MOLECULAR AND CELLULAR BIOLOGY, Feb. 1991, p. 795–802
0270-7306/91/020795-08$02.00/0

Vol. 11, No. 2

Molecular Cloning and Functional Characterization of the Pathway-Specific Regulatory Gene *nirA*, Which Controls Nitrate Assimilation in *Aspergillus nidulans*

GERTRAUD BURGER,[1]†* JOAN TILBURN,[2]‡ AND CLAUDIO SCAZZOCCHIO[1]

Institut de Microbiologie, Université de Paris-Sud, F-91405 Orsay, France,[1] and Department of Biology, University of Essex, Colchester CO4 3SQ, England[2]

Received 17 July 1990/Accepted 2 November 1990

We have cloned an 11-kbp segment of the genomic DNA of *Aspergillus nidulans* which complements mutations in *nirA*, the pathway-specific regulatory gene of the nitrate assimilation pathway. Gene disruption in the corresponding region of the nuclear DNA leads to a phenotype and a gene complementation pattern indistinguishable from that observed in known noninducible *nirA* mutants. Transformation studies with subclones of the 11-kbp genomic segment showed that a nonreverting null mutation, *nirA87*, maps to a 1.5-kbp stretch within that segment. These data confirm that the cloned segment contains the *nirA* gene. The gene is completely encompassed in the 11-kbp genomic segment, as a plasmid carrying the corresponding insert gives rise to multicopy transformants exhibiting better growth than wild type on nitrate or nitrite as the sole nitrogen source. Southern and genetic analyses of transformants obtained with various plasmid subclones established a gene size of at most 5.9 kbp. Northern (RNA) hybridization experiments revealed a 4-kb *nirA* transcript which is barely visible in the wild type but clearly seen in a transformant carrying about 10 gene copies. In both strains, *nirA* mRNA is synthesized constitutively. Upstream of *nirA*, a neighboring transcript about 2.8 kbp in length which is transcribed from the opposite strand with respect to *nirA* was localized. The transcript levels of *niaD* and *niiA*, encoding the nitrate and nitrite reductase core proteins, respectively, were investigated in *nirA* mutants and a *nirA* multicopy transformant. The results show that the *nirA* product regulates the transcript steady-state level of these structural genes and that it is a limiting factor for their expression.

In the plant kingdom, nitrate is the main nitrogen source, whereas the fungi are heterogenous with regard to nitrate assimilation. Filamentous fungi like *Neurospora*, *Fusarium*, and *Aspergillus* species as well as a number of budding "yeasts," e.g., *Hansenula* and *Candida* species, possess the enzyme system to assimilate nitrate and even nitrite, while *Saccharomyces* and *Schizosaccharomyces* species, for example, are unable to utilize this nitrogen source.

Nitrate assimilation has been well studied in plants, algae, and filamentous fungi and has been investigated with particular emphasis in *Aspergillus nidulans* by means of genetic and physiological analyses (for a review, see reference 5). In this ascomycete, the structural genes coding for the core proteins of nitrate and nitrite reductase (*niaD* and *niiA*, respectively) and a gene coding for a permease, *crnA*, are clustered in linkage group VIII, whereas a number of *cnx* genes, necessary for the synthesis of the molybdenum cofactor of nitrate reductase (and other molybdoflavoproteins), are scattered throughout the genome. The *nirA* gene is needed for expression of both nitrate and nitrite reductase and is therefore considered the pathway-specific regulatory gene (22).

The regulation pattern of the nitrate assimilation enzymes in *A. nidulans* had been established by enzymatic and immunological studies of the wild type and various mutants (for a review, see reference 5). Expression of the genes encoding the nitrate and nitrite reductase core proteins, *niaD* and *niiA*, respectively, is subject to a double control: they are induced by nitrate or nitrite and repressed by ammonium. Induction is mediated by the pathway-specific regulatory gene *nirA*, mentioned above, and repression is mediated by the wide-range regulatory gene *areA*, which is necessary for the utilization of all nitrogen sources except ammonium and glutamine (for reviews, see references 5 and 25). A double control also exists in *Neurospora crassa*, in which *nit-4* mediates nitrate induction and *nit-2* mediates ammonium repression (8, 30).

In *A. nidulans*, the nitrate assimilation gene cluster has been cloned (17, 20) and sequenced (17). The recently published DNA sequence of *areA* indicates that this gene encodes a DNA-binding protein including a peculiar Zn^{2+} finger structure (18).

The pathway-specific regulatory gene *nirA* is particulary attractive. A great number of mutant alleles mapping to this regulatory gene have been isolated. The majority confer inability to grow on nitrate or nitrite as the sole nitrogen source and have been classified as noninducible, presumably loss-of-function mutations (22). Of special interest are the *nirA* alleles, which confer an altered response to induction and repression of this pathway. Rare constitutive mutations which map to this gene and allow the synthesis of nitrate and nitrite reductase even in the absence of inducers have been selected. Even more interestingly, a number of mutations mapping in *nirA* result in nitrogen metabolite derepression, characterized by the synthesis of the nitrate-assimilating enzymes even in the presence of ammonium, thus bypassing the absence of a functional *areA* protein specifically for nitrate assimilation (24, 28). Therefore, *nirA* is a choice gene

* Corresponding author.
† Present address: Département de Biochimie, Université de Montréal, C.P. 6128, Succursale A, Montréal, Quebec H3C 3J7, Canada.
‡ Present address: Department of Bacteriology, Royal Postgraduate Medical School, Hammersmith Hospital, London W12 OHS, United Kingdom.

