The Formamidase Gene of *Aspergillus nidulans*: Regulation by Nitrogen Metabolite Repression and Transcriptional Interference by an Overlapping Upstream Gene

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ABSTRACT

The ability to utilize formamide as a sole nitrogen source has been found in numerous fungi. We have cloned the *fmdS* gene encoding a formamidase from *Aspergillus nidulans* and found that it belongs to a highly conserved family of proteins separate from the major amidase families. The expression of *fmdS* is primarily regulated via AreA-mediated nitrogen metabolite repression and does not require the addition of exogenous inducer. Consistent with this, deletion analysis of the 5′ region of *fmdS* has confirmed the presence of multiple AreA-binding sites containing a characteristic core GATA sequence. Under carbon starvation conditions the response to nitrogen starvation is eliminated, indicating that the lack of a carbon source may result in inactivation of AreA. Sequence analysis and isolation of cDNAs show that a gene of unknown function lies directly 5′ of *fmdS* with its transcript overlapping the *fmdS* coding region. Disruption of the 5′ gene and analysis of the effects of overexpression of this gene on *fmdS* expression has shown that expression of this upstream gene interferes with *fmdS* transcription, resulting in a strong dependence on AreA activation for expression. Therefore the relative position of these two genes is essential for normal regulation of *fmdS*.

The hydrolysis of amides via amidase activities has been characterized in the filamentous fungus *Aspergillus nidulans* (Hynes 1975a; Hynes and Pateman 1970). A wide variety of amides can be hydrolyzed via a number of distinct enzyme activities (Hynes 1975a). The acetamidase enzyme encoded by the *amdS* gene allows growth on acetamide as a carbon and/or nitrogen source by producing ammonium and acetate. Regulation of *amdS* expression is complex, involving multiple induction signals as well as control by carbon and nitrogen metabolites (for a review, see Hynes and Davis 1996). The general amidase encoded by *gmdA* contributes to growth on longer chain amides, producing ammonium and the corresponding carboxylic acid (Hynes 1975a). A formamidase enzyme (formamide amidohydrolase, EC 2.5.1.49) mediates the highly specific hydrolysis of formamide to produce formate and ammonium (Hynes 1975a). Formamide therefore serves only as a nitrogen source as formate is a single carbon molecule and hence not a carbon source for *Aspergillus* spp. The *fmdS* gene on chromosome III was defined by isolation of the formamide nonutilizing *fmdS*1 mutant lacking detectable formamidase activity (Hynes and Pateman 1970).

In filamentous fungi the activities of many nitrogen catabolic enzymes, including the amidases, have been shown to be highly regulated in response to nitrogen levels in the cell through nitrogen metabolite repression, which results in the preferential utilization of the nitrogen sources ammonium and L-glutamine (for a review, see Marzluf 1997). In *A. nidulans* the positively acting product of the *areA* gene is the major regulatory factor involved in this control mechanism (Arst and Cove 1973; Kudla et al. 1990). Loss-of-function *areA* mutants are unable to grow on most nitrogen sources other than ammonium, including formamide (Hynes 1972; Arst and Cove 1973; Hynes 1975b). In the absence of a preferred nitrogen source this GATA zinc-finger-type DNA-binding protein recognizes and activates expression through the consensus sequence HGA TAR in the promoters of over 100 genes encoding permeases and enzymes required for the catabolism of secondary nitrogen sources (Wilson and Arst 1998).

Regulation by AreA gives rise to a hierarchy of gene expression, with the expression of some genes being more highly activated than others. Mutations within the DNA-binding domain of AreA have been isolated that alter this hierarchy. The *areA*102 (L683V) mutation gives rise to a product that has a higher affinity for TGA TAR sites, allowing stronger growth on acetamide and reduced growth on uric acid (Hynes 1972, 1975b; Kudla et al. 1990; Ravagnani et al. 1997). Analysis of the *amdS* promoter has revealed the presence of TGA TAR sites (Corrick et al. 1987), while the promoters of the purine permeases contain primarily CGATA TAR sites (Gorfinkel et al. 1993; Diallinas et al. 1995).
Mirror-image mutants have been isolated (L683M) that show an opposite-yet-weaker phenotype with better growth on substrates dependent on transcription of genes whose promoters contain CGATAR sites (Ravagnani et al. 1997).

The expression of areA itself is controlled by auto-regulation and by regulation at the mRNA stability level, with the areA transcript half-life being significantly reduced under nitrogen-sufficient conditions through an element in the 3′ untranslated region (UTR) of the transcript (Langdon et al. 1995; Platt et al. 1996a). The activity of AreA is also affected by the NmrA protein (Andrianopoulos et al. 1998). In Neurospora crassa, the NmrA homologue NMRI has been shown to be required for nitrogen metabolite repression through interaction with the zinc finger and 12 carboxyl-terminal residues of the AreA homologue NIT2 (Dunn-Coleman et al. 1981; Xiao et al. 1995). Mutations affecting nmr-1 or altering the interacting residues of NIT2 result in nitrogen-derepressed phenotypes. The relevant regions of both AreA and NmrA are highly conserved in A. nidulans, suggesting both organisms share this regulatory mechanism (Platt et al. 1996b; Andrianopoulos et al. 1998). Deletion of the C-terminal amino acids of AreA thus partially relieves nitrogen metabolite repression while almost complete relief of repression is obtained when the stability-mediating element in the 3′ UTR of the areA transcript is also mutated (Platt et al. 1996a).

In addition, the product of the tamA gene is believed to act as a coactivator of AreA function and to contribute to the activation of a subset of nitrogen metabolite repression regulated genes and has also been shown to interact with the carboxyl terminus of AreA (Davis et al. 1996; Small et al. 1999). The formamidase of A. nidulans was previously shown to be strongly regulated by AreA-dependent nitrogen metabolite repression (Hynes 1972, 1975b). No evidence for induction by exogenous formamide was found and furthermore the fmdS transcript half-life being significantly reduced. The fmdS transcript is also mutated (Platt et al. 1996a).

