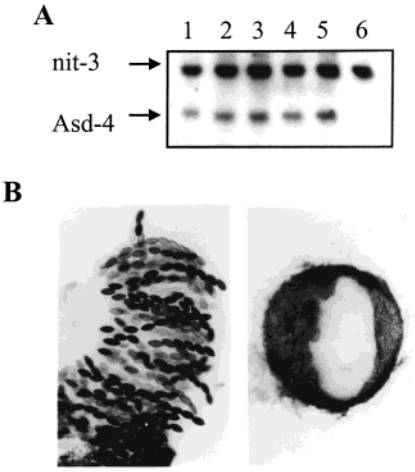

FIGURE 3: (A) Southern analysis of Asd-4 Rip mutant. Genomic DNA from the Rip progeny was restricted with *Bam*HI. The Southern blots were probed with ^{32}P-labeled DNA from both the nit-3 and Asd-4 genes (identified by arrows). Lanes 1−6 represent part of the Southern results. Lane 1, wild-type strain; lanes 2−6, progeny from Rip cross. (B) Effect on Asd-4 disruption on ascus development. Ascus development was examined by microscopy following each cross. Representative results are shown. Left panel, a dissected rosette structure from a day 7 WT × WT cross; right panel, an empty fruiting body from a day 20 Asd-4Rip × WT cross. No asci or ascospores were observed in the latter.

with wild-type. These results strongly suggest that disruption of the Asd-4 gene was responsible for the severe developmental defects in ascus and ascospore development.

Asd-4 may represent a key regulatory protein that is involved in the control of ascus development. It is possible that Asd-4 corresponds to one of several previously identified genetic loci involved in sexual development of *Neurospora*, identified by a mutation that resulted in failure of the sexual cycle. To determine whether Asd-4 represents an entirely newly identified gene or one recognized previously, we mapped the chromosomal location of Asd-4 using the RFLP method (*43*). Digestion with *Kpn*I of genomic DNA of the two parental strains used for RFLP with *Kpn*I revealed distinctive restriction patterns when probed with Asd-4. The Asd-4 gene was then mapped via RFLP to a novel location on linkage group 4 near met-5 and nit-3, a region that does not include previously identified ascus developmental mutants.

ASD4 Specifically Recognizes the 'GATA' Core DNA Element. ASD4 contains a Cys2/Cys2-type zinc finger near the N-terminus. This type of zinc finger is believed to recognize 'GATA' core DNA sequences in their target promoters. To determine whether ASD4 possesses sequence-specific DNA binding activity, a truncated ASD4 protein consisting of residues 1−102 which contains the zinc finger motif was overexpressed in *E. coli* as a GST fusion protein and purified. No downstream genes controlled by ASD4 have yet been identified. However, the upstream region of the Asd-4 gene itself contains eight GATA sequences. A 34-mer double-stranded DNA probe containing the forth and the fifth 'GATA' elements from the 5′ promoter region of Asd-4 was tested for ASD4 binding in a mobility shift assay. The GST-ASD4(1−102) fusion protein containing the zinc finger region demonstrated strong binding to this DNA fragment that contains two GATA elements (Figure 4A). An otherwise identical DNA fragment, except that the two GATA sequences were replaced by GAAA, was used as a

