Complementation of areA\(^-\) regulatory gene mutations of Aspergillus nidulans by the heterologous regulatory gene nit-2 of Neurospora crassa

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Abstract

Loss-of-function mutations in the regulatory gene areA of Aspergillus nidulans prevent the utilization of a wide variety of nitrogen sources. The phenotypes of nit-2 mutants of Neurospora crassa suggest that this gene may be analogous to the areA gene. Transformation has been used to introduce a plasmid containing the nit-2 gene into A. nidulans. The nit-2 gene of Neurospora complemented mutations in the areA gene, restoring the ability to use a variety of nitrogen sources. This indicated that the activator function of nit-2 and areA gene products was retained across these two fungal species. Southern blot analysis revealed both single-copy and multicycopy integrations and, in at least one case, integration appeared to generate a nit-2 mutation. Integration of the transforming plasmid appeared to be by nonhomologous events at a number of different sites in the Aspergillus genome. The transformants were less sensitive to nitrogen-metabolite repression of extracellular protease activity and nitrate reductase (EC 1.6.6.3) than were wild-type A. nidulans. This indicated that nitrogen control was not completely normal in the nit-2 transformants.

Gene transfer techniques have been used recently to investigate the regulation of heterologous genes in eukaryotes. In some cases evidence for essentially normal regulation has been found, for example, in transgenic mice and tobacco (1–3). This implies that regulatory genes and their sites of action may be conserved during evolution. Here we show directly that a regulatory gene from one fungal species can function in another species.

Nitrogen-metabolite repression is a widespread regulatory phenomenon in microorganisms. Studies with fungi have revealed a complex situation in which mutations in a variety of genes alter the response of the cell to changes in nitrogen status (4, 5). In Aspergillus nidulans, previous work has suggested that the areA gene plays a central role in nitrogen control. The areA gene product has been shown to be a protein (6), and mutants are available that result in loss of function, altered activator specificities, or derepression (7–9). Loss-of-function (areA\(^-\)) mutants have lost (partially or totally) the ability to use nitrogen sources other than ammonium, whereas mutations that affect areA specificities result in allele-specific alterations of growth responses on a variety of nitrogen sources. The properties of these mutants indicate that the areA protein controls the expression of unlinked structural genes by binding at a recognition site to activate their expression. The derepressed class of areA mutants comprises two mutations generated by chromosomal rearrangements (5), and, therefore, their status in relation to areA function is unclear.

Studies in Neurospora crassa have identified the nit-2 gene, which is postulated to work in an analogous fashion to the areA gene. The nit-2 gene has been characterized by loss-of-function mutations, and although derepressed alleles have been sought, none have been isolated (4). The isolation of nonsense suppressible nit-2 mutations indicates that the nit-2 gene product is a protein (10). Studies in both organisms have pointed to glutamine as the key effector of nitrogen-metabolite repression (4, 5).

Given the apparent functional similarity between the areA gene of A. nidulans and the nit-2 gene of N. crassa, we have used transformation studies to determine whether the nit-2 gene product could replace the function of the areA gene in areA\(^-\) mutants of A. nidulans. Our results show that the nit-2 gene complements areA\(^-\) mutations allowing transformant colonies to utilize a variety of nitrogen sources. Further analysis has shown that nitrogen-metabolite repression in the transformants differs from nitrogen control in the wild-type strain.

Materials and Methods

Strains, Media, and Genetic Methods. The areA\(^-\) strains used in transformation experiments were yaI areA2/7 prnA\(^-\) riboB2 or yaI areA9 pyroA4 nicB8. The areA2/7 and areA9 mutations have been described previously (8, 11). Transformants were crossed to the wild-type strain bial niiA4 and to a bial areA102 niiA4 strain. The wild-type strain used for nitrate reductase assays was bial1. For haploidization analysis, a pabA1 galA1 pyroA4 facA303 nicB8 prnC61 strain was used. All markers have been described previously (12). Genetic analysis was by standard techniques (13), and basic media have been described (14).