TABLE 1. Genotypes and origins of *A. nidulans* strains

Genotype	Notes and reference
biA1	Used as wild type
pabaA1	Used as wild type
nirA87 biA1 fwA-1	27
nirA1 sB43 amdS368 prn309 alcR125 amdA7 cbxC34 oxpA5 uaY207 fpaD43	5
argB2 biA1 pyroA4 hxA1 wA4	22
nirA502 nirA509 nirA592 nirA593 nirA614 nirA620 pabaA1	This report
nirA601 argB2 biA1 pyroA4 hxA1 wA4	This report
niaD52 pabaA1	13, 29
niiA/niaDΔ506 biA1 hxnR10 amdSΔ368 amdA7	13, 29
niiA/niaDΔ616 puA2 yA2	29
cnxF24 puA2 wA4	5

TABLE 2. Crosses between noninducible *nirA* mutants

Parents[a]	No. of:	
	Tested progeny	Recombinants
nirA87 × *nirA1*	3.7×10^5	0
nirA87 × *nirA502*	9.2×10^5	12
nirA87 × *nirA509*	1.1×10^5	2
nirA87 × *nirA592*	3.0×10^4	1
nirA87 × *nirA593*	5.1×10^5	61
nirA87 × *nirA614*	3.0×10^4	0
nirA87 × *nirA620*	2.7×10^5	10

[a] All listed *nirA* mutant alleles except *nirA1* and *nirA87* are newly isolated. Ascospores were plated onto solid medium containing nitrite as the sole nitrogen source plus deoxycholate to induce colonial growth.

for investigating the interaction between a pathway-specific and a wide-domain regulatory gene in *A. nidulans*.

In order to study the regulation of nitrate assimilation at the molecular level, we have cloned the *nirA* gene, identified its transcript, and characterized the *nirA* product, which was found to be a positive-acting regulation factor necessary for *niaD* and *niiA* transcription.

MATERIALS AND METHODS

***A. nidulans* strains and genetic analyses.** All *A. nidulans* strains used in this work are listed in Table 1. Mutants carrying the *nirA87* and *nirA1* alleles were used as recipients in transformation experiments. *nirA1* originally was *niiB1* (22); *nirA87* is a spontaneous nonrevertible allele (2a); and *nirA502*, *nirA509*, *nirA592*, *nirA593*, *nirA614*, and *nirA620* are spontaneous alleles recently obtained in our laboratory which show the growth and complementation patterns typical of *nirA* mutations (5). A strain carrying the *biA1* mutation (biotin requirement) served as a wild-type control in growth and Southern analyses, and a strain containing the *pabaA1* mutation (*p*-aminobenzoic acid requiring) was used as a wild type in crosses with transformants. In cotransformation experiments which disrupted the wild-type *nirA* gene, the recipient strain had the genotype *argB2 nirA*$^+$. Complementation analyses of the resulting noninducible transformants and of their automeiotic products were performed as previously described (2, 23), using strains carrying the alleles *nirA1*, *cnxF24*, *niaD52*, *niiA/niaDΔ506*, and *niiA/niaDΔ616*. (The last two mutations are deletions comprising *niiA* and *niaD*.)

Media growth conditions, and transformation of *A. nidulans*. Standard media and growth conditions for *A. nidulans* were used (3). For RNA isolation, mycelia were grown in the presence of urea at 25°C for 12 h, nitrate was added for induction, and mycelia were harvested after 2 h. Nitrate and nitrite reductase are fully induced after this period (3). The protocols for protoplast preparation and DNA-mediated transformation are described elsewhere (26).

Genomic libraries, plasmids, and bacterial strains. The gene library containing a *Sau*3A partial digest of genomic DNA from *A. nidulans* cloned into EMBL4 was kindly provided by C. M. Lazarus and J. Turner. The genomic library containing an *Mbo*I partial digest cloned into the plasmid vector pMA2 which carries the *argB2* gene was kindly provided by W. Timberlake (21). Plasmid pnir2A was isolated from the *Mbo*I library, and the phages EM4-1, EM4-3, and EM4-4 were isolated from the *Sau*3A library. The phage inserts were subcloned into Bluescript KS+ and KS− (Stratagene), yielding the bs series of plasmids. The plasmids pSF5, pucBam12, pAN501, and pALS were used as hybridization probes and contain the *A. nidulans* actin gene, the *niaD* gene cloned in pUC19, the *niiA* gene cloned in Bluescript, and the *alcR* gene cloned in pUC8, respectively. These plasmids were kindly provided by R. Morris (10), T. Langin, L. Malardier, and B. Felenbok. *Escherichia coli* Q359 served as a bacterial host for lambda clones, strain DH5α was a host for the pnirA2 plasmid, and strain XL1-Blue was a host for the Bluescript recombinant clones.