Using published sequences for formamidases from Methylphilus methylotrophus and Mycobacterium smegmatis we have identified an A. nidulans expressed sequence tag (EST) sequence with high similarity. This has enabled us to characterize the structure and regulation of the fmdS gene of A. nidulans.

## MATERIALS AND METHODS

### A. nidulans strains, growth media, and transformation:

Strains used in this study are shown in Table 1. Growth media and conditions were as described by Cove (1986). Nitrogen sources were used at a final concentration of 10 mM and carbon sources at 1% w/v unless stated otherwise. Mycelia for assays were grown at 37°C for 16 hr, washed with carbon-free media, and transferred to the assayed growth condition for 4 hr. Genetic analysis was carried out using techniques as described by Clutterbuck (1974). A. nidulans protoplast preparation and DNA transformation was performed using the method of Andrianopoulos and Hynes (1988). Arabidopsis thaliana media was prepared as described in Cobbett et al. (1998) with nitrogen sources added at 5 mM. Growth tests used the Columbia ecotype. Schizosaccharomyces pombe Edinburgh minimal media was prepared as described in Alfa et al. (1993) with nitrogen sources added at 100 mM.

### Molecular techniques:

Standard methods were as described by Sam brook et al. (1989). Restriction enzymes and Mung Bean Exonuclease (Promega, Madison, WI) were used with the supplied buffers. DNA fragments were purified from agarose gels using the BresaClean DNA purification kit (Gene) while almost complete relief of repression is obtained when the stability-mediating element in the 3′ UTR of the areA transcript is also mutated (Platt et al. 1996a).

### Plasmid construction:

The 7-kb BamHI and 3.5-kb Ccl fmdS-hybridizing fragments from cosmid L19H02 were cloned into pBluescript SK+ to create pJAF4136 and pJAF4510, respectively. A minimal fmdS subclone was created by subcloning the 1.7-kb Scal fragment from pJAF4136 into pBluescript SK+ cut

### PCR protocols:

Sequences of primers referred to in the text are as follows:

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
</tr>
</thead>
</table>
| BTUB2      | GCC CAG CAC CCA CCA AC | 5′ rapid amplification of DNA ends (RACE) was performed using the 5′ RACE System v2 kit (Gibco BRL, Gaithersburg, MD) with the nested gene specific primers fmdSP1 and fmdSP4, using total RNA from A. nidulans strain MH1 collected after transfer to nitrogen starvation, carbon-sufficient conditions. Semiquantitative RT-PCR was performed as described in Ha et al. (1999) using the Superscript One-step RT-PCR system (Gibco BRL). A total of 22 PCR cycles were used for the benA control (BTUB2 and BTUB3), 26 for usgS (USGT1 and USGT2), and 28 or 30 for fmdS (fmdSrt1 and FMD2) with all reactions stopped while in their linear phase.

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### TABLE 1:

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
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<tbody>
<tr>
<td>MH1</td>
<td>biA1</td>
</tr>
<tr>
<td>MH50</td>
<td>yA1 areA102 riboB2 pyroA4</td>
</tr>
<tr>
<td>MH341</td>
<td>yA1 riboB2 su-adE20 adE20areA217</td>
</tr>
<tr>
<td>MH3018</td>
<td>yA1 pabaA1 argB2</td>
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<tr>
<td>MH8375</td>
<td>yA1 riboB2 fmdS1 alX</td>
</tr>
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<td>MH9467</td>
<td>yA1 areA102 pyroA4 usgS(pJAF4473)</td>
</tr>
<tr>
<td>MH9469</td>
<td>yA1 areA102 pyroA4 usgS(pJAF4472)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genotype</th>
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</thead>
<tbody>
<tr>
<td>biA1</td>
</tr>
<tr>
<td>yA1 areA102 riboB2 pyroA4</td>
</tr>
<tr>
<td>yA1 riboB2 su-adE20 adE20areA217</td>
</tr>
<tr>
<td>yA1 pabaA1 argB2</td>
</tr>
<tr>
<td>yA1 riboB2 fmdS1 alX</td>
</tr>
<tr>
<td>yA1 areA102 pyroA4 usgS(pJAF4473)</td>
</tr>
<tr>
<td>yA1 areA102 pyroA4 usgS(pJAF4472)</td>
</tr>
</tbody>
</table>

Gene symbols are as described in Clutterbuck (1974).
with EcoRV to create pJAF4155. usgS inactivation plasmids were created by inserting a 2.4 kb BglII/SmaI riboB fragment from pPLI (Oakley et al. 1987b) into pJAF4510 digested completely with BglII and partially with HinII. The HinII site at -121 was used to create pJAF4472 and the HinII site at -1023 to create pJAF4473. Nucleotide positions are with reference to the fmdS ATG at +1. usgS cDNA clones were isolated from a AZAP library constructed by Dr. Rodolfo Aramayo at Texas A&M University, which used RNA from a mixed vegetative and 24-hr asexual development culture of A. nidulans strain FGSC A26. Plaque lifts were probed with the 3.3-kb ClaI fragment from pJAF4510. Clones were excised as described in Stort et al. (1988).

All fmdS::lacZ constructs were created through fusion of fmdS at the BglII site at +472 in frame to the BamHI site in the lacZ reporter construct pSS524. pJAF4241 was constructed using the XhoI site at -599 bp, inserting into pJS3524 cut with XhoI and BamHI. pJAF4504 was created by inserting the 2.5-kb XhoI fragment, including the rest of usgS, into the XhoI site of pJAF4241. pJAF4242 and pJAF4243 were created by digesting pJS3524 with KpnI, blunting with mung bean endonuclease, cutting with BamHI, and inserting fmdS fragments starting at the EcoRV site at -123 and the Scal site at -62, respectively. The remaining fmdS fusion constructs were created by introducing a KpnI site via PCR within the promoter that was used (in conjunction with the BglII site) to clone the sequenced PCR products into pJS3524. All PCR reactions used primer FMD2 in conjunction with a construct-specific primer. Constructs included pJAF4503 (primer FM1lac1), pJAF4502 (FMlac2), pJAF4501 (FMlac3), pJAF4500 (FMlac4), and pJAF4499 (FMlac5). A similar approach was taken to create the usgS::lacZ construct pJAF4622, using primer USG1 to introduce a KpnI site at -1303 and USG2 to create a BamHI site at +144 relative to the usgS start codon, with this fragment sequenced and subcloned into pJS3524.