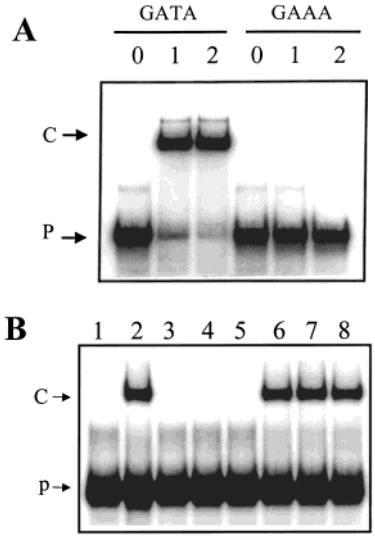

FIGURE 4: Mobility shift assay: (A) Recognition of 'GATA' sequence by ASD4. Two DNA probes containing 'GATA' or 'GAAA' were made from single-stranded oligos. GATA: '5′-GCATTTATT**TATC**AAGGTTGGCT**TATC**GCCAGCA'; GAAA: '5′-GCATTTATT**TTTC**AAGGTTGGCT**TTTC**GCCAGCA'. For GST-ASD4 protein added: lanes 0, no protein added; lanes 1, 120 ng; lanes 2, 360 ng. C, DNA−protein complex; P, free DNA probe. (B) Competition of ASD4 DNA binding with GATA or GAAA double-stranded oligos. Ten nanograms of a fragment containing GATA elements annealed from complementary strands (5′-AAATCGGATAAGATCGTGTCTCGCAGTTTTGATCTGATAAAAAG-3′) was used as the DNA probe. Lane 1, no protein added; lanes 2−8 each contained 40 ng of GST-ASD4; for lanes 3, 4, and 5, the unlabeled GATA fragment from (A) was added at an amount 30, 60, and 120 times that of the labeled probe, respectively; in lanes 6, 7, and 8, the unlabeled GAAA fragment from (A) was added at 30, 60, and 120 times that of the labeled probe, respectively. As measured by PhosphoImager scanning, the amount of the shifted probe in lanes 2, 6, 7, and 8 was 22.5%, 20.6%, 20.9%, and 19.4%, respectively.

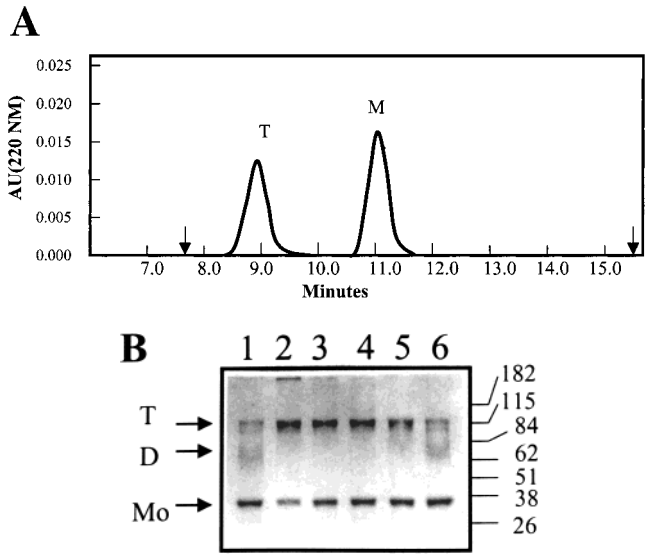

FIGURE 5: Oligomer formation by ASD4 protein. (A) Gel filtration. Two micrograms of purified ASD4 (amino acids 103−319)-his6 was loaded onto a HPLC gel filtration column (Water 200 SW, 8.0 × 300 mm, phosphate saline buffer, pH 7.0, 0.8 mL/min). Two elution peaks were detected. $V_T = 8.947$; $V_M = 11.008$; $V_{void} = 7.9$, $V_{total} = 15.2$. The BioRad HPLC molecular mass marker set was used as control. Their masses and corresponding elution times are 150 kDa, 8.78; 44 kDa, 10.77; 17 kDa, 12.33; 1.35 kDa, 15.24, respectively. The apparent molecular mass for ASD4 monomer (M) is 34−36 kDa, and for the oligomeric species that elutes as a tetramer (T) 129−135 kDa. The ratio is approximately 3.75. (B) Chemical cross-linking assays. Approximately 2 μg of ASD4-his6-tagged protein was cross-linked in 20 μL of imidazole buffer (60 nM imidazole, pH 8.0, 150 mM KCl) with 0.05% glutaraldehyde for 10 min. Lanes 1−5: 0, 500, 70, 35, and 17.5 μM $CaCl_2$ were added, respectively; lane 6 contained 1 mM $MgCl_2$. Samples were heated in 1× SDS−PAGE loading buffer and resolved on 10% SDS−PAGE. The positions of molecular mass markers are shown in kilodaltons. ASD4 monomer (Mo), dimer (D), and tetramer (T) positions are shown by arrows.