Isolation of nucleic acids from *A. nidulans*. After the mycelia were ground in liquid nitrogen, nucleic acids were extracted with guanidine chloride (6). The RNA was isolated from the guanidine chloride solution by precipitation with 2 M LiCl, and the DNA was collected from the supernatant by precipitation with isopropanol. Poly(A) RNA was purified by mAP paper (Orgenics Ltd).

Southern and Northern (RNA) hybridizations. DNA was separated on 0.7% agarose gels and alkali transferred under vacuum onto Hybond N membranes (Amersham). RNA was separated in a 0.66 M formaldehyde gel (11) and capillary transferred to Hybond N (Amersham) or nitrocellulose (Schleicher & Schuell). Conditions for transfer and hybridization (in formamide) were as recommended by the membrane suppliers. Probes were radiolabeled with a random priming kit (Boehringer) using [α-^{32}P]dCTP. Hybridization with single-stranded probes in Northern blot experiments were performed by the sandwich method: membranes were hybridized with the unlabeled single-stranded DNA of a Bluescript recombinant clone, washed, and then hybridized with the labeled double-stranded vector DNA.

RESULTS

Linkage of *nirA* mutants. Table 2 shows the recombination frequencies in crosses involving *nirA87*, which is a spontaneous nonrevertible mutation (5a), and a number of other, newly isolated *nirA* mutant alleles. All mutations map very close to *nirA87*, the longest distance being 0.033 centimorgans. In the region of the proline utilization gene cluster of *A. nidulans*, 1 centimorgan corresponds to between 3 and 4 kbp (15). If this correlation applies in the region of the *nirA* gene, the two most distant alleles, *nirA87* and *nirA593*, would be separated by about 100 bp only. Two of seven mutations failed to recombine with the *nirA87* allele. Thus it is possible that *nirA87* results from a small deletion or from tightly linked multiple point mutations. Physical localization of *nirA87* was attempted as outlined below.

Isolation of *nirA*-containing clones. Cloning of *nirA* was performed by complementation of *A. nidulans nirA* mutants.

TABLE 3. Transformation efficiency of subcloned plasmids

Plasmid[a]	No. of *nirA*$^+$ transformants/μg of DNA
pnirA2	202
bs4	93
bs14	39
bs41	5
bs42	0
bs43	0
bs44	20
bs44r	11
bs41	26
bs411	17
bs412	0
bs413	0
bs18-4	6
bs13-2	2
bs14-1	8
bsr5-2	1

[a] Plasmids were tested for their abilities to transform the *A. nidulans nirA87* mutation to a NirA$^+$ phenotype. The physical map positions of the corresponding plasmid inserts are depicted in Fig. 1. Transformed protoplasts were spread on plates containing nitrite as the sole nitrogen source and incubated for up to 7 days. The values given for plasmids bs18-4, bs13-2, bs14-1, and bsr5-2 represent single experiments, whereas values for the other plasmids represent the means of two or three independent experiments with approximately 30% variation.

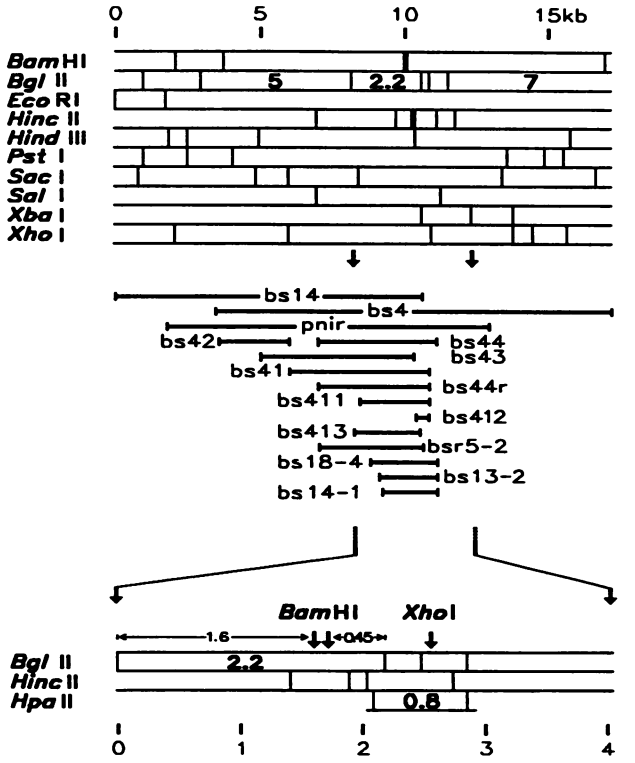

FIG. 1. Physical map of the *nirA* locus of *A. nidulans*. Top, Restriction map of a 17-kbp genomic region of chromosome 8; middle, positions of cloned fragments; bottom, the stretch between *Bgl*II (map position 8.2) and *Xba*I (map position 12.2) enlarged fourfold. The position of the 800-bp *Hpa*II fragment has been mapped with a precision of ±100 bp.