Transformation. Protoplasts were prepared and transformed as described by Tilburn and coworkers (15). Transformed protoplasts were plated on selective protoplast medium containing the appropriate nitrogen source as described under Results.

Nitrate Reductase Assay and Protein Determination. Nitrate reductase (EC 1.6.6.3) was assayed as described (14), with modified controls (16). Results are expressed as milliunits per milligram of soluble protein, where a unit is defined as the amount of enzyme catalyzing the reduction of 1 \(\mu\)mol of nitrate to nitrite per minute. Soluble protein was estimated by using the Bradford procedure (17) and commercially available reagents (Bio-Rad).

Molecular Methods. Isolation of Aspergillus DNA and Southern blot analysis were as described (18), except that the prehybridization solution contained 50% deionized formamide, 4 mM EDTA, 32 mM NaOH, 40 mM NaH\(_2\)PO\(_4\), 0.72 M NaCl, 1% NaDodSO\(_4\), 0.5% skim milk powder, and 0.05 mg of denatured herring sperm DNA per ml. Hybridization solution contained 47% deionized formamide, 3 mM EDTA, 24 mM NaOH, 30 mM NaH\(_2\)PO\(_4\), 0.54 M NaCl, 1% NaDod-
SO₄, 0.5% skim milk powder, and 10% dextran sulfate. Nitrocellulose filters were hybridized with ³²P-labeled nick-translated pNIT2 plasmid for 18 hr at 42°C and were washed at 65°C under stringent conditions (18).

RESULTS

Transformation of areA⁻ Mutants. The nit2⁺ gene of N. crassa was cloned on a cosmid that carried an insert of at least 40 kilobases (kb) of Neurospora DNA (19). An EcoRI fragment of ≈6 kb was subcloned into the EcoRI site of plasmid pUC8 to make pNIT2 (G. Marzluf, personal communication). pNIT2 was transformed into several different areA⁻ mutants of A. nidulans. The Neurospora gene was found to complement the areA217 mutation (8) and the areA19 mutation (11), both of which prevent the utilization of most nitrogen sources by A. nidulans. Transformants were obtained by direct selection for growth on either nitrate or glutamate as sole nitrogen sources. In addition, the pNIT2 plasmid was introduced into an areA217 strain by cotransformation with a second selectable plasmid. The cotransforming plasmids used were pAN22 (20), containing the genes of the prn cluster, which allowed selection of transformants on proline as a sole nitrogen source in the areA217 prnΔ309 strain or p3SR2 (18), containing the amdS gene, which allowed selection of cotransformants on acetamide as a sole nitrogen source. Transformants obtained by proline or acetamide selection grew on a variety of nitrogen sources. Control transformation experiments in which areA217 strains were transformed with either pAN22 or p3SR2 alone yielded transformants able to grow only on proline or acetamide, respectively. These transformants retained an areA217 phenotype on all other media.

Growth Properties of pNIT2 Transformants. A more detailed analysis was undertaken on pNIT2 transformants of an areA217 strain obtained by direct selection for growth on nitrate as a sole nitrogen source. The twelve transformants chosen were representative of the phenotypic classes obtained in the transformation experiment. The growth properties of these strains on a variety of media are given in Table 1, and examples are shown in Fig. 1. In the majority of cases, the nit-2-containing transformants showed growth properties resembling those of the wild type (areA⁺) such as strong growth on nitrate, glutamate, and uric acid and poor growth on nitrogen sources such as histidine and acetamide, which are used poorly by wild type A. nidulans. Slight differences in growth or colony morphology distinguished the transformants from the wild-type strain. By contrast, three transformants, TNIT7, TNIT20, and TNIT22 showed a restoration of growth only on particular nitrogen sources in addition to nitrate (Table 1). The TNIT22 strain was the only one that did not grow as strongly as the wild type on nitrate as a nitrogen source. Additional growth tests showed that TNIT1 and TNIT22 were temperature sensitive for nitrate utilization at 42°C.