The usgS overexpression plasmids were made using a three-step cloning procedure. The usgS overexpression plasmid pJAF4869 was generated by inserting a 400-bp EcoRI/BamHI aca promoter fragment from pAL3 (Fillinger et al. 1995) into pJAF4510 digested with EcoRI/BamHI. A 1.4-kb EcoRI/BamHI pycG-containing fragment from pARB4342 was then inserted into EcoRI/BamHI-digested pJAF4869 to give pJAF4870. The gene replacement plasmid pJAF4871 was created by inserting the 3.8-kb BamHI/ClaI fragment from pJAF4870 into the usgS subclone pJAF4510 digested fully with BglII and partially with ClaI.

lacZ reporter gene assays: Reporter gene studies used strains carrying a single copy of the relevant construct in single copy at the argB locus as described by Punt et al. (1990). β-Galactosidase assays were carried out by the method of Davis et al. (1988).

Nucleotide sequence accession number: The sequence for the fmdS and usgS genes has been deposited in GenBank under accession no. AF274009.

RESULTS

Cloning of fmdS: A BLAST search performed on the University of Oklahoma A. nidulans EST database (http://www.genome.ou.edu/asper.html) revealed a sequence (EST c8h05a1.r1) with a high level of similarity to the formamidases of the bacteria M. methylotrophus and M. smegmatis (Mahenthiralingam et al. 1993; Wyborn et al. 1996). Primers FMD1 and FMD2 based upon the EST sequence were used to amplify a 317-bp product from A. nidulans genomic DNA, which was then used to probe colony lifts of a chromosome III specific cosmid library (Brody et al. 1991). Two hybridizing cosmids were identified, L19H02 and L20G07. Both cosmids were shown to complement the A. nidulans fmdS mutant (MH8375) in transformants directly selected on formamide as the sole nitrogen source. Neither of these cosmids lay within the region of the ordered minimal library indicated to contain the fmdS region of the chromosome (Prade et al. 1997), nor were these cosmids adjacent to each other.

A 7-kb BamHI fragment capable of complementing the fmdS mutation was subcloned from cosmid L19H02. Sequencing 3.5 kb of the end of the clone to which the PCR fragment hybridized in Southern blots revealed that the fmdS gene contained four introns that were confirmed by cDNA sequence. Complementation of the fmdS mutation with the minimal 1.7-kb Scal subclone (pJAF4155) confirmed the function of this sequence.

Two major startpoints of fmdS transcription within the fmdS promoter region were identified in multiple independent clones generated by 5’ RACE. The startpoints of transcription (at -21 and -12 relative to the predicted ATG) corresponded to the existence of two possible TATA boxes (both with the sequence TTA-

fmdS belongs to a highly conserved gene family: The predicted 45-kD 411-amino-acid FmdS does not share significant similarity with AmdS (Corrick et al. 1987) but has 56% identity and 77% similarity to the formamidase of M. methylotrophus, with conservation across the entire protein. Similarity to the M. smegmatis enzyme is lower (66%), with no homology at the carboxyl terminus (Figure 1). Database searches using the predicted FmdS protein sequence identified previously uncharacterized highly conserved formamidase-like sequences in the genomes of prokaryotic, eukaryotic, and archaeal species (Figure 1). Full sequences have been isolated as part of the genome sequencing projects of Bordetella pertussis, B. bronchiseptica, Aeropyrum pernix, S. pombe, Candida albicans and two (in tandem) from A. thaliana. Tests on defined media showed that S. pombe could use formamide as a sole nitrogen source and that formamide enhanced growth of A. thaliana relative to growth in the absence of added nitrogen source, indicating that these formamidase sequences are likely to be functional. Incomplete sequences were also identified as ESTs from the plant species Oryza sativa (accession nos. C72046, D49000, D49028, and D46854), Zea mays (AI714639 and AI739746), Maligica truncatula (AW256497), Lotus japonicus (AW720090, AW720252, and AW719264), Sorghum bicolor (AW564921), and Lycopersicon esculentum (AI782257). All of these proteins are distinct from the two major amidase families (the nitrilase and amidase...
Regulation of \textit{fmdS} by nitrogen metabolite repression:

Previous studies of the regulation of formamidase activity showed a lack of induction by formamide yet strong regulation by nitrogen metabolite repression (Hynes 1970, 1972). In an attempt to localize the promoter elements responsible for this response, an \textit{fmdS}\textsubscript{::lacZ} promoter deletion series was constructed and integrated in single copy at \textit{argB} as described by Punt \textit{et al.} (1990). Each strain was assayed under growth conditions of nitrogen and carbon starvation or sufficiency (Figure 2). The longest \textit{fmdS}\textsubscript{::lacZ} fusion (pJAF4241, with 599 bp of \textit{fmdS} promoter) displayed a 10-fold increase in expression during nitrogen starvation. Four potential AreA recognition sequences (HGATAR) were identified within the \textit{fmdS} promoter, and the deletion series was used to determine the relative contribution of each site. The sites at −70 (TGATAG), −145 (CGATAA) and −160 (AGATAA) relative to the predicted \textit{fmdS} start codon all contribute to \textit{fmdS}\textsubscript{::lacZ} expression under nitrogen-limiting conditions, with the site at −145 only playing a minor role. The site at −315 (TGATAA) appeared to have little effect on the regulation of \textit{fmdS}, with the deletion of this region actually corresponding to a slight increase in the basal levels of β-galactosidase activity, perhaps through the removal of other regulatory sequences (Figure 2).