A strain carrying *nirA1* was transformed with a genomic library made in the plasmid pMA2 (see Materials and Methods), and transformants able to grow on medium containing nitrite as the sole nitrogen source were selected directly. A number of stable transformants were analyzed by Southern hybridization, and one, TpnirA2, was shown to have arisen through one single integrative event (results not shown). To rescue the transforming plasmid (16), *E. coli* was transformed with total uncut DNA from the transformant Tpnir2A. Plasmid DNA extracted from several ampicillin-resistant *E. coli* clones was used to retransform an *A. nidulans* strain containing the *nirA1* mutation. Plasmid pnirA2 yielded stable *nirA*$^+$ transformants and also transformed a strain carrying the nonrevertible *nirA87* allele (25a). This plasmid was therefore selected for further studies.

Plasmids rescued from *A. nidulans* DNA preparations arise presumably by recombination events which take place either in the fungus or in *E. coli* after introduction of the linear DNA. In either case, rearrangements are possible. To avoid the risk of working with rearranged sequences, independent clones were selected by using pnirA2 as a probe for screening a second *A. nidulans* genomic library. Three lambda clones, EM4-1, EM4-3, and EM4-4, which hybridized with the rescue plasmid but not with the pMA2 vector were isolated. Restriction analysis strongly suggested that EM4-1 and EM4-3 are identical. The 10- and 14-kbp inserts isolated by *Eco*RI digestion from EM4-1 and EM4-4, respectively, were cloned into the phagemid Bluescript yielding the plasmids bs14 (10-kbp insert) and bs3 and bs4 (14-kbp insert in both orientations). All three plasmids were able to transform the strain carrying the *nirA87* allele to a NirA$^+$ phenotype, albeit at a lower rate than the rescued plasmid, pnirA2 (Table 3). The presence of the *argB* gene in pnirA2 could account for the higher transformation efficiency of this plasmid.

Plasmids bs3, bs4, and bs14 served to establish a restriction map covering 17 kbp of the chromosomal DNA (Fig. 1, top). The map is consistent with the restriction patterns of both the rescued plasmid and *A. nidulans* genomic DNA as derived from Southern analysis (results not shown).

Localization of *nirA87* mutation. A number of subclones derived from bs3, bs4, and bs14, with insert sizes of between 6 and 0.6 kbp (Fig. 1, bottom), were tested for their abilities to transform *A. nidulans* strains carrying the *nirA87* mutation (Table 3). The smallest plasmid which did repair this mutation was bs411, containing a 2.7-kbp *Sac*I-*Xho*I fragment. Two other plasmids, bs14-1 and bsr15-2, which also transform *nirA87* (though at a lower rate), carry inserts which partially overlap with that of bs411. It follows that the *nirA87* mutation is located within the stretch common to the inserts of these three plasmids, i.e., between map positions 9.2 and 10.7 kbp (Fig. 1). Although genetic data (see above) suggested that *nirA87* could be a small deletion, Southern hybridization analysis with a number of restriction enzymes failed to reveal any obvious size alteration (>50 bp) (data not shown). When the DNA was cut with *Hpa*II and hybridized with small probes, a 950-bp band instead of an 800-bp band appeared in *nirA87*, strongly indicating that one *Hpa*II recognition site is missing in the mutant (Fig. 2A).

The relative signal intensity of the variable *Hpa*II band increased when bs411 instead of bs413 was used as a hybridization probe, obviously because of a greater overlap of bs411 with the *Hpa*II fragment (Fig. 1). This allowed us to pinpoint the fragment between the *Bgl*II and *Xho*I sites at map positions 2.2 and 2.7 kbp, respectively (Fig. 1, bottom). As the mutation was now tentatively mapped, targeted DNA