negative control. ASD4 completely failed to bind to this DNA probe. The ability of ASD4 to bind to an entirely different DNA fragment which contains GATA sequences in the presence of potential competitors was examined in order to confirm its specificity. A DNA fragment containing GATA sequences strongly competed with ASD4 binding to the probe, whereas a DNA fragment with an identical sequence except for a single base change from GATA to GAAA showed no competition (Figure 4B). These results clearly demonstrate that ASD4 specifically recognizes DNA fragments that contain the 'GATA' core sequence. The fact that the Asd-4 promoter itself contains multiple 'GATA' sites which are recognized by the ASD4 protein suggests the possibility that the Asd-4 gene may be subject to autogenous regulation, or that Asd-4 might be controlled by still another GATA factor. These results, combined with the fact that the GATA proteins in diverse eukaryotes serve many different control functions, suggest that ASD4 is a major transcriptional regulator controlling ascus development in *Neurospora*.

ASD4 Exists as a Homotetramer in Solution. A central region of the ASD4 protein, consisting of residues 180−300, was predicted to form a coiled-coil structure (*44*), which suggests that ASD4 may exist as an oligomeric species essential for its regulatory function. Residues 103−319 of ASD4 were expressed in *E. coli* as a his-tagged protein and purified. Gel filtration of ASD4 revealed that a significant amount of this protein existed as a tetramer, with the remainder occurring as a monomer. A chemical cross-linking assay was used as described under Materials and Methods to further test the possibility that ASD4 assumes an oligomeric form in solution. ASD4(103−319) occurs in solution as a tetrameric species, which was readily detected after treatment with the cross-linking reagent for 10 min (Figure 5B). The predicted coiled-coil region exhibited homology to a similar motif in various motor proteins such as myosin, and kinesin with calcium binding ability. Addition of calcium ion (from 17.5 μM to 1 mM) stimulated tetramer formation (Figure 5B, lanes 2−5). It is noteworthy that a calcium concentration of 17.5 μM is well within the physiological range. In contrast, addition of 1 mM magnesium ion did not enhance ASD4 oligmer formation. Under certain conditions, cross-linking experiments revealed that ASD4 could also form dimers (not shown).

DISCUSSION

GATA factors, a large family of proteins that possess one or two highly conserved Cys2/Cys2 zinc fingers, are widely distributed in eukaryotic organisms. In mammals and other higher organisms, GATA factors are responsible for differentiation and function of a variety of specific tissues and cell types. In *Neurospora*, a model lower eukaryote, the four previously identified GATA factors all act in response to