The \textit{areA}217 loss-of-function allele abolished the response to nitrogen starvation for all constructs showing that the response was AreA dependent. Expression of \textit{fmdS}\textsubscript{::lacZ} was unaffected by the \textit{tamAΔ} mutation (results not shown) in agreement with previous studies (Arst and Sheerins 1996).

Effects of carbon starvation on \textit{fmdS} expression:
The absence of a carbon source led to loss of response to nitrogen starvation of \textit{fmdS}\textsubscript{::lacZ} expression (Figure 2). Earlier studies on formamidase levels showed a reduced response to nitrogen starvation in the absence of an added carbon source (Hynes 1972). This was observed with all fusion constructs, indicating that the effect was not due to a specific site in the \textit{fmdS} promoter. Reduced \textit{fmdS}\textsubscript{::lacZ} expression was observed only upon complete carbon starvation, with the poorer carbon sources, lactose, fructose, or limiting glucose (0.1% w/v), giving expression equivalent to 1% glucose (results not shown). In \textit{A. nidulans} carbon catabolite repression (CCR) is mediated by the zinc-finger repressor protein CreA (Dowzer and Kelly 1991). Analysis of the \textit{fmdS} promoter identified possible tandem CreA recognition sites.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{alignment.png}
\caption{Alignment of 10 predicted formamidase-type enzymes. Identical residues are indicated by dark shading; similar residues are indicated by light shading. The alignment was generated using Pileup (GCG software package, Devereaux \textit{et al.} 1984) and viewed with Boxshade (Bioinformatics group, ISREC). At1, \textit{A. thaliana} formamidase 1 (accession no. CAB38294); At2, \textit{A. thaliana} formamidase 2 (CAB38295); An, \textit{A. nidulans}; Ap, \textit{A. pernix} (BAA79495); Bb, \textit{B. bronchiseptica}; Bp, \textit{B. pertussis}; Ca, \textit{C. albicans}; Mm, \textit{M. methylotrophus} (Q50228); Ms, \textit{M. smegmatis} (Q07238); Sp, \textit{S. pombe} (CAB60014). Preliminary sequence data for \textit{C. albicans}, \textit{B. pertussis}, and \textit{B. bronchiseptica} was obtained from The Institute for Genomic Research website at http://www.tigr.org.}
\end{figure}
Regulation of the *fmdS* Gene of *A. nidulans*

**Figure 2**—Deletion analysis of *fmdS::lacZ* regulation. Reporter gene constructs with varying *fmdS* promoter lengths were targeted in single copy at the *argB* locus (Punt et al. 1990). Constructs were designed to sequentially remove potential regulatory elements (represented by the indicated symbols) with the expression of each construct assayed in *areA*⁺ and *areA*⁻ genetic backgrounds under different growth conditions. Mycelium was grown in 100 ml of 1% glucose and 10 mM ammonium tartrate medium at 37°C for 16 hr, washed with carbon-free/nitrogen-free media, and then transferred to the indicated growth media for 4 hr. When present after transfer, glucose was at 1% w/v. NH₄ indicates 10 mM ammonium tartrate; -N is nitrogen free. Values are given in units per minute per milligram of protein and represent the results of at least three separate experiments with standard errors shown.

Equivalent sites with almost identical flanking sequence are also seen in the *niaD* promoter (Johnstone et al. 1990). Hynes (1973) showed that nitrate reductase levels are also low under carbon starvation conditions. Introduction of the *creA*²₀⁴ loss-of-function allele led to a reduction of *fmdS::lacZ* expression although the response to AreA was still apparent (Table 2). Removal of the tandem CreA sites had no detectable effect on expression of the reporter construct (Figure 2).

A time course analysis of the effect of carbon starvation on *fmdS::lacZ* expression was performed (Figure 3). Following transfer of mycelia to carbon-sufficient (1% glucose), nitrogen-free media, a short phase of no detectable change in expression was followed by β-galactosidase levels increasing over a 3-hr period. Transfer of mycelia to carbon-free media at 2.5 hr led to a rapid decrease in the response, comparable to that caused by the protein synthesis inhibitor cycloheximide. This effect suggests that nitrate reductase levels are also low under carbon starvation conditions. Introduction of the *creA*²₀⁴ loss-of-function allele led to a reduction of *fmdS::lacZ* expression although the response to AreA was still apparent (Table 2). Removal of the tandem CreA sites had no detectable effect on expression of the reporter construct (Figure 2).

**Table 2**

<table>
<thead>
<tr>
<th>Mutant genotype</th>
<th>Glucose</th>
<th>Carbon free</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>NH₄</td>
<td>-N</td>
</tr>
<tr>
<td><em>areA</em>²¹⁷</td>
<td>9 ± 1</td>
<td>111 ± 7</td>
</tr>
<tr>
<td><em>creA</em>²⁰⁴</td>
<td>2 ± 0</td>
<td>31 ± 4</td>
</tr>
<tr>
<td><em>areA</em>²¹⁷</td>
<td>3 ± 0</td>
<td>5 ± 1</td>
</tr>
<tr>
<td><em>creA</em>²⁰⁴</td>
<td>2 ± 0</td>
<td>75 ± 7</td>
</tr>
<tr>
<td><em>areA</em>²¹⁷</td>
<td>13 ± 1</td>
<td>113 ± 11</td>
</tr>
<tr>
<td><em>creA</em>²⁰⁴</td>
<td>10 ± 2</td>
<td>12 ± 0</td>
</tr>
<tr>
<td><em>creA</em>²¹⁷</td>
<td>9 ± 1</td>
<td>111 ± 7</td>
</tr>
<tr>
<td><em>creA</em>²⁰⁴</td>
<td>2 ± 0</td>
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<tr>
<td><em>areA</em>²¹⁷</td>
<td>3 ± 0</td>
<td>5 ± 1</td>
</tr>
<tr>
<td><em>creA</em>²⁰⁴</td>
<td>2 ± 0</td>
<td>75 ± 7</td>
</tr>
</tbody>
</table>

A strain containing the *fmdS::lacZ* fusion plasmid pJAF4241 with 599 bp of *fmdS* promoter integrated at the *argB* locus was crossed into the relevant mutant genetic background. Assay conditions were as described in Figure 2. Wild-type (WT) and *areA*²¹⁷ data are reproduced from Figure 2.
was also observable irrespective of the time of transfer (data not shown). The nonmetabolizable glucose analogue 2-deoxyglucose (2-DOG) is sufficient at 0.5% w/v to cause CCR of *amdS* expression mediated by CreA (M. J. Hynes, unpublished results). Transfer of mycelia to carbon-free/nitrogen-free media containing 0.5% 2-DOG for 4 hr did not simulate carbon sufficiency and allow *fmdS::lacZ* expression (data not shown). The lack of effect of the creA204 mutation on the response to nitrogen starvation combined with the inability of 2-DOG to substitute for glucose implies that the loss of AreA control of *fmdS* expression is in response to a signal independent of carbon catabolite repression.

**Regulation of *fmdS* by AnCF:** The 5’ region of *fmdS* has a CCAAT sequence (−112) that was shown to bind the *A. nidulans* CCAAT-binding factor (AnCF; Papagiannopoulos et al. 1996; Steidl et al. 1999) by electromobility shift assay (data not shown). The same site has been shown to be required in an AnCF-dependent manner for the formation of a nucleosome-free region in the *fmdS* promoter (Narendja et al. 1999). A strain deleted for the *hapC* gene encoding one of the subunits of AnCF (Papagiannopoulos et al. 1996) displayed reduced growth on formamide as the sole nitrogen source. A *hapC*Δ background resulted in slightly reduced expression of the *fmdS::lacZ* fusion (Table 2). However, deletion of the CCAAT sequence had no noticeable effect on *fmdS::lacZ* expression (compare pJAF4472 with pJAF4499 in Figure 2). This could have been due to the deletion fortuitously bringing other regulatory sequences into the proximity of the *fmdS* promoter or more probably this is due to loss of *usgS* in this construct (see Discussion).

The 5’ end of *fmdS* contains an overlapping gene: Previous studies of formamidase activity indicated a reduction of *fmdS* expression in *areA*Δ strains. Exons are shown as shaded boxes (*usgS* dark, *fmdS* light). The presence of a divergent EST is indicated. Directions of transcription (arrows) are shown. B, BamHI; Bg, BglII; C, ClaI; H, HincII; S, ScaI; and X, XhoI. (B) *usgS* deletion constructs. Constructs allowed disruption of *usgS* through deletion of the first two exons (pJAF4472) and first three exons (pJAF4473) of *usgS* while still maintaining an uninterrupted *fmdS* promoter region. (C) Nucleotide and conceptual protein sequence of the area surrounding the *usgS/*fmdS intergenic region, showing *UsgS* residues 238–362 and *FmdS* residues 1–65. Putative *fmdS* TATA boxes are underlined and AreA recognition sites boxed. A CCAAT site shown to be required for the formation of a nucleosome-free region (Narendja et al. 1999) is doubly underlined. *UsgS* transcript polyadenylation sites are indicated with down arrows and *fmdS* transcriptional startpoints as determined by RACE with solid circles. Nucleotides are numbered with reference to the +1 at the start of the *fmdS* coding sequences. Lowercase letters indicate intron sequences.
sponding with a reduction in growth on formamide as a sole nitrogen source (Hyne 1972). However, expression of the fmdS::lacZ construct was equivalent to wild type in an areA102 mutant background (Table 2). To investigate this discrepancy further analysis of the fmdS promoter was undertaken. Sequence analysis and searches of the A. nidulans EST database revealed the existence of a gene within the promoter of fmdS transcribed in the same direction as the fmdS transcript. To sequence the entire gene an additional 1.3 kb of fmdS upstream sequence was obtained through cloning of a 3.3-kb ClaI fragment (pJAF4510) from cosmid L19H02.

Isolation of three independent cDNA clones confirmed that the upstream gene (usgS) contained four introns and encoded a predicted 362-amino acid highly hydrophobic 41-kD product (Figure 4A). The usgS/fmdS intergenic region (from the stop codon of usgS to the start codon of fmdS) is only 242 bp and the usgS transcript overlaps that of fmdS by up to 150 bp, terminating within either the first exon or first intron of fmdS (Figure 4C). Database searches with usgS revealed a single homologue of unidentified function as an EST from the fungus Botrytis cinerea (accession nos. CNS01D74 and CNS019CV). Application of a hidden Markov topology prediction algorithm (Tusnady and Simon 1998) indicated that usgS is likely to encode a six-transmembrane helix protein with a long extracellular C-terminal tail.

Characterization of a usgS deletion strain: Two disruption constructs of usgS were created by replacing either the first two (pJAF4472) or first three exons (pJAF4473) of usgS/inplasmid pJAF4510 with a riboB+ fragment from plasmid pPL1 (Oakley et al. 1987b; Figure 4B). The riboB+-containing fragment was inserted in reverse orientation to ensure that no novel usgS transcripts were generated. For each construct a linear ClaI fragment containing the usgS/riboB insert was used to transform an areA102; pyroA4; riboB2 strain (MH50), selecting for riboflavin prototrophy. No obvious morphological phenotype could be seen in any RiboB+ transformants. Southern blot analysis of 30 transformants for each construct revealed one disruptant for each containing the desired integration event at usgS (data not shown).

Plate tests were used to determine if loss of usgS function gave rise to an altered growth phenotype on different carbon or nitrogen sources. Apart from formamide utilization, no other phenotypes were detected. Growth on either 10 mM or 50 mM formamide was noticeably stronger, particularly when comparing the usgS/areA102 double mutant (MH4473) to the parental areA102 strain (MH50), which showed poor growth and reduced mycelial density on formamide as the sole nitrogen source (Figure 5). Phenotypes of disruptants generated with either of the deletion constructs were identical, indicating that the new phenotype was not due to the particular positioning of the riboB+ fragment. The usgSΔ mutation was introduced by genetic crosses into an areA+ background. usgSΔ areA+ (MH9510) colonies grew slightly better than wild type (MH1). Therefore loss of usgS results in increased formamide utilization in both areA102 and areA+ backgrounds.

The plasmid pJAF4510 bearing usgS was cotransformed with the pyroA+ plasmid pI4 (May et al. 1989) into the areA102 usgS strain. All cotransformants were phenotypically identical to the usgS strains. Southern blot analysis indicated that the usgS+ plasmid had integrated ectopically into the genome and the genomic usgS was still disrupted, showing that the effects of the usgSΔ could not be restored in trans (MH9487, Figure 5). To confirm the lack of a trans-acting regulatory role for the usgS product on fmdS expression, the usgSΔ was introduced into an fmdS::lacZ background and no change in fmdS::lacZ expression was observed (results not shown). These results are compatible with usgS having a cis-acting effect on fmdS expression.

Analysis of transcription of fmdS and usgS by semi-quantitative RT-PCR showed that the fmdS product increased with nitrogen starvation and that levels were lower with carbon starvation (in agreement with the data presented in Figure 2). The usgS transcript was present in high levels on ammonium and showed only a slight response to nitrogen starvation and no change in response to carbon starvation (Figure 6). The loss-of-function areA1217 allele resulted in levels of fmdS product not responding to nitrogen starvation. In agreement with usgS having a cis-acting effect, the fmdS product level was increased under both nitrogen limitation and sufficiency in the usgSΔ mutant. This response was most...
weakly regulated by AreA. No major effects of carbon starvation were evident.

Inclusion of the upstream gene in the usgS fmdS::lacZ construct (pJAF4504) led to a reduction in \( \beta \)-galactosidase levels under all growth conditions assayed compared to those observed for the construct lacking the entire usgS gene (pJAF4241, Figure 7). Under nitrogen-sufficient conditions expression of fmdS::lacZ was easily observed in the absence of usgS, whereas inclusion of the entire usgS gene reduced fmdS::lacZ expression to almost undetectable levels. The levels of usgS fmdS::lacZ considered in conjunction with the phenotype of the usgS strain on formamide suggested that transcription of the upstream gene was interfering with expression of fmdS. Despite little change in usgS::lacZ expression, expression of usgS fmdS::lacZ was highly dependent on an AreA-mediated response to nitrogen starvation (Figure 7). Inclusion of usgS therefore changed fmdS from having leaky expression to being tightly regulated by nitrogen metabolite repression.

**Alteration of usgS regulation affects fmdS expression:** The areA102 mutation was previously found to result in a \( \sim50\% \) of wild-type formamidase activity (Hynes 1972, 1975b). This was not found for the assays of fmdS::lacZ fusions lacking usgS (Table 2). The areA102 mutant background resulted in increased usgS::lacZ expression under conditions of nitrogen limitation (Figure 7), consistent with the usgS promoter containing three potential TGATAR AreA recognition sites (at \( -305, -676, \) and \( -821 \) relative to the usgS predicted start codon). When usgS was present in the fmdS promoter (as in pJAF4504) the presence of the areA102 allele resulted in an \( \sim50\% \) reduction in fmdS expression (Figure 7), consistent with the effects of areA102 on formamide utilization being due to increased usgS transcription that interferes with fmdS expression.

<table>
<thead>
<tr>
<th>Construct</th>
<th>( \beta )-galactosidase Activity</th>
<th>glucose</th>
<th>carbon free</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>areA allele</td>
<td>NH(_4)</td>
<td>-N</td>
</tr>
<tr>
<td>pJAF4241</td>
<td>fmdS::lacZ</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>9 ± 1</td>
<td>111 ± 7</td>
</tr>
<tr>
<td></td>
<td>102</td>
<td>13 ± 1</td>
<td>113 ± 11</td>
</tr>
<tr>
<td></td>
<td>217</td>
<td>9 ± 1</td>
<td>10 ± 9</td>
</tr>
<tr>
<td>pJAF4504</td>
<td>usgS: fmdS::lacZ</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>+</td>
<td>1 ± 0</td>
<td>52 ± 6</td>
</tr>
<tr>
<td></td>
<td>102</td>
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<td>1 ± 0</td>
</tr>
<tr>
<td>pJAF4622</td>
<td>usgS: lacZ</td>
<td></td>
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<td></td>
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<td>72 ± 9</td>
<td>127 ± 14</td>
</tr>
<tr>
<td></td>
<td>102</td>
<td>79 ± 4</td>
<td>168 ± 9</td>
</tr>
<tr>
<td></td>
<td>217</td>
<td>84 ± 2</td>
<td>92 ± 6</td>
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Figure 6.—Semiquantitative RT-PCR analysis of fmdS and usgS transcription. All reactions were stopped while in their linear phase as described in Ha et al. (1999). Twenty-two PCR cycles were used for the benA control (using the primers BTUB2 and BTUB3), 26 cycles for usgS (USGrt1 and USGrt2), and 28 cycles for fmdS (fmdSrt1 and FMD2) except for the areA217 mutant where 30 cycles were used to enhance the signal. Wild-type RNA was isolated from strain MH1, areA217 RNA from strain MH341, and usgS RNA from MH9510. Transcription of the constitutively expressed \( \beta \)-tubulin gene benA (May et al. 1987) was used as a loading control. Growth conditions were as described in Figure 2.

noticeable in repressing (glucose and ammonium grown) conditions.