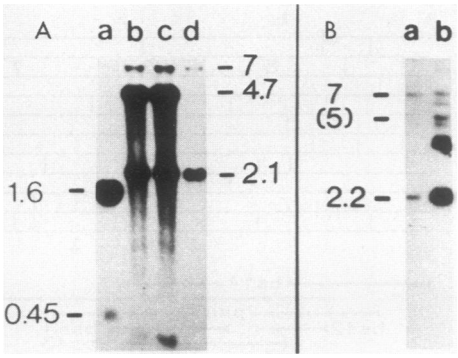

FIG. 2. Southern hybridization analyses of transformant T4⁻, and *nirA* multicopy transformant Tp22. (A) Disruption of the *nirA* gene. Nuclear DNA was digested with *Bgl*II and *Bam*HI and probed with the radiolabeled insert of bs411. Lane a, Wild type carrying *argB2*, which served as recipient strain in these transformation experiments; lane b, transformant T4⁻ obtained with plasmid bs4111 plus pMA2; lanes c and d, strains 600 and 601, respectively, derived from ascospores resulting from automeiosis of T4⁻. The *nirA* allele of strain 601 is referred to as *nirA601*. In the wild type, the 2.2-kbp *Bgl*II fragment is cut by *Bam*HI into two major fragments of 1.6 and 0.45 kbp plus a 100-bp fragment, which is not visible on the exposure. The corresponding *Bgl*II fragments in transformant T4⁻ and derived strains lack a 100-bp *Bam*HI fragment as well as both adjacent *Bam*HI sites. That *Bgl*II fragment is therefore only 2.1 kbp long and not cut by *Bam*HI. The faint 7-kbp band comprises the vector inserted between map positions 2.7 and 2.8 (Fig. 1) plus flanking sequences which are recognized by the probe. (B) Tp22 is a *nirA* multicopy transformant. Nuclear DNAs of a *nirA87* strain (lane a) (from which Tp22 was derived by transformation with pnirA2) and of transformant Tp22 (lane b) were digested with *Bgl*II and hybridized with a *Stu*I fragment of 4.3 kbp (map position 8.0 to 12.3 kbp; Fig. 1). The hybridization signal of the 5-kbp restriction fragment is relatively faint because it overlaps the probe only partially. In Tp22, the 2.2-kbp *Bgl*II fragment, which corresponds to the original *A. nidulans Bgl*II fragment, is amplified. The amplified 3.8-kb fragment corresponds to a *Bgl*II fragment of the pnirA2 plasmid and consists of vector and insert sequences.

sequencing of that region could be undertaken to determine the exact nature of the *nirA87* mutation.

Disruption of *nirA* sequence. The fact that a particular DNA sequence transforms a mutant to the wild-type phenotype does not necessarily imply that the introduced sequence contains the respective wild-type allele. In order to exclude the possibility that the appearence of a wild-type phenotype is due to an allele-specific or general suppressors, we transformed an *A. nidulans* wild-type strain with a plasmid carrying a disrupted version of the cloned gene.

A *Bam*HI fragment of 120 bp was deleted from plasmid bs411. In addition, the *Bam*HI site was destroyed by filling in the protruding ends and then ligating the generated blunt ends. The resulting plasmid, bs4111, was cotransformed into an arginine-requiring *A. nidulans* strain (*argB2*) together with vector pMA2, which carries the *argB*⁺ gene. Among 50 Arg⁺ transformants, 1 (T4⁻) displayed a NirA⁻ phenotype. This phenotype was found to be stable at automeiosis, as no NirA⁺ strains were recovered in 5,000 progeny obtained from a selfed cleistrothecium. When crossed with *nirA1*, no *nirA*⁺ recombinants were found in >50,000 viable ascospores, demonstrating that the new mutation is tightly linked to the classically defined locus. T4⁻ also exhibited a complementation pattern typical of noninducible *nirA* mutants (5); i.e., it failed to complement other *nirA* mutant strains, showed reduced complementation with strains carrying mutations in the *niiA* or *niaD* gene, and complemented fully with strains containing mutations in the *cnx* genes.

Southern hybridizations using bs4111 as a probe demonstrate that in T4⁻ the wild-type sequence has been replaced by the shortened bs4111 conformation (Fig. 2A, lanes a and b). Instead of the three bands of 1.6, 0.45, and 0.1 kbp found in the recipient strain, there is only one band, of 2.1 kbp, in the transformant. However, the presence of an additional strong 4.7-kbp band and a faint 7-kbp band (lane b) indicates that a more complex integration-replacement event that included tandem integration of two copies of the transforming plasmid had occurred (results not shown).

In *A. nidulans*, integrated sequences are often excised during meiosis (7, 9, 26). The meiotic stability of the NirA⁻ phenotype of T4⁻, mentioned above, implies that the inserted sequences of this transformant cannot be excised precisely. Eight strains which were derived from ascospores resulting from T4⁻ automeiosis and displaying an Arg⁻ NirA⁻ phenotype were analyzed by Southern hybridization. All contain the shortened *Bgl*II fragment, and one, strain 601, had lost the additional 4.7-kbp band (Fig. 2A, lanes c and d). Further Southern analysis (results not shown) established that this strain carries a 2.5-kbp insertion essentially consisting of vector sequences integrated just between the *Xho*I and *Sal*I sites (map positions 2.7 and 2.8; Fig. 1, bottom), whereas no changes are detectable outside this region. Finally, strain 601 showed exactly the same complementation pattern as the original transformant T4⁻. This newly created *nirA* mutant allele has been designated *nirA601*.

The disruption experiment and the nature and position of the *nirA87* mutation demonstrate that the cloned DNA fragment indeed corresponds to the *nirA* gene.

Boundaries of *nirA* gene. In order to specify where the gene starts and ends, *nirA*⁺ transformants obtained with various plasmids were analyzed. Three possible types of integration originally found in bakers' yeast (14) and later found in *A. nidulans* (32) were expected: (i) replacement, (ii) homologous insertion, and (iii) heterologous insertion. The different types were distinguished by Southern hybridization and by analysis of the genetic linkage between the introduced gene and its resident allele. Growth tests on nitrate as the sole nitrogen source were particularly useful, allowing us to identify multiple integration events and/or heterologous insertions placing the introduced gene under the control of another promoter. Either case would prove that the transforming plasmid comprises the complete coding sequence of the gene in question. Table 4 summarizes the results of crosses between the wild type and 24 transformants which were obtained with seven different plasmids (Fig. 1). All transformants obtained with the small plasmids bs411, bs14-1, and bsr5-2 failed to yield any progeny displaying a NirA⁻ phenotype. The meiotic stability indicated that sequence replacement must have occurred, because homologous integration events generate direct repeats which are meiotically unstable in *A. nidulans* (e.g., see reference 26). Those transformants obtained with the five larger plasmids (pnirA2, bs14, bs4, bs44, and bs41) did segregate NirA⁻ progeny at rates lower than 3.5%, which is consistent with homologous plasmid insertion (25% NirA⁻ segregants would be expected for unlinked heterologous plasmid insertion (see, for example, reference 31). We conclude that these five large plasmids contain at least one end of the gene, whereas the three small ones do not contain either end.