Southern Blot Analysis. Each of the transformants produced a unique Southern blot pattern in EcoRI and Pvu II digests of genomic DNA probed with nick-translated pNIT2 plasmid (Fig. 2 A and B). A preliminary restriction map of the pNIT2 plasmid (G. Marzluf, personal communication) is shown in Fig. 2C. Evidence from Southern analysis indicated that the pNIT2 plasmid was present either in a single copy (TNIT1, 7, 30, and 32) or in tandem arrays, with or without additional rearrangements in multicopy transformants (TNIT3, 5, 6, 19, 20, 27, and 28). Multicopy tandem integrations were shown by the presence of plasmid-sized bands in both digests. The TNIT22 transformant appeared to contain multicopies of a rearranged plasmid. In single-copy transformants, a plasmid-sized band is replaced by different bands generated by integration into the Aspergillus genome. From the EcoRI digests, integration was found to be via the insert sequences of the pNIT2 plasmid in TNIT1, TNIT7, and TNIT32 and via vector sequences in TNIT30. Pvu II digests showed that in TNIT30, integration was adjacent to the insert. Preliminary data (not shown) suggest that the TNIT7 transformant was generated by an integration event in the small Pst I/Pst I fragment of the pNIT2 plasmid (see Fig. 2C). The unusual phenotype of this transformant suggested that the nit-2 gene extends into this region and that the integration event has generated a novel nit-2 "allele," possibly with an altered 3' sequence. It is likely that the rearrangement of the plasmid in TNIT22 also generated an altered nit-2 sequence.

Genetic Analysis. The pattern of integration of the pNIT2 plasmid into the Aspergillus genome in the transformants was

### Table 1. Growth properties of transformants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Nitrate</th>
<th>Glutamate</th>
<th>Uric acid</th>
<th>Formamide</th>
<th>Histidine</th>
<th>Acetamide</th>
<th>Milk</th>
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Growth was scored relative to the wild-type strain on each medium. The symbols used range from +++ (strong growth) to − (negligible growth). Scoring across media is not strictly comparable. Milk clearing was scored on a numerical system from 0 (no detectable clearing) to 8. Plates containing skim milk protein were scored both for growth (A) and milk clearing (B). A halo of milk clearing was used as an indicator of extracellular protease levels (9). All growth tests were carried out at 37°C on glucose minimal media with the exception of acetamide plates that contained sucrose as the carbon source. Nitrogen sources were used at a concentration of 10 mM except skim milk protein, which was added at a final concentration of 1%.
investigated by genetic analysis. The phenotypic properties of the transformants (Table I) were found to segregate together in the progeny of outcrosses to a wild-type strain. The appearance of areA217-type progeny in the outcrosses suggested that the plasmid had not integrated into the areA gene in any of the transformed strains. However, in the majority of cases where the growth properties of the transformants were similar to those of the wild-type strain, it was not possible to reliably identify areA+ nit-2 recombinant progeny from the parental areA217 nit-2 types. Therefore, plasmid instability through meiosis generating the areA phenotype could not be excluded in these crosses. Mitotic stability was found to be high for the transformants, although multicopy transformants occasionally produced areA− sectors under nonselective conditions. Haploidization analysis confirmed the nonhomologous integration sites for some of the transformants; TNIT1, TNIT7, and TNIT19—chromosome VII, TNIT20—chromosome VIII, TNIT32—chromosome IV. Only TNIT22 was found to carry an integration on the same chromosome as the areA gene (III); however recombinational studies showed that the integration was unlinked to the areA locus.