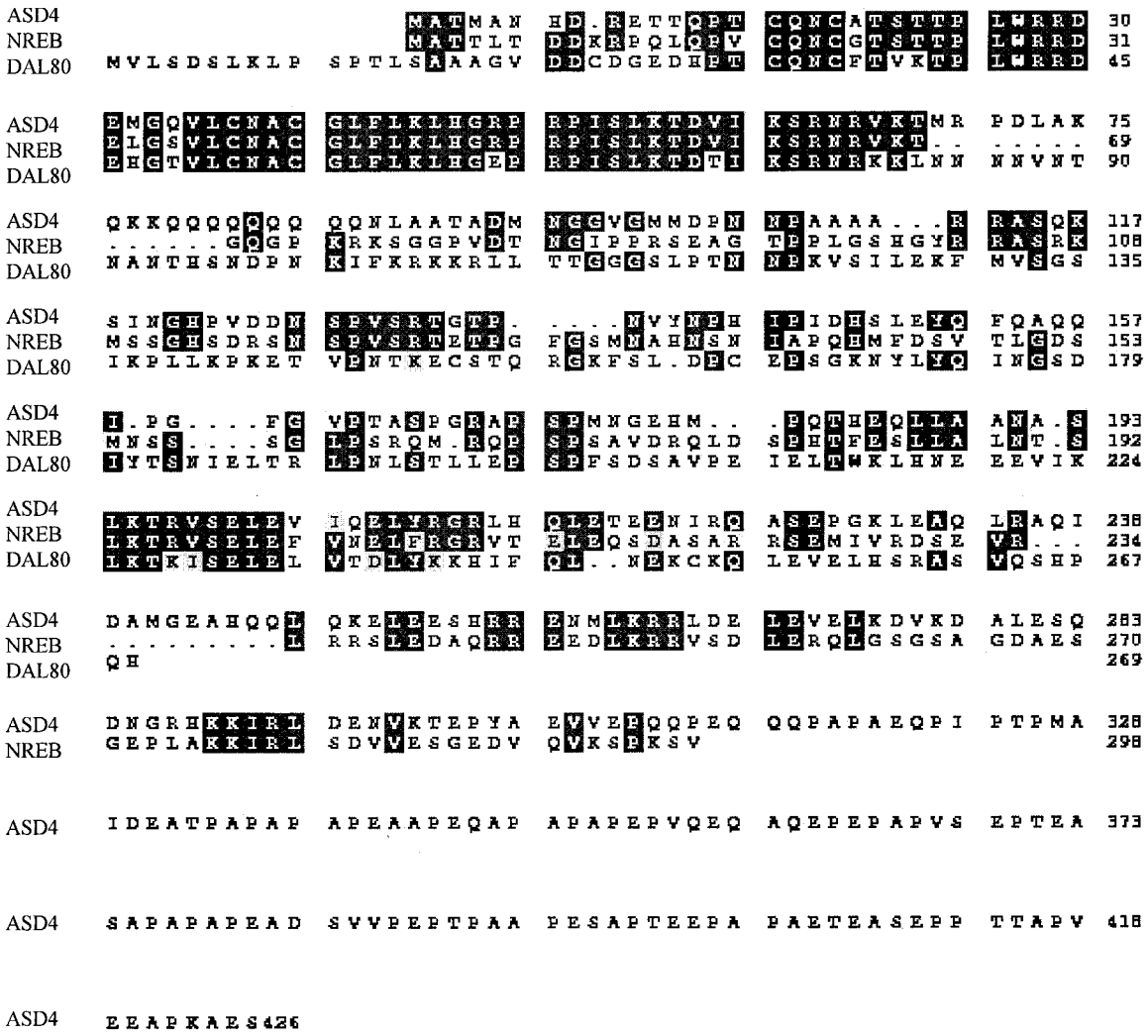

FIGURE 6: Sequence alignment of ASD4 with DAL80 and NREB was carried out with Prettybox(GCG).

environmental cues, e.g., the abundance of nitrogen sources, the availability of iron, and in sensing and responding to blue light. Here we show that a fifth GATA factor of *Neurospora*, Asd-4+, appears to play a significant role in development of the ascus and ascospores, specialized structures and cells that result from a sexual cross. The fact that Asd-4+ has a well-defined GATA-type zinc finger and displays sequence-specific binding to DNA molecules that contain GATA sequence elements suggests that it functions as a regulatory protein, controlling genes involved in ascus development. Several genetic loci that function in ascus development have been recognized. Mutants of the asd-1 gene are recessive, and in a homozygous cross, asd-1 × asd-1, large numbers of normal-appearing asci form, but are completely devoid of ascospores (*45*, *46*). The asd-1+ gene has been cloned and encodes a protein that appears to possess rhamnogalacturonase enzyme activity (*45*). Downstream genes which may be regulated by Asd-4+ have not yet been identified, although asd-1+ represents an interesting gene involved in ascus development which might be subject to Asd-4+ control.