Seven of the transformants described above obtained with

TABLE 4. Analyses of *nirA*$^+$ transformants

Transforming plasmid	Integration event[a]	No. of transformants			No. of:		
		Tested for growth on NO_3^- + CsCl[b]	CsCl resistant	Crossed with wild type	Analyzed cleistrothecia	Tested progeny	NirA$^-$ segregants[c]
pnirA2	2:(repl)						
	3:(ho.int)	22	3	1	2	87	3
bs14		39	0	9	9	1,324	7
bs4		6	0	1	3	253	2
bs44		25	0	3	5	1,227	3
bs41		23	0	1	2	309	3
bs411	7:(repl)	18	0	3	15	678	0
bs14-1				3	5	2,666	0
bsr5-2				1	2	550	0

[a] The types of plasmid integration were deduced from Southern hybridizations. repl, Sequence replacement; ho.int, homologous insertion by single crossover.
[b] Transformants were tested for growth compared with that of wild type on plates containing 40 mM CsCl plus 10 mM sodium nitrate as the sole nitrogen source.
[c] After transformants were crossed with the wild type, the progeny were screened for *nirA* mutant segregants by replica plating them from plates containing urea (plus deoxycholate) onto plates containing nitrate as the sole nitrogen source.

plasmid bs411 (2.7-kbp insert) were analyzed by Southern hybridization. In all of them, gene replacement had occurred; i.e., the restriction patterns are identical to that of the recipient strain (results not shown). This confirms that the 2.7-kbp fragment lacks both gene ends.

A great number of transformants originating from various transformation experiments was screened for more vigorous mycelial growth on nitrate and nitrite as the sole nitrogen source (Table 4). CsCl, which partially inhibits growth and permits a more sensitive estimation of the degree of nitrate utilization, was added to the medium. The mechanism of CsCl inhibition is not fully understood (24), but it has been established that the resistance to CsCl is proportional to the efficiency with which a given nitrogen source is utilized. Out of 90 transformants, the only 3 isolates with increased resistance to CsCl originated from plasmid pnirA2. The most resistant transformant (Tp22) was analyzed by Southern hybridization and clearly showed the insertion of about 10 complete copies of the pnirA2 plasmid in tandem configuration, integrated most probably at the *nirA* locus (Fig. 2B). The copy number was estimated from DNA dot blots calibrated with the single-copy gene *alcR* (result not shown). The CsCl resistance phenotype is apparently caused by an elevated copy number of the pnirA2 plasmid, and this increase results in a higher expression of the structural genes *niaD* and *niiA*. Therefore we conclude that pnirA2 contains the entire *nirA*-coding region.

The transformants obtained from plasmid pnirA2, bs44, and bs411 were particularly useful in delimiting the *nirA* gene. The insert in bs44, which contains one end of the gene (see above), exceeds the internal gene fragment contained in bs411 by 1.4 kbp on the left, therefore placing the left end of *nirA* between map positions 6.8 and 8.2 (*Sal*I and *Sac*I sites). As defined by pnirA2, the maximum right-hand end is at map position 12.7 (for map positions, see Fig. 1). Consequently, the 5.9-kbp segment must encompass the entire *nirA* gene, including any control elements strictly necessary for its expression.

Transcript analysis. A plasmid containing the 450-bp *Bam*HI-*Bgl*II fragment (Fig. 1, map position 10.0 to 10.45 kbp) was used as a gene-specific probe for identifying the *nirA* transcript. Repeated experiments in which poly(A) RNA of the wild-type strain, grown under inducing and noninducing conditions, was probed with this plasmid revealed only a very faint band of about 4 kb. This band was clearly seen in the multicopy transformant Tp22, which contains around 10 copies of plasmid pnirA2 in tandem (Fig. 3A). Our results indicate that *nirA* is transcribed at a very low rate and/or is highly unstable, its steady-state level being even lower than that of transcripts of other regulatory genes of *A. nidulans* (e.g., *alcR*, which regulates ethanol utilization [19]).

Two observations prove that the 4-kb transcript indeed

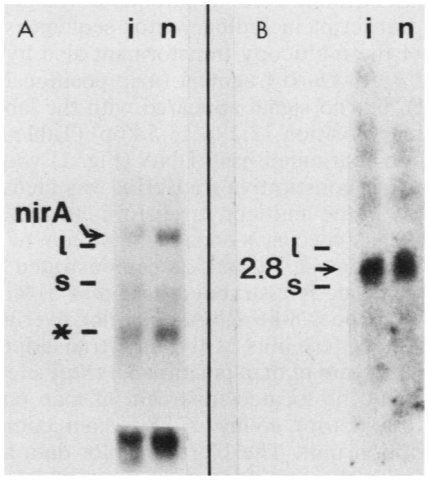

FIG. 3. Transcripts arising from the cloned region. (A) *nirA* transcript levels of the *nirA* multicopy transformant Tp22. Northern analysis of poly(A) RNA prepared from Tp22 grown with urea (lane n, noninduced) or with urea plus nitrate (lane i, induced) as the sole nitrogen source is shown. Single-stranded DNA derived from plasmid bs450(+) was hybridized to 10 μg of filter-bound RNA and sandwiched with a radiolabeled Bluescript vector. The positions of the large and small rRNAs, 1.8 and 3.7 kb in size, respectively, are indicated by l and s, respectively. The broad band marked with an asterisk is an artifact, as it is also visualized when the Bluescript vector alone or plasmid bs450(−) was used as probe. The boxes at the bottom of the lanes show the signals obtained after identical blots were hybridized with an actin probe, serving as quantification of membrane-bound poly(A) RNA. (B) Unassigned neighbor transcript upstream of *nirA*, read from the opposite strand. Northern analysis of poly(A) RNA prepared from wild type grown under induced (lane i) and noninduced (lane n) conditions is shown. Single-stranded DNA from plasmid bs44 (Fig. 1) served as a probe.

TABLE 5. *nirA* transcript mapping[a]

mRNA transcript (length)	Presence in:					
	bs44	bs411	bs450	bs412	BX1.1	XX1.3
nirA (4 kb)	+	+	+	+	+	−
Adjacent (2.8 kb)	+	−	−	−	−	−

[a] Northern blots of poly(A) RNA prepared from the *nirA* multicopy transformant Tp22 grown under induced conditions were hybridized with various double-stranded DNA probes. For details on bs44, bs411, and bs412, see Fig. 1. bs450 contains the 450-bp *Bam*HI-*Bgl*II fragment (map position 10.0 to 10.45 kbp; Fig. 1), BX1.1 is a 1.1-kbp *Bgl*II-*Xba*I restriction fragment (map position 11.1 to 12.2 kbp), and XX1.3 is a 1.3-kbp *Xba*I fragment (map position 12.2 to 13.5).

corresponds to *nirA*: the increased steady-state concentration of the transcript in the multicopy transformant and the lack of the 4-kb signal in *nirA601* strains carrying the disrupted gene (result not shown). The *nirA* transcript does not appear to be inducible, like that of the *amdR* gene regulating acetyl coenzyme A-producing enzymes (1). This is in contrast to *alcR* transcription, which is inducible by ethanol (19).

The direction of transcription was determined in the multicopy transformant by using single-stranded probes derived from two plasmids containing the 450-bp *Bam*HI-*Bgl*II fragment in different orientations (Fig. 1, bottom). One probe hybridized to the 4-kb transcript, showing that *nirA* is transcribed from left to right in the restriction map in Fig. 1 (Fig. 3A). Both complementary single-stranded probes and a double-stranded vector probe detected in the transformant a diffuse band of around 1 kb, apparently corresponding to an artifactual transcript including vector sequences. The *nirA* transcript of the multicopy transformant also hybridized to the labeled *Bgl*II-*Xba*II fragment (map position 11.1 to 12.2 kbp; Fig. 1), but no signal appeared with the labeled *Xba*II fragment (map position 12.2 to 13.5 kbp) (Table 5).

When double-stranded bs44 DNA (Fig. 1) was used as a probe, a further constitutive transcript was identified in the wild type and in the multicopy transformant Tp22 (Table 5). This 2.8-kb RNA species is encoded upstream of *nirA* and is read from the opposite strand, as demonstrated by probing with single- and double-stranded bs44 and bs44r (Fig. 3B). Assuming that the 2.8-kb RNA does not overlap the *nirA* transcript, the 5′ terminus of the *nirA* transcript should be located downstream of map position 6.8 (*Sal*I site) and the 3′ terminus should be located upstream of map position 13.5 (*Xba*I site; Fig. 1, top), giving 6.7 kbp as a maximum size for the transcription unit. The Northern blot data are in good agreement with the transformation data, which positioned the gene's left end between positions 6.8 and 9.2 and the maximum right end at position 12.7 (Fig. 1), therefore suggesting a maximum *nirA* gene size of 5.9 kbp.

In order to investigate at which level the *nirA* product mediates induction of the structural genes encoding nitrate and nitrite reductase, we determined the transcript concentrations of *niaD* and *niiA* in the wild type, in mutants carrying the *nirA601*, *nirA1*, and *nirA87* alleles, and in the *nirA* multicopy transformant Tp22. Figure 4 shows that the *nirA* mutants grown under induced conditions have a very low steady-state level of *niaD* transcript compared with that of the wild type, the level being similar to that of the wild type grown under noninduced conditions. The same result was obtained when we probed for the *niiA* transcript (data not shown). In Tp22, the transcript concentrations of the structural genes were elevated under both induced and noninduced conditions and were roughly proportional to the number of *nirA* copies compared with the ratio in the wild type (Fig. 5). This is in agreement with the CsCl resistance growth phenotype of that transformant and also consistent with the gene dosage effect observed in *nirA1*-*nirA*$^+$ diploids (4). We conclude that the *nirA* product is involved in the regulation of the transcript levels of *niiA* and *niaD* and is a limiting factor for their expression.

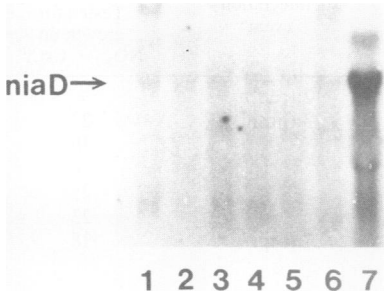

FIG. 4. The transcript level of *niaD* is reduced in *nirA* mutants. Northern analysis was of poly(A) RNA prepared from strains carrying *nirA1* (lane 1), *nirA87* (lane 2), *nirA601* (lanes 3 to 6), and wild type (lane 7) grown under induced conditions (see the legend to Fig. 3). A 2.8-kbp DNA fragment comprising the entire *niaD* gene was amplified by polymerase chain reaction from plasmid pucBam12 and used as a probe. The amount of membrane-bound poly(A) RNA was similar in all lanes. It was quantified by hybridization with an actin-specific probe (see the legend to Fig. 3).

DISCUSSION

The evidence presented here proves that we have cloned the *nirA* gene, which is at most 5.9 kbp in length. Its

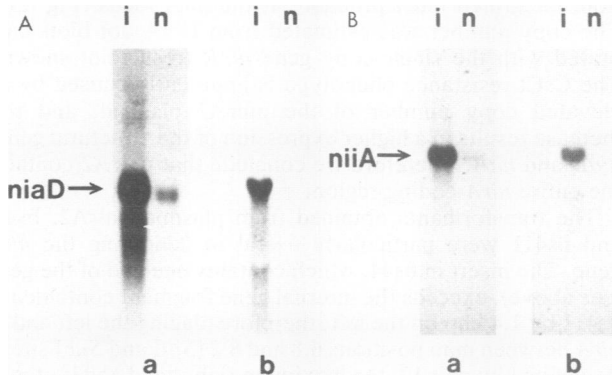

FIG. 5. The transcript levels of *niaD* and *niiA* are elevated in the *nirA* multicopy transformant Tp22. Northern analysis was of poly(A) RNA prepared from Tp22 and wild type grown under induced (lanes i) and noninduced (lanes n) conditions (see the legend to Fig. 3). (A) Blots were probed with an *niaD*-specific DNA fragment (see the legend to Fig. 4). (B) The same blots as in panel A were stripped and then probed with an *niiA*-specific DNA fragment, i.e., the 1.6-kbp *Sal*I-EcoRI fragment isolated from pAN501. Lane a, *nirA* multicopy transformant Tp22; lane b, wild type. The amount of membrane-bound poly(A) RNA was similar in each lane and was quantified by hybridization with an actin-specific probe (see the legend to Fig. 3).

transcript is approximately 4 kb long and very scarce in the wild type. The *nirA* product regulates the transcript level of the *niaD* and *niiA* structural genes. Further work will determine whether it acts directly as a transcription factor.

nirA transcription is apparently not induced by nitrate. According to our data, the simplest regulation mode is the direct activation of the putative *nirA* protein by nitrate or nitrite. A more complex hypothesis, proposed on the basis of genetic and physiological studies of various mutants, implies interaction of the *nirA* gene product with nitrate reductase (see references 5 and 25 and references therein). The cloned *nirA* gene of *A. nidulans* will now permit us to investigate at the molecular level the mechanism of nitrate induction and its interplay with ammonium repression, i.e., the possible interactions of the *nirA* gene product with nitrate, nitrite, nitrate reductase, and the *areA* protein.

Recently, cloning of *nit-4*, the pathway-specific regulatory gene mediating nitrate induction in *N. crassa*, has been reported, but the presented data suggest a smaller gene than that in *A. nidulans* (12). DNA sequencing is presently in progress to clarify whether *nirA* and *nit-4* of these two ascomycetes are indeed homologous.

Mutants exhibiting a NirA$^-$-like phenotype have been also isolated in *Fusarium oxysporum* (5b). According to recent molecular data, this imperfect fungus is closely related to members of the class *Pyrenomycetes* and is taxonomically as distant from *A. nidulans* as *N. crassa* is from *A. nidulans* (13a). We have succeeded in complementing a *Fusarium nirA* mutant with the *A. nidulans nirA* gene, indicating a strong conservation of the regulatory gene products despite the evolutionary distance between members of the classes *Plectomycetes* and *Pyrenomycetes* (5b).

ACKNOWLEDGMENTS

We thank B. Lang (Montreal) for continuous discussions and helpful suggestions concerning the experiments and the preparation of the manuscript; Nicole Baur (Montreal) for excellent technical help and for critically reading the paper; V. Sophianopoulou and G. Diallinas (Orsay) for their support in Northern experiments; and T. Langin, L. Malardier, and B. Felenbok (Orsay) for providing the plasmids pucBam12, pAN501, and pALS, respectively.

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