The progeny of the cross between TNIT1 and the wild-type strain further demonstrated the complementarity between the nit-2 and areA gene products. The temperature sensitivity of the TNIT1 “allele” meant that at 37°C, but not at 42°C, the nit-2 gene product allowed areA− strains to utilize nitrate. In recombinant progeny containing the areA+ allele, the deficiency in the TNIT1 nit-2 product at 42°C was, in turn, compensated for by a functional areA product, allowing growth on nitrate. Crosses were also made between some transformant strains and strains carrying another areA mutation, areA102 (21). The areA102 mutation results in an areA product with altered specificities leading to increased growth on acetamide and acrylamide and reduced growth on uric acid as sole nitrogen sources compared with the wild-type strain. In progeny of cross between TNIT1, TNIT3, TNIT7, or TNIT19 and areA102 strains, the double mutants had regained the ability to use uric acid but were slightly weaker on acetamide or acrylamide than the areA102 strain alone. It is possible that the phenotype of these double mutants is indicative of an interaction between the areA and nit-2
products or competition at their site of action. In all crosses, the milk clearing phenotype of the transformants was retained independent of the status of the areA gene (areA17, areA", or areA102).

Nitrogen Metabolite Repression Control. As evidenced by the growth properties of most of the transformants, the nit-2 gene product of *N. crassa* has similar affinities for a variety of *Aspergillus* structural genes as does the areA gene. However, one distinguishing property of many of the transformants was an increased level of extracellular protease production compared to wild-type *A. nidulans* (Table 1). Plate tests also indicated that these transformants produced substantial, although reduced, levels of milk clearing in the presence of either ammonium or glutamine, conditions under which extracellular protease activity is not detectable in the wild type. This is illustrated in Fig. 3. Extracellular protease production is under areA control in *Aspergillus* and is abolished in areA" mutants. The phenotypes of the transformants suggested that the nit-2 gene product may promote a greater level of expression than the areA gene product and, more significantly, may not be as sensitive to the signals for repression.

To further quantify differences between the areA and nit-2 gene products, the nitrate reductase enzyme (EC 1.6.6.3) was assayed in the transformants under repressed and derepressed conditions (Table 2). The results indicated clearly that all nit-2-containing strains were partially derepressed for the synthesis of the nitrate reductase enzyme in the presence of either ammonium or glutamine. Furthermore, the induced levels were also found to be higher (up to 2-fold) in the transformants than in the wild-type strain. The high nitrate reductase levels of the TNIT22 strain suggested that the poorer growth of this strain on nitrate must be due to effects at another step of nitrate utilization or other unknown metabolic defects. The elevated levels in the transformants were not due to altered inducibility. The uninduced nitrate reductase levels, as measured in alanine-grown mycelia, were comparable to the wild-type levels in all transformants tested (Table 2). Nitrate reductase levels were also assayed in derivatives of the TNIT1 and TNIT3 strains containing the areA" allele. The properties of these strains were indistinguishable from those of the original strains carrying the areA127 mutation (data not shown). The level of expression therefore appears to be a property of the nit-2 allele carried by the strain rather than any interaction with the resident areA allele.

**DISCUSSION**

Transformation of areA" strains with the pNIT2 plasmid carrying the nit-2 gene of *N. crassa* showed that the heterologous regulatory gene can be expressed in *A. nidulans* and that the regulatory protein functions to activate expression of nitrogen-regulated genes in this organism. The growth properties of the transformants suggest that the nit-2 gene product recognizes the appropriate regulatory sequences adjacent to the various structural genes in a way comparable to the native areA protein. The functional similarity between the regulatory proteins was also apparent where one product substituted for deficiencies in the other when present in the same nucleus. An example of this was described above where a functional areA product restored the ability of the temperature-sensitive TNIT1 strain to utilize nitrate at 42°C. Clearly there are differences in the apparent efficiencies with which the nit-2 product and areA products activate expression of many, if not all, nitrogen-regulated genes. However, relative affinities appear to be similar for both regulatory gene products.

It appears that the similarities between these products can be disturbed if the integration event involves the integrity of the nit-2-coding sequence. In these instances, the transformation event would appear to be generating nit-2 mutations in vivo. In this context, it is interesting to note that Perrine and Marzluf (10) were able to generate a nonsense mutation in the nit-2 gene that resulted in a truncated protein with altered affinity for a variety of *N. crassa* structural genes.