It is somewhat surprising that the Asd-4 mutant is dominant to Asd-4+ since a cross between them yields fruiting bodies devoid of asci. It is possible that the mutant acts dominant negative and produces a defective ASD4 protein or an amino-terminal region of it which associates with wild-type ASD4 subunits, inhibiting their activity. Alternatively, Asd-4+ function may be required in both parental nuclei in order for the sexual cycle to progress. This possibility would indicate that ASD4 acts at a very early step during the mating process, perhaps at meiosis or even preceding this step. The dominant effect of the Asd-4 mutant also suggests the possibility that this locus displays transvection, namely, that expression of a gene requires a pairing interaction with a homologous sequence (*47*). The Asm-1 (ascospore maturation) gene, which encodes an abundant nuclear-localized DNA binding protein, was demonstrated to show transvection; a deletion of the Asm-1 gene acted as a dominant to the wild-type, and two wild-type Asm-1 genes must be located at identical genomic positions in order to function. However, in the case of the Asd-4 locus, the fact that function was restored when the mutant strain was transformed with a wild-type Asd-4+ gene at ectopic sites argues against transvection.

Significant homology between ASD4 and the yeast Dal80p and *Penicillium* NREB exists (Figure 6) and is in contrast to their different cellular functions, since the later two appear to act only in nitrogen gene expression (*13*, *14*). The Asd-4 mutant displayed completely normal control and expression level of nit-3, the highly regulated nitrate reductase structural gene. In addition, Northern blot analysis showed that various nitrogen sources did not affect Asd-4+ expression.

Since *Penicillium* does not possess a sexual cycle, it is obvious that NREB cannot be involved in spore development. The ASD4 protein possesses an acidic carboxyl-terminal region which is not present in NREB or in Dal80p, and this feature may be significant for the novel physiological function of the Asd-4 gene (Figure 6). It is noteworthy that GATA factors play a central role in the sexual cycle or reproductive stages of diverse organisms, e.g., in development of egg yolk protein in *Drosophila* (*48*), and a maternal transcription factor in Xenopus (*49*).

The homology observed between regions of ASD4 with a number of proteins with calcium binding activity provided the rationale to examine the effect of Ca^{2+}, if any, upon the formation of ASD4 oligomeric species. Very low concentrations of Ca^{2+}, but not Mg^{2+}, strongly stimulated the ability of ASD4 to form a tetrameric species. However, it is unknown whether this reflects a physiological aspect of ASD4 function. It is noteworthy that a substantial number of nuclear DNA binding proteins have a calcium binding site, including, among others, HMG1 (*50*), chromokinesin (*51*), and the *Drosophila* nod protein (*52*). Some of these nuclear calcium binding proteins appear to function in chromosome integrity or movement (*51*, *52*). In view of these characteristics, it seems possible that the ASD4 protein is involved in a very early step during the sexual cycle and may depend on an ability to bind calcium.

Neurospora possesses at least five GATA factors, all of which recognize GATA sequence elements and show overlapping DNA binding activities. Moreover, promoters of some genes which are not regulated by any known GATA factor nevertheless contain several GATA elements. These features raise a puzzling question, namely: How do each of these GATA factors specifically regulate a distinct set of downstream genes without interfering with each other? At least one major factor in GATA factor specificity depends on distinct protein−protein interactions that occur in the context of a regulated promoter. Thus, NIT2, the *Neurospora* global-acting nitrogen regulatory GATA protein, specifically interacts with NIT4, a pathway-specific DNA binding protein. Significantly, the NIT2−NIT4 protein−protein interaction is required for expression of genes that encode nitrate assimilatory enzymes (*36*). Moreover, other than NIT2, none of the other *Neurospora* GATA factors have any ability to interact with NIT4 (*36*). These features imply that regulatory specificity of the NIT2 GATA factor depends on its ability to interact with NIT4 or other pathway-specific proteins with which it functions and suggest that protein−protein interactions may indeed be essential for the specific regulatory responses elicited by each of the GATA factors of *Neurospora*.

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