As none of the fmdS fusion constructs studied thus far had included the upstream gene, additional constructs were generated: a usgS::lacZ fusion (pJAF4622) and a usgS fmdS::lacZ fusion (pJAF4504) that contained 3.1 kb of fmdS promoter, incorporating the upstream gene and its promoter (Figure 7). The expression of usgS::lacZ (pJAF4622) was not strongly regulated in agreement with the RT-PCR data (Figure 7). \( \beta \)-Galactosidase levels increased less than twofold under nitrogen starvation conditions, and this increase was eliminated by the areA217 mutation, indicating that usgS expression is
Constructs were generated to determine the effects of overexpression of usgS by inserting a hybrid promoter fragment from the alcA gene (Fillinger et al. 1995) fused to the pyrG promoter and coding region (Oakley et al. 1987a; Figure 8A). Transformation of the usgSΔ/riboB+ pyrG+ strain MH9755 with pJAF4870 yielded a large number of pyrG+ transformants, all of which had a wild-type phenotype on 0.1% glucose with the inducer 1% ethanol present. No phenotype produced by usgS overexpression was observed, indicating that expression of ectopic copies of usgS via the alcA promoter did not affect fmdS expression. The gene replacement plasmid pJAF4871 was then linearized via Clal digestion and transformed into the same strain. Scoring 200 trans- of overexpression of usgS by inserting a hybrid promoter fragment from the alcA gene (Fillinger et al. 1995) formants for growth on 1% ethanol/10 mM formamide media and on media lacking riboflavin revealed three classes of pyrG+ transformants: wild type, those that were still riboB+ and displayed reduced formamide utilization on ethanol, and a third class that was riboB- with reduced formamide utilization. Southern analysis revealed that the second class had been generated by integration of circular pJAF4871 by a crossover event within usgS, introducing the pyrG/alcA::usgS fusion while retaining the deletion allele upstream in tandem. The third class was shown to be produced by a gene replacement event, with linearized pJAF4871 replacing usgSΔ/riboB+ with the pyrG/alcA::usgS fusion (Figure 8B). Both classes containing the alcA promoter fused to usgS in cis to fmdS were greatly impaired in formamide utilization (Figure 8C), indicating that increased usgS expression decreases fmdS expression.

DISCUSSION

The ability to utilize formamide as a nitrogen source via formamidase activity has been detected in both fungal and bacterial species (Draper 1967; Friedrich and Mitterenga 1981; Munoz and Agosin 1993; Wyborn et al. 1994). We have found that the fmdS gene encodes a protein conserved within prokaryotes, eukaryotes, and archaeb. Formamide has been shown to be a degradation product of both histidine and cyanide in various microorganisms (Ferber et al. 1988; Kunz et al. 1994). Cyanide hydratase activity converts hydrogen cyanide to formamide (Wang et al. 1992) and has been detected during infection of cyanogenic Sorghum spp. by the phytopathogenic fungus Gloeocercospora sorghi (Myers et al. 1996).
and Fry 1978a; Fry and Myers 1981; Wang et al. 1999). No formamidase activity is detected in this fungus but the formamide produced is not detectable as it is rapidly metabolized by other organisms (Myers and Fry 1978b). Cyanide originating from cyanide-producing bacteria or cyanogenic glucosides in plants is likely to be a source of formamide for utilization by saprophytes. In the phytopathogenic fungi Leptosphaeria maculans and Fusarium solani, both cyanide and formamide can serve as a nitrogen source (Barclay et al. 1998; Sexton and Howlett 2000). Our finding that formamidase homologues occur in many plant species may indicate a detoxification role for these enzymes in plants.

As had been shown previously, \( fmdS \) expression was strongly regulated in response to the nitrogen state of the cell (Hynes 1972, 1975b). Deletion analysis of the \( fmdS \) promoter revealed multiple GATA sequences through which this effect was modulated by AreA, as found for other genes required for alternative nitrogen source utilization (Davis et al. 1993; Punt et al. 1995; Gonzalez et al. 1997; Hutchings et al. 1999). Induction of \( fmdS \) expression by exogenous formamide does not occur and it is unlikely that formamide is generated by the cell as an endogenous inducer (Hynes 1970, 1975a). In support of this the promoter deletion series shows dependence only on the GATA sites. This independence from pathway-specific induction has been observed for other nitrogen catabolic enzymes, e.g., histidase (Polkinghorne and Hynes 1982) and the general amidase (Hynes 1975b). It is likely that, upon nitrogen starvation, the expression of many genes producing enzymes responsible for nitrogen scavenging is activated, irrespective of the presence of the relevant substrates.

Reduced formamidase expression in response to carbon starvation (Hynes 1972) was further confirmed by this work. This has also been observed for nitrate reductase, NADP-GDH, and histidase (Hynes 1973, 1974; Polkinghorne and Hynes 1982). Expression of the \( fmdS:\ lacZ \) fusion follows the same trend as formamidase levels and, together with the RT-PCR data, is consistent with an effect at the transcriptional level. The time-course analysis of \( fmdS:\ lacZ \) expression suggests that carbon starvation results in loss of activation by AreA. This is supported by the loss of dependence on AreA for expression under carbon starvation conditions and the finding that the element responsible for this regulation could not be localized to any specific sites in the \( fmdS \) promoter deletion series. It is highly unlikely that this is a general effect of carbon starvation on gene expression. Carbon starvation leads to increased levels of acetamidase activity even in \( areA \) loss-of-function mutants and relieves ammonium repression (Hynes 1972, 1975b). This has been supported by more recent data using an \( amdS:\ lacZ \) fusion that shows that the response to carbon starvation is independent of AreA but dependent on an element in the 5′ region of \( amdS \) (M. J. Hynes, M. A. Davis and J. A. Sharp, unpublished data).

These results suggest that AreA is rapidly inactivated in response to carbon starvation. Genes dependent on AreA, such as \( fmdS \) and other genes solely involved in nitrogen source utilization, will not respond to nitrogen starvation in the absence of a carbon source. Genes such as \( amdS \), which are also involved in carbon source utilization, will respond to carbon starvation by activation by one or more other regulatory pathways. The mechanism for signaling AreA inactivation due to carbon starvation is yet to be determined. Due to its simplicity \( fmdS \) regulation provides an excellent system for the further study of this phenomenon. It is likely that in nature fungi may often face severe nutrient deprivation and derepress appropriate enzymes for the scavenging of trace sources of carbon and nitrogen.

Analysis of the \( fmdS \) promoter revealed the presence of an overlapping gene (\( usgS \)) whose transcript terminated within \( fmdS \). The deletion of \( usgS \) resulted in increased growth on formamide and cis/trans tests showed that the effect of \( usgS \) was apparent only when in cis with \( fmdS \). When considered in conjunction with the data obtained when \( usgS \) expression was increased (in an \( areA102 \) background and when overexpressed from the \( ppyG/alcA \) promoter), the results strongly suggest that expression of \( usgS \) results in transcriptional interference of the \( fmdS \) promoter. The \( fmdS:: lacZ \) and RT-PCR analysis clearly indicate that the \( usgS \) effect is at the transcriptional level.

As discussed by Eggermont and Proudfoot (1993) a genuine interference effect should be seen only in cis and not in trans, which is clearly shown in the \( usgS/ fmdS \) system and indicates that the intergenic region between \( usgS \) and \( fmdS \) lacks interference blocking elements such as transcriptional pause or polyadenylation sites. In fact, the \( usgS \) poly(A) sites within \( fmdS \) show little similarity to the characterized diffuse fungal polyadenylation sites rich in A and U bases (for a review, see Proudfoot 1991). Multiple polyadenylation sites for the \( usgS \) transcript exist and by definition must be inefficient, because the \( fmdS \) transcript itself must pass through these sites. In contrast the \( fmdS \) gene appears to contain an efficient polyadenylation signal, although the definition of a fungal poly(A) site and the mechanisms involved are not as well understood as in mammalian systems (Levitt et al. 1989; Proudfoot 1991).

One model that can be proposed is that there is a direct effect of transcription through a downstream promoter on transcription initiation of the downstream gene. Positive and negative supercoils generated by RNA polymerase could prevent access to the downstream promoter by RNA polymerase (Wu et al. 1988). This phenomenon, which has been called promoter occlusion (Adhya and Gottesman 1982), has been characterized in the compact genomes of phage and bacteria (e.g., Adhya and Gottesman 1982). There are also rare eukaryotic examples. In Saccharomyces cerevisiae it has been shown that expression from the promoter of the actin
gene completely occludes a cryptic promoter in the first intron (Krügener et al. 1992). The Drosophila melanogaster Adh larval promoter is also occluded in this case by transcription from the upstream adult promoter (Corbin and Maniatis 1989). In these situations transcriptional interference is absolute with the downstream promoter being completely inactive.

A second but not mutually exclusive model is that expression of an upstream gene affects the chromatin structure of the downstream regulatory region and promoter such that access by transcription factors and/or RNA polymerase is altered. Studies in S. cerevisiae have generally indicated that transcript overlap of convergent genes has little effect on transcriptional levels (e.g., Peterson and Myers 1993), although some exceptions have been observed (Puig et al. 1999). Recent studies of the convergent POT1 and YIL161w open reading frames (ORFs; with 84–113-bp transcript overlap) showed that deletion of the YIL161w promoter leads to a twofold increase in POT1 expression and alteration of the intergenic chromatin structure (Puig et al. 1999). If this imposed alteration in nucleosome structure occurred instead over the promoter of a tandemly arrayed gene, the effects could be expected to be more significant through the alteration of transcription factor accessibility. In the unr/N-ras system in Mus musculus, the unr transcript, although shown to interfere with expression of the downstream N-ras promoter, actually terminates 150 bp before the N-ras transcription initiation sites (Boussadai et al. 1997). The effect observed in this instance is proposed to occur through interference with upstream promoter elements, and this could be via the enforcement of an altered chromatin structure.

The fmdS promoter has been shown to have an ordered nucleosome structure centered around the CCAAT sequence, which may be required for overcoming such an effect (Narendja et al. 1999). Reporter gene assays in this study revealed that the CCAAT-binding complex AnCF has a relatively minor role when usgS is absent. In a genomic context the role of AnCF may be much greater and be required, in conjunction with AreA, to open up the fmdS promoter to overcome the negative effects of the upstream gene. Expression of usgS altering nucleosome positioning would enforce a greater dependence on both AnCF and AreA binding, resulting in tightly controlled fmdS expression. It has been recently shown that the effects of readthrough transcripts from the S. cerevisiae GAL10 gene into the adjacent GAL7 promoter result from displacement of the Gal4p transcriptional activator (Gregger et al. 2000).

The effects of transcriptional interference result in expression of the usgS and fmdS genes being functionally linked. Alterations in expression of usgS result in altered fmdS expression. In an evolutionary sense selection might maintain the tandem gene arrangement in order to retain appropriate controlled expression of fmdS. Alteration of this gene arrangement would result in position effects on fmdS expression as demonstrated here. Position effects are often seen with ectopic integration of genes and with directed gene rearrangements that result in alterations of gene regulation (e.g., Miller et al. 1987). In general, in lower eukaryotes genes can be relatively close together. In S. cerevisiae the average distance between tandemly oriented open reading frames is only 517 bp (Dujon 1996), indicating that transcription overlap and interference may occur in some cases. Gene clusters occur commonly in fungi, an example of which is in A. nidulans sterigmatocystin biosynthesis where the 25 genes involved are clustered within only 50 kb (Brown et al. 1996). The relative positions of genes within these clusters may affect their expression via transcriptional interference.

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LITERATURE CITED


Curbera, B., and C. Scanzocchio, 1994 Two different, adjacent and


Hyne, M. J., 1974 The effects of carbon source on glutamate dehy-


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