The ability of the nit-2 gene to complement areA" mutants suggests that there has been a strong conservation of conformation and functionality between these two regulatory proteins despite the evolutionary distance between the two
species. However, probing of total *A. nidulans* genomic DNA with the pNIT2 plasmid has revealed only weak homology that has been insufficient for use in screening an *Aspergillus* gene library. The possibility remains that there are small areas of sequence conservation that would be detected in direct hybridization between cloned *areA* and *nit-2* genes as has been reported for the *gdhA* and *am* genes of *A. nidulans* and *N. crassa* (22).

Plate tests and enzyme assays have provided direct evidence that, at least for those systems studied, the *nit-2* gene product leads to partial derepression of enzyme synthesis in *A. nidulans*, irrespective of whether a wild-type *areA* product is simultaneously produced. This is suggestive that the *areA* product does not assume a repressor function under repressing conditions. The interpretation of the *nit-2* result is difficult, as the mechanism by which repression is achieved has not been established. In the presence of metabolites derived from growth on ammonium or glutamine, the *areA* product may assume a conformation incompatible with activator function, or, alternatively, the synthesis of the *areA* product itself may be curtailed by a means of autoregulation or the intervention of a second regulatory product that controls *areA* synthesis. Given this level of uncertainty, a number of possibilities exist. The *nit-2* product may not register the signal for the onset of repression conditions. If this signal is received via direct interaction between the *nit-2* or *areA* product and a low molecular weight effector, there is no reason to assume that this signal would not be recognized in *Aspergillus* as it is in *Neurospora*, unless the *nit-2* product itself affects the distribution of this metabolite. Similarly, if the *nit-2* gene is autoregulated, there is no obvious reason why such control should not be retained when the intact gene (see below) is expressed in *Aspergillus*. The involvement of an additional hierarchy of regulatory gene(s) controlling the synthesis of *nit-2* product could therefore be suggested. This would imply that such regulatory genes are either absent from *Aspergillus* or do not interact correctly with the *nit-2* gene or *nit-2* gene product. Mutations in the *Neurospora* gene defined by the *nmr-1* and *ms-5* mutations may identify such a higher-order regulatory locus (ref. 23; G. Marzluf, personal communication). A further alternative is that the partial derepression of enzyme synthesis in the transformants may indicate that the pNIT2 plasmid does not contain the entire *nit-2* gene and that regions adjacent to the structural gene are necessary for the regulated expression of the *nit-2* gene.

The finding that the heterologous regulatory gene can be expressed and can function in *A. nidulans* has opened up new possibilities of studying the activation and repression of enzyme synthesis. The properties of the pNIT2 transformants may allow these aspects to be considered separately, an approach that is not easily taken with the currently available *areA* mutants in which derepression is associated with gross chromosomal rearrangements. The *xprDI* mutation, for example, is thought to have arisen by an inversion event which has placed the *areA* gene under the control of a new promoter (24). Such gross alterations make the function of the *areA* gene in nitrogen metabolite repression more difficult to dissect. Clearly much remains to be understood about how cells register their nitrogen status and how this is translated into effective activation or repression of a variety of unlinked structural genes subject to nitrogen control. The finding that the cloned *nit-2* gene of *N. crassa* can complement mutations in the *areA* gene of *A. nidulans* will provide an additional approach to gaining this understanding.

Transformation in *Aspergillus*, as in *Neurospora* and mammalian systems, is integrative and is not strongly dependent on homology (25). Although the transformants described here appear to be generated by such nonhomologous events, it is possible that transformants could be obtained in which the integration event generates a hybrid *areA–nit-2* gene. Furthermore, the *areA* gene has recently been cloned (26), allowing a detailed comparison of these genes to be undertaken as well as reciprocal experiments to those reported here in which expression of the *areA* gene in *N. crassa* is studied. Such approaches will provide valuable information about the conservation of regulatory sites and indicate sequences of functional importance to nitrogen control of gene expression by trans-acting regulatory molecules.

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