Multiple Nuclear Localization Signals Mediate Nuclear Localization of the GATA Transcription Factor AreA

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The Aspergillus nidulans GATA transcription factor AreA activates transcription of nitrogen metabolic genes in response to nitrogen limitation and is known to accumulate in the nucleus during nitrogen starvation. Sequence analysis of AreA revealed multiple nuclear localization signals (NLSs), five putative classical NLSs conserved in fungal AreA orthologs but not in the Saccharomyces cerevisiae functional orthologs Gln3p and Gat1p, and one putative noncanonical RXR3RXR bipartite NLS within the DNA-binding domain. In order to identify the functional NLSs in AreA, we constructed areA mutants with mutations in individual putative NLSs or combinations of putative NLSs and strains expressing green fluorescent protein (GFP)-AreA NLS fusion genes. Deletion of all five classical NLSs individually or collectively did not affect utilization of nitrogen sources or AreA-dependent gene expression and did not prevent AreA nuclear localization. Mutation of the bipartite NLS conferred the inability to utilize alternative nitrogen sources and abolished AreA-dependent gene expression likely due to effects on DNA binding but did not prevent AreA nuclear localization. Mutation of all six NLSs simultaneously prevented AreA nuclear accumulation. The bipartite NLS alone strongly directed GFP to the nucleus, whereas the classical NLSs collaborated to direct GFP to the nucleus. Therefore, AreA contains multiple conserved NLSs, which show redundancy and together function to mediate nuclear import. The noncanonical bipartite NLS is conserved in GATA factors from Aspergillus, yeast, and mammals, indicating an ancient origin.

Eukaryotic transcription factors are synthesized in the cytoplasm but function in the nucleus to regulate gene expression. This provides a logistical problem for these proteins, as they must be imported into the nucleus for function. Proteins enter the nucleus through the nuclear pore complex (NPC) (1). Depending on the size of the protein, they can either passively diffuse through the NPC (<30 kDa), or they must be actively transported through the NPC (≥30 kDa) (2, 3). The NPC has been thoroughly studied in Aspergillus nidulans and is a dynamic structure of essential and nonessential proteins (4–6). Transport of large proteins through the NPC is facilitated by nuclear importins (karyopherins), which recognize short stretches of positively charged sequences on the cargo proteins that serve as nuclear localization signals (NLSs) (7–10). A. nidulans has 17 karyopherins to actively transport proteins between the cytoplasm and the nucleus (11, 12). There are two main types of classical NLSs found in eukaryotes, monopartite NLSs and bipartite NLSs. The monopartite NLSs conform to the classical simian virus 40 (SV40) large T-antigen NLSs and are the most commonly found NLS type (9). The classical bipartite NLSs comprise two distinct lysine-rich parts generally separated by 10 to 12 amino acids (13). Other NLS types are known, including tripartite NLSs associated with the zinc binuclear clusters of Saccharomyces cerevisiae Lys14p, A. nidulans PrnA, AlcR, and NirA (14–17), and a noncanonical arginine-based bipartite NLS (RRX3RXR) discovered in mammalian GATA-4 (10).

Although most nuclear proteins contain a single NLS, some nuclear proteins lacking a NLS are thought to enter the nucleus by piggybacking as a preassembled complex with a protein containing a NLS, as proposed for A. nidulans HapC and HapE, which associate with the NLS-containing protein HapB for nuclear import (18). In other cases, two or more NLSs occur within a single protein. Two NLSs were found in A. nidulans HapB, S. cerevisiae Gln3p, S. cerevisiae Mcm10p, human BRCA1, and human BRCA2 (19–23), whereas three NLSs were found in mammalian 5-lipxygenase, human S1-1/RBM10, human Dot1a, and human Tra2β (24–27). As far as we know, there is no reported example of a nuclear protein with more than three NLSs.

While localization to the proper subcellular compartment constitutes a logistical problem, it also provides a platform for regulating protein function. A number of examples of regulated nuclear localization of transcription factors are known, e.g., S. cerevisiae Gln3p, Msn2p, and Mig1p (28, 29), Neurospora crassa NUC1 (30), and A. nidulans AmyR, PacC, and NirA (17, 31, 32). Regulated localization of nuclear proteins can be achieved by altering the balance of nuclear import and nuclear export using multiple mechanisms including direct covalent modification of targeting sequences to prevent or promote transport, cytoplasmic or nucleoplasmic anchoring, and by intramolecular or intermolecular masking of the NLS or nuclear export signal (NES) (33). Covalent modification by phosphorylation and cytoplasmic anchoring both regulate nuclear import of S. cerevisiae Gln3p (20, 21), which is conserved in S. cerevisiae Gln3p, whereas three NLSs were found in mammalian 5-lipxygenase, human S1-1/RBM10, human Dot1a, and human Tra2β (24–27). As far as we know, there is no reported example of a nuclear protein with more than three NLSs.

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Received 11 February 2014 Accepted 19 February 2014
Published ahead of print 21 February 2014
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This article is contribution 14-064-J from the Kansas Agricultural Experiment Station.
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doi:10.1128/EC.00040-14
Nuclear import is clearly critical for AreA function. Despite the importance, the mechanism of AreA nuclear import is not known. Herein we identify and characterize the nuclear localization signals in AreA. We show that the AreA protein contains five classical monopartite NLSs, which are conserved in most ascomycete AreA orthologs, and a noncanonical bipartite NLS conserved with the RXR3/RXR NLS of mammalian GATA-4 (10). We determine the effects of mutations affecting these NLSs on AreA function and nuclear localization. When these classical NLSs are deleted separately or when point mutations are introduced in place of the four key arginines in the bipartite NLS, nuclear accumulation is not attenuated (46). This rapid response identifies nuclear export as the mechanism of regulation of AreA nuclear accumulation. Regulated nuclear localization has now also been shown for the AreA ortholog in *Fusarium fujikuroi* (47).

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### MATERIALS AND METHODS

**A. nidulans** genetic manipulations and growth tests. *A. nidulans* crosses and genetic analysis were performed as described previously (48). *A. nidulans* transformations were conducted as described previously (49). Growth tests were performed as described in reference 48 at 37°C using *Aspergillus* nitrogen-free minimal medium (ANM) with nitrogen sources added at 10 mM (50). The *A. nidulans* strains used in this study are shown in Table 1.

**Sequence analysis.** Sanger DNA sequencing to confirm constructs and gene replacements was performed at the Kansas State University DNA Sequencing and Genotyping Facility. DNA sequence analysis was performed using Geneious Pro 5.3.4 (A. J. Drummond, B. Ashton, S. Buxton, M. Cheung, A. Cooper, C. Duran, M. Field, J. Heled, M. Kearse, S. Markowitz, R. Moir, S. Stones-Havas, S. Sturrock, T. Thierer, A. Wilson, 2011) (Biomatters Ltd, Auckland, New Zealand). The AreA protein sequence was examined for NLs using PSORTII (51) and was examined manually to identify the noncanonical bipartite NLS conserved with GATA-4 (10). Protein sequence alignments were done using ClustalW (52), and protein sequences were obtained from AspGD (http://aspergillusgenome.org/) (53), SGD (www.yeastgenome.org/), or the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/): *Aspergillus niger* AreA (CAA57524) (54), *Aspergillus oryzae* AreA (CAA50776) (55), *Aspergillus parasiticus* AreA (AAD37409) (56), *A. nidulans* AreA (CAA36731) (37), *F. fujikuroi* AreA (CA71897) (57), *Magnaporthe oryzae* NUT1 (AA034158) (58), *N. crassa* NIT2 (P21912) (59), Penicillium chrysogenum Nrc (AAA38400) (60), *Penicillium roqueforti* Nmc (CA604815) (61), *S. cerevisiae* Gap1p (P43574) (62), *S. cerevisiae* Gln3p (P18494) (63), *S. cerevisiae* Dal80p (SGD no. YKR034W) (64), *S. cerevisiae* Gze3p (SGD no. YLI110C) (65), *A. nidulans* AreB beta (AspGD no. AN6221) (66), *A. nidulans* Lrea (AspGD no. AN3435) and Lreb (AspGD no. AN3607) (67), *A. nidulans* Sre (AspGD no. AN0176) (68), and *A. nidulans* NsdD (AspGD no. AN3152) (69), *Homo sapiens* GATA-1 (P19576) (70, 71), *H. sapiens* GATA-2 (P23769) (72, 73), *H. sapiens* GATA-3 (P23771) (74, 75), *H. sapiens* GATA-4 (P43094) (76), *H. sapiens* GATA-5 (NP_536721) (77), *H. sapiens* GATA-6 (Q92098) (78), and mouse GATA-4 (Q08369) (79).

**Molecular techniques.** Standard molecular techniques were performed as described in reference 80 or according to instructions from the manufacturer. PCR to generate gene replacement constructs used proof-reading enzymes: Pfu (Agilent), PfuTurbo (Stratagene), Phusion (Thermo Scientific), or *Esxu* TaKaRa. Southern blot analysis to confirm gene replacements was performed using the DIG (digoxigenin) High Prime DNA Labeling and Detection Starter kit II (Roche). Oligonucleotide primers used in this study are shown in Table 2.

**Construction of gpd(p)[areA]HA NLS gene replacement mutants via direct selection.** (i) Deletion of NL51, NL52, and NL53 in combination. To delete in-frame *areA* codons 60 to 423, which contain NL51, NL52, and NL53, a *gpd(p)[areA]HA,Δ0-423* (HA stands for hemagglutinin) construct (pJAF5203), in which expression is driven by the constitutive promoter *gpd(p)*, was generated by digestion of the *gpd(p)[areA]HA* (truncated at position +1475) plasmid pJAF2000 (46) with Xhol (complete)/Sall (partial) and religation, deleting a 1.15-kb internal *areA* fragment. This construct was linearized with Apal and used to transform *A. nidulans* MH9922 [*areA::ribB(3') ribB2*] (46), directly selecting for growth on 10 mM nitrate as the sole nitrogen source. A transformant with the correct gene replacement generating *gpd(p)[areA]HA,Δ0-423* as confirmed by Southern blotting (data not shown) and restored ribonuclease auxotrophy, was outcrossed to generate *A. nidulans* MH10041.

(ii) Deletion of NL54 and NL55 separately and in combination. First, *A. nidulans* MH10266 [*gpd(p)[areA]HA,:ribB(3') ribB2*] in which 3' *areA* sequences of the *gpd(p)[areA]HA* fusion gene were replaced with ribB was made by gene replacement of the 3' *areA* sequences in strain MH9883 [*gpd(p)[areA]HA,ribB2*] (46) with a *JFareA1-JFareA2* PCR-amplified *areA:ribB(3') gene replacement cassette. The *areA:ribB(3')* construct was made from pJAF5458 by digestion with *SphI* (Klenow-blunted) and *Stul*, and the 0.6-kbp *SphI*-*Stul* mutation-containing fragment (+1951 to +2520) was replaced by the 2.6-kbp *XbaI* (partial, end-filled)-*SmaI* of pBluescript SK(+)/H9004 to generate the *areA::ribB(3')* fusion gene of interest. This construct was linearized with Apal and used to transform *A. nidulans* MH9922 [*areA::ribB(3') ribB2*] (46), directly selecting for growth on 10 mM nitrate as the sole nitrogen source. A transformant with the correct gene replacement generating *gpd(p)[areA]HA,Δ0-423* was used to generate *A. nidulans* MH10041.
TABLE 1 A. nidulans strains used in this study

| Strain | Relevant genotype | Source or reference |
|--------|------------------|---------------------|
| MH1072 | direct selection of transformants on nitrate. For NLS5Δ, the PCR product (+1591 to +2526) was generated using the oligonucleotides JFareA1 and RTareANLS5Del with wild-type areA pJAF4689 (46) as the template; the PCR product was cloned into pBluescript SK (+) (pSL190). The AvrII-Stul fragment from pSL190 was subcloned into the AvrII and Stul sites of pJAF5458, which contains JFareA1-2areA PCR product (+1591 to +2881) from strain MH150 genomic DNA template in pGEM-T Easy, to generate pSL1791. The NLS5Δ mutant [MH1668 (gpd)[areA1(A.A))[D.H1-116-816]] was generated by transformation of strain MH1072 with JFareA1-2areA areA NLS5Δ PCR product, amplified from pSL1791 as the template, and direct selection on nitrate. For NLS4ΔNLS5Δ, PCR product containing both NLS4Δ and NLS5Δ, made with JFareA1 and RTareANLS5Del primers and pCW6606 (NLS4Δ) as the template was cut with AvrII and Stul and cloned into the AvrII and Stul sites of pCW6606 to generate pCW6607, containing both NLS4Δ and NLS5Δ. The AvrII-Stul fragment of pCW6607 was cloned into pCW6590, which contains the SnaBI- EcoRV areA fragment of pJAF4689 (46) inserted into the Smal site of the Aspergillus fumigatus pyra A (AfpyrA) selectable marker plasmid pSM6363 [AfpyrA pst-HindIII (blunted) in Spel of pBluescript SK (+)], to generate pCW6609. The NLS4ΔNLS5Δ mutant [MH11967 (gpd) [areA1(A.A.[H1-116-816])] was generated by transformation of a JFareA1-2areA PCR product amplified from pCW6609 into strain MH1072 and direct selection on nitrate. Construction of gpd)[areA1(A.A)] NLS mutants via two-step gene replacement. The recipients for two-step gene replacement, strain MH11131 [gpd)[areA1(A.A)] pyra A Δnkua::argB] or MH11457 [gpd)[areA1(A.A.[D.H1-116-816])] were constructed by meiotic crossing to introduce this study. Several studies have demonstrated the importance of nuclear localization signals (NLS) in the import of proteins into the nucleus (11-16). These signals are typically composed of basic amino acids and are recognized by nuclear localization factor (NLC) proteins, which are responsible for the transport of proteins into the nucleus. NLSs are often found in transcription factors, such as the GATA factor AreA, which plays a crucial role in nitrogen metabolism in fungi. The AreA protein contains two NLSs, NLS4 and NLS5, which are important for its nuclear localization and function. In this study, the authors investigated the role of these NLSs in the import of AreA protein into the nucleus by using various strategies such as gene deletion, site-directed mutagenesis, and plasmid-based expression. The results showed that NLS4 and NLS5 are essential for the nuclear localization of AreA protein and that mutations in these NLSs lead to a decrease in the nuclear localization of the protein. Furthermore, the authors demonstrated that the bipartite NLSs of AreA are important for the efficient import of the protein into the nucleus. These findings highlight the importance of NLSs in the regulation of protein localization and provide insights into the mechanisms governing nuclear import in fungi. Overall, this study contributes to our understanding of the role of NLSs in the import of proteins into the nucleus and opens up new avenues for the development of strategies to manipulate nuclear localization in fungi. |
TABLE 2 Oligonucleotides used in this study

| Oligonucleotide | Sequencea | Coordinatesb |
|-----------------|-----------|--------------|
| JFareA1         | 5'-CGACTCGGATGTTAAGATG-3' | +1591–+1610 |
| areA NS4-invR   | 5'-GTCTTGTATGCCGATGTCGACCTGCG-3' | +1885–+1860 |
| RTareANSS5Del   | 5'-CGAGGGCTTTACAGGGCCACTTGA-3' | +2525–+2510, +2491–+2481 |
| JFareA2         | 5'-GGTCTACCTGTAACCATGA-3' | +2881–+2862 |
| areANSS4-invF   | 5'-GCCACCTGTCATCCATCAACAGC-3' | +1907–+1932 |
| DeltaZnF3       | 5'-AACAGGGCCAAATGCCTTGC-3' | +2006–+2012, +2235–+2257 |
| ArcR720AR722A   | 5'-GGGTTAGCCTTTGATCA-3' | +2227–+2208 |
| newAreAR685ARB686A | 5'-GTTAAGGCAGCAGACGC-3' | +2122–+2105 |
| newAreAR688invF | 5'-CTCGAAGGGTACGCGCTG-3' | +2123–+2141 |
| NotI78         | 5'-TTGCGGCGCTAATAGCCTATATAGG-3' | N/A |
| T3             | 5'-AATTAACCTCTAATAGG-3' | N/A |
| CCHNLS123F1    | 5'-AAAAAGCTTCACAAGGAGGCTCCGGAGGCC-3' | +670–+691 |
| CCHNLS123R1    | 5'-AAAAAGCTTCACAAGGAGGCTCCGGAGGCC-3' | +922–+903 |
| KSNLS4F        | 5'-AAAAAGCTTCACAAGGAGGCTCCGGAGGCC-3' | +1839–+1876 |
| KSNLS4R        | 5'-AAAAAGCTTCACAAGGAGGCTCCGGAGGCC-3' | +1951–+1933 |
| CCHNLS5BglIF   | 5'-AAAAAGCTTCACAAGGAGGCTCCGGAGGCC-3' | +1839–+1876 |
| KSNLS5F        | 5'-AAAAAGCTTCACAAGGAGGCTCCGGAGGCC-3' | +2461–+2481 |
| KSNLS5R        | 5'-AAAAAGCTTCACAAGGAGGCTCCGGAGGCC-3' | +2520–+2539 |
| KSNLS8BglIF    | 5'-AAAAAGCTTCACAAGGAGGCTCCGGAGGCC-3' | +2462–+2479 |
| KSNLS9F        | 5'-AAAAAGCTTCACAAGGAGGCTCCGGAGGCC-3' | +1996–+2013 |
| KSNLS9R        | 5'-AAAAAGCTTCACAAGGAGGCTCCGGAGGCC-3' | +2353–+2335 |
| whitecodF      | 5'-TATGGTGCACATCACCGGC-3' | N/A |
| whitecodR      | 5'-TGGATGGAGAAGATCTGGCC-3' | N/A |

a Mismatches with the wild-type areA sequence are underlined in areA oligonucleotides.
b The coordinates are relative to the A of the areA ATG at position +1. N/A, not applicable.

The NLS mutants that contained Δ60–423 (i.e., ΔNLS1,2,3) were made by two-step gene replacement in a MH11457 [gpd(p)areA(Δ60–423) pyroA(ΔnkaA:Bar)] recipient isolated from a cross of MH11004 × MH11072. In each case, the constructs contained areA sequences in the AfpyroA vector pSM6363, and transformants were selected for pyridoxine prototrophy. One transformant was selfed, and the progeny were screened for loss of pyridoxine prototrophy. The second step of the gene replacement was confirmed by sequencing the PCR products amplified with JFareA1 and JFareA2. Strain RT175 [gpd(p)areA(Δ60–423) pyroA(ΔnkaA:Bar)] was made by transformation with the construct pCH225 which carried a JFareA1-JFareA2 PCR product from strain MH11099. pNLSΔ4 genomic DNA cloned into the Smal site of pSM6363. Strain RT168 [gpd(p)areA(Δ60–423) pyroA(ΔnkaA:Bar)] was made by transformation with the construct pKS139, which carried a JFareA1-JFareA2 PCR product from a pSL7191 (NLS5) construct. Strains RT167 [gpd(p)areA(Δ60–423) pyroA(ΔnkaA:Bar)] and RT37 [gpd(p)areA(Δ60–423) pyroA(ΔnkaA:Bar)] were made by transformation with the construct pRT7309. Strains RT30 [gpd(p)areA(Δ60–423) pyroA(ΔnkaA:Bar)] and RT73 [gpd(p)areA(Δ60–423) pyroA(ΔnkaA:Bar)] were made by transformation with the construct pRT145. The positions of the crossovers were different in RT30 and RT73 as revealed by sequencing. Strain RT237 [gpd(p)areA(Δ60–423) pyroA(ΔnkaA:Bar)] was made by transformation with the construct pKS138.

Construction of GFP-NLS fusions. (i) Constructs. We constructed gfp-NLS fusions in wa-targeting vectors in two steps. First, pDFC6917, which was derived by filling in the ends of the unique BglII site in the polylinker of the gpd(p)gfp targeting plasmid pALX213 (pAA3462 [83]), was used to construct gfp-NLS fusions expressed from the gpdA promoter. The NLSs were amplified with primers containing BamHI and HindIII sites, and the amplicons were digested with BamHI and HindIII and ligated into the BamHI and HindIII sites of pDFC6917. Second, the gpd(p)gfp-NLS sequence was PCR amplified with primers NotI78 and T3, and the NotI- BamHI restriction fragment was subcloned into the NotI and BamHI sites of the wa-targeting vector pCW6500, which was constructed by insertion.
Nuclear Localization Signals in the GATA Factor AreA

### A

| NLS | Amino acid coordinates | Sequence |
|-----|------------------------|----------|
| NLS1 | 216 – 222 | PIKARRD |
| NLS2 | 252 – 258 | PRRVRK7 |
| NLS3 | 271 – 277 | P5RKPQ |
| NLS4 | 609 – 615 | PPRQKQ |
| NLS5 | 811 – 816 | PPRQKQ |

Classical NLS consensus: PKKKRKV

### B

- **bipNLS**: 685 – 722

### C

#### AnAreA

- **AnAreA**
  - 204: TTKASEATTGAIPIK.ARRDQSASEATPVPASFPHPAQDQRRE.SEFGYVPRRVRKTSIDDERQFFN.LQIPSRKRPAESSP.HVPPVSTSMLAHDP 295
  - 323: TTTKASEATTGAIPIK.ARRDQXKESATPVPASFPHPAQDQRRE.SEFGYVPRRVRKTSIDDERQFFN.LQIPSRKRPAESSP.HVPPVSTSMLAHDP

**Noncanonical bipartite NLS**

- **bipNLS**: 685 – 722

### D

- **ScGAT1**: RNSSVRKK...KPALKKIKSS...LSSSVPIEAETFSSFRPDMNMTMNL
- **ScGLN3**: SNTVTGNFRRS...SRRSSTSSNTSSS                      LSQQLQNSESNSFISNHKFNNRLSS..
- **ScGAT1**: KQKLAECN...LPVLPK...SRRSSTSSNTSSS                      LSSSVPIEAETFSSFRPDMNMTMNL

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**FIG 1** Nuclear localization signals in AreA. (A) Sequences of the five classical NLSs identified by similarity to the consensus sequence PKKKRKV using PSORTII analysis. The bipartite NLS (bipNLS) conforms to the noncanonical bipartite arginine NLS RRX₃RXR. (B) Positions of the NLSs in the AreA protein. The NLSs are indicated as black bars, the GATA zinc finger DNA-binding domain is indicated as a dark gray box, and the HA tag is indicated as an open box. (C) Partial protein sequence alignment of AreA homologs showing the conservation of the nuclear localization signals across species. The first two or three letters indicate
of an internal 2.15-kbp EcoRI fragment of the wA (white) gene, amplified with primers whitecodIF and whitecodIR, into the NruI site of pSM6363. For the GFP control, gpd(p)gfp was PCR amplified from pDFC6917 and subcloned into pCW6500 to generate pCH183. For GFP-NLS1,2,3, areaA codons 204 to 287 were amplified from M1 homologous DNA using CCHNL523F1 and CCHNL523R1 primers, cut with BamHI and HindIII, and ligated into pDFC6917. The gpd(p)gfpNL523 sequence was subcloned into pCW6500 to generate pCH59. For GFP-NLS4, KSNL54F and KSNL54R primers were used to amplify areaA codons 600 to 630 using the pKS5 [JFareA1-JFareA2 areaA amplified from M1 homologous DNA cloned into the Smal site of pBluescript SK(+) template. The gpd(p)gfpNL54 sequence was subcloned into pCW6500 to generate pCH12. For GFP-NLS5, KSNL55F and KSNL55R primers were used to amplify areaA codons 801 to 826 from the pK5 template. The gpd(p)gfpNL55 sequence was transferred into pCW6500 to generate pCH62. For GFP-bipNLS, KsAreAxF and KsAreAxF primers were used to amplify areaA codons 646 to 746 from the pK5 template. The gpd(p)gfpbipNL5 sequence was transferred into pCW6500 to generate pCH64. For GFP-bip14-NLS, KsAreAxF and KsAreAxF primers were used to amplify areaA codons 646 to 746 from pRT7309. The gpd(p)gfp14-NLS sequence was transferred into pCW6500 to generate pCH65. For GFP-NLS1,2,3,5, the NLS5 encoding codons 600 to 630 were amplified with primers CCHNL54BglIIF and KSNL54R using the pK5 template, digested with BglII and BamHI, and ligated into the BamHI site of pCHS9 to generate pCH180. For GFP-NLS1,2,3,4, the NLS4 encoding codons 600 to 630 were amplified with primers CCHNL54BglIIF and KSNL54R using pK5 as the template, digested with BglII and BamHI, and ligated into the BamHI site of pCHS9 to generate pCH227. For GFP-NLS4,5, the NLS5 encoding codons 801 to 826 were amplified with primers CCHNL55BglIIF and KSNL55R using the pK6 template, digested with BglII and BamHI, and ligated into the BamHI site of pCHS9 to generate pCH228.

(ii) GFP strains. The gpd(p)-gfp-NLS plasmids pCH59, pCH121, pCH62, pCH64, pCH65, pCH180, pCH226, pCH227, and pCH228 and the gpd(p)gfp control pCH183 were transferred into strain RT96 [pyroA4 nukA::Bar]. Transformants were selected for pyridoxine prototrophy, and transformants with targeted integration were confirmed by Southern blot analysis (data not shown).

Immunostaining, immunofluorescence, and GFP microscopy. Immunostaining was conducted as described previously (46). Indirect UV immunofluorescence microscopy was performed using an Olympus BX51 upright biological reflected fluorescence microscope equipped with Nomarski differential interference contrast (DIC), an EXFO X-Cite 120 Q fluorescence illumination system and a UPlanFLN Plan Semi Apochromatic (field number FN26.5) Fluorite 100× oil objective with a numerical aperture of 1.30. Alexa Fluor 488 immunofluorescence was detected using a BrightLine fluorescein isothiocyanate (FITC) filter set (excitation wavelength band pass, 482/35 nm; dichroic mirror, 506 nm; emission, 536/40 nm; ZPIXEL). DAPI (4′,6′-diamidino-2-phenylindole) fluorescence was detected using a BrightLine DAPI Hi Contrast filter set (excitation wavelength band pass, 387/11 nm; dichroic mirror, 409 nm; ZPIXEL). For direct visualization of GFP, germlings were prepared for UV fluorescence microscopy as described previously (83). GFP fluorescence was detected in fixed cells with the same microscope and camera as used for immunofluorescence but using a BrightLine GFP filter set (excitation wavelength band pass, 473/31 nm; dichroic mirror, 495 nm; emission 483/32 nm; ZPIXEL). At least 30 nuclei from each of two independent experiments were analyzed for each growth condition. Photomicrographs were captured using an Olympus DP72 12.8 Megapixel digital color camera and DP2-BSW digital camera software. Images were manipulated similarly within and between experiments using Adobe Photoshop CS4. Images were cropped, and the tonal range was increased by adjusting highlights and shadows without altering the color balance. GFP or α-hemagglutinin (α-HA) fluorescence was quantified with ImageJ (W. S. Rasband, U.S. National Institutes of Health, Bethesda, Maryland, USA; http://imagej.nih.gov/ij; 1997 to 2012) using representative raw images. The nuclear fluorescence to cytoplasmic fluorescence ratio per unit area was calculated using the mean of 25 randomly paired ratios of nuclear to cytoplasmic regions, allowing the standard error of the mean (SEM) to be calculated. Significance was tested with a two-sample, unequal variance t test (P value <0.025).

β-Galactosidase assays. Strains for fim5-lacZ assays were constructed by meiotic crossing (48). β-Galactosidase assays were performed as described previously (84). Specific activity is expressed as A420/10 min -1 mg -1 of soluble protein. Protein concentrations were calculated as described previously (85) using Bio-Rad protein assay reagent (Bio-Rad) following the manufacturer’s instructions. Significance was tested using a two-sample, unequal variance t test (P value <0.05). t tests were done comparing the wild type and each mutant for each condition, within each strain comparing NH4 to either alanine (ALA) or nitrogen-free medium (−N), as well as within strains comparing ALA to −N.

RESULTS AreA has multiple conserved nuclear localization signals. The A. nidulans AreA sequence was analyzed for nuclear localization signals using the PSORTII program (51), which identifies sequences with similarity to consensus targeting signals. Five classical NLSs were identified within AreA by their adherence to the PKKKRKV consensus sequence (86): NLS1 (residues 216 to 222), NLS2 (residues 252 to 258), NLS3 (residues 271 to 277), NLS4 (residues 609 to 615), and NLS5 (residues 811 to 816) (Fig. 1A and B).

We aligned the protein sequences of AreA orthologs (Fig. 1C). NLS1, NLS2, NLS3, and NLS5 were strongly conserved in most ascomycete homologs of AreA. NLS4 was conserved in many ascomycetes but showed poor conservation with F. fujikuroi AreA, M. oryzae NUT1, and N. crassa NIT2. None of the classical NLSs were conserved in either Gln3p or Gat1p from S. cerevisiae. Gln3p contains two predicted NLSs, only one of which is required for nuclear localization (20). Neither of these sequences corresponds in position with any of the classical NLSs in the AreA orthologs.

Effects of mutation of AreA classical NLSs on AreA activity. We constructed a battery of hemagglutinin (HA) epitope-tagged AreA mutant strains by direct selection or by two-step gene replacement (see Materials and Methods) in order to determine the effects of the loss of NLSs on AreA function and localization. The AreAHA variants were expressed from the constitutive gpdA pro-

the species as follows: An, A. nidulans; Ani, Aspergillus niger; Ao, Aspergillus oryzae; Ap, Aspergillus parasiticus; Fa, F. fujikuroi; Mo, M. oryzae; Nc, N. crassa; P, Penicillium chrysogenum; Pr, Penicillium roqueforti; Sc, Saccharomyces cerevisiae; Hs, Homo sapiens; Mm, Mus musculus. For the fungal NLSs including the noncanonical bipartite NLS, dark gray shading represents >60% identity and light gray shading represents >60% similarity. Within the gaps, the aligned sequences are indicated as periods. The arginine-based bipartite NLS has the arginine residues shown in bold white type and the cysteine residues for the GATA zinc finger binding domain shown in bold black type. For the human and mouse GATA transcription factors, dark gray shading represents identity with the fungal amino acids, and light gray shading represents identity within the mammalian GATA factors but not with the fungal zinc finger-binding domain. Coordinates are shown for A. nidulans AreA.
moter \[ gpd(p) \] to uncouple any effects of the mutations on autogenous control of \( \text{areA} \) transcript levels (46). We deleted NLS1, NLS2, and NLS3 together in a single deletion mutation of residues 60 to 423 (ΔNLS1,2,3). NLS4 (residues 609 to 615) and NLS5 (residues 811 to 816) were deleted individually to generate the NLS4Δ and NLS5Δ mutations. We also made all of the possible double and triple combinations of the ΔNLS1,2,3, ΔNLS4, and ΔNLS5 mutations. The seven mutants, with wild-type and \( \text{areA} \Delta \) controls, were tested for growth on a range of sole nitrogen sources, including the preferred nitrogen sources ammonium and glutamine, and various alternative nitrogen sources (Fig. 2). Mutation of the classical NLSs in all combinations, including all the mutations together, resulted in growth comparable to the wild-type controls for all nitrogen sources tested.

In order to determine the effects of these mutations on AreA-dependent gene expression, the \( \text{fmdS-lacZ} \) reporter gene (87) was introduced into the NLS mutants by meiotic crossing. We assayed \( \beta \)-galactosidase activity of the \( gpd(p)\text{areA}^{\text{HA}} \)-NLS mutant \( \text{fmdS-lacZ} \) progeny grown on ammonium or grown on ammonium and then transferred to either alanine medium or nitrogen-free medium (Fig. 3). The wild-type \( gpd(p)\text{areA}^{\text{HA}} \) control showed low levels of gene expression when grown on ammonium, increased AreA-dependent expression on the alternative nitrogen source alanine, and even higher AreA-dependent expression following transfer to medium lacking nitrogen, as observed previously (46). Deletion of the classical NLSs (NLS1,2,3, NLS4, and NLS5) in all combinations had no effect on the expression of FmdS-LacZ.

Analysis of the effects of mutations in AreA classical NLSs on nuclear accumulation. The wild-type AreAHA protein accumulates in the nucleus after nitrogen starvation but not after growth in nitrogen-sufficient or nitrogen-limiting conditions (46). To determine which of the NLSs was necessary for nuclear accumulation of AreA, we immunostained the HA epitope-tagged AreA NLS mutant strains (Fig. 4). Deletion of the five classical NLSs individually (ΔNLS1,2,3, ΔNLS4, or ΔNLS5), in combinations...
A noncanonical bipartite NLS in AreA is conserved with mammalian GATA-4. The mouse transcription factor GATA-4 has a noncanonical arginine-based bipartite NLS RRX33RXR within the GATA zinc finger DNA-binding domain (10). As the PSORTII algorithm does not include this noncanonical bipartite NLS, we identified by manual inspection an RRX33RXR motif within the AreA zinc finger domain at residues 685 to 722 as a sixth bipartite NLS (bip) (Fig. 1A and B). The AreA bipartite NLS is highly conserved within the zinc finger across most fungal AreA homologs, with S. cerevisiae Gln3p and Gat1p being the notable exceptions showing only partial conservation of this motif (RRX33RXS and RRX33RXR, respectively). The bipartite NLS is also conserved in the A. nidulans GATA factors AreA, the C-terminal zinc finger of A. nidulans SreA, the S. cerevisiae GATA factors Dal80p and Gzf3p, and the C-terminal zinc finger of all six mammalian GATA factors, suggesting that this NLS is ancient in origin (Fig. 1C). The bipartite NLS was not conserved in the other A. nidulans GATA factors, LreA, LreB, or NsdD. An additional RRX33RXR motif separated from the zinc finger is also found in SreA.

As the bipartite NLS spans the GATA zinc finger, we mutated the bipartite NLS by point mutation of the four key arginine residues R685A, R686A, R720A, R722A (bip<sup>ALA</sup>). We also made all of the possible double, triple, and quadruple combinations of bip<sup>ALA</sup> with the ΔNL5<sub>1,2,3</sub>, ΔNL5<sub>4</sub>, and ΔNL5<sub>5</sub> mutations. Deletion of all five classical NLSs in conjunction with the bip<sup>ALA</sup> bipartite NLS mutation (ΔNL5<sub>1,2,3</sub>-ΔNL5<sub>4</sub>-bip<sup>ALA</sup>-ΔNL5<sub>5</sub>) prevented nuclear accumulation of AreA during nitrogen starvation (Fig. 4), implicating the bipartite NLS as a major nuclear localization sequence. However, the bip<sup>ALA</sup> single mutant strain showed nuclear accumulation (Fig. 4), indicating that the five classical NLSs together can mediate AreA nuclear import without the bipartite NLS. The bip<sup>ALA</sup> mutant alone or in combination with any of the classical NLSs showed a loss-of-function phenotype with growth comparable to growth of the areAΔ strain (Fig. 2). Furthermore, mutation of the bipartite NLS affected FmdS-LacZ activity as severely as areAΔ, indicating that the four arginine residues comprising the bipartite NLS are critical for AreA function, presumably for AreA DNA binding (Fig. 3). We found that the ΔNL5<sub>1,2,3</sub>-bip<sup>ALA</sup>-ΔNL5<sub>5</sub> mutant AreA protein lacking all NLSs except NL5<sub>4</sub> weakly accumulated in the nucleus, showing that NL5<sub>4</sub> is able to direct AreA nuclear accumulation by itself. NL5<sub>1,2,3</sub> (i.e., in the ΔNL5<sub>4</sub>-bip<sup>ALA</sup>-ΔNL5<sub>5</sub> mutant) and NL5<sub>5</sub> (i.e., in the ΔNL5<sub>1,2,3</sub>-ΔNL5<sub>4</sub>-bip<sup>ALA</sup> mutant) did not individually confer AreA nuclear accumulation, but in the ΔNL5<sub>4</sub>-bip<sup>ALA</sup> mutant, which has NL5<sub>1,2,3</sub> and NL5<sub>5</sub> intact, we observed strong nuclear accumulation. Therefore, although NL5<sub>1,2,3</sub> and NL5<sub>5</sub> are separately insufficient for nuclear accumulation, they appear to work together to signal AreA nuclear localization.

Identification of AreA nuclear localization signals sufficient for nuclear localization. The mutational analysis above strongly suggests that all the identified NLSs in AreA are functional and show redundancy. In order to dissect the nuclear import function of the six NLSs, we fused the AreA NLSs to the C terminus of GFP expressed from the constitutive gpdA promoter in a wA-targeting vector. The gpd<sub>p</sub>/gfp-NLS constructs and a gpd<sub>p</sub>/gfp control construct lacking sequences encoding a NLS were targeted in single copy at the A. nidulans wA gene (see Materials and Methods). Subcellular localization of the GFP-NLS fusion proteins was determined by direct UV fluorescence microscopy. Similar patterns

(ΔNL5<sub>1,2,3</sub>-ΔNL5<sub>4</sub>, ΔNL5<sub>1,2,3</sub>-ΔNL5<sub>5</sub>, or ΔNL5<sub>4</sub>-ΔNL5<sub>5</sub>), or together (ΔNL5<sub>1,2,3</sub>-ΔNL5<sub>4</sub>-ΔNL5<sub>5</sub>) did not abolish nuclear accumulation of AreA, indicating that other noncanonical nuclear localization signals are involved in AreA nuclear import.
The GFP control was weakly localized outside the nucleus after 4 h of nitrogen starvation
and was not directed specifically to the nucleus during nitrogen starvation. However, when we deleted all five of the classical NLSs and mutated the bipartite NLS target AreA to the nucleus, the lack of nuclear accumulation of GFP-NLS4 coupled with the weak nuclear accumulation we observed for the ΔNLS1,2,3-bipALA,ΔNLS5 mutant suggests that NLS4 has weak or context-dependent activity. We tested whether the classical NLSs might be separately weak NLSs that could function in combination. GFP was fused to both NLS4 and NLS5, to NLS1, NLS2, NLS3 and either NLS4 or NLS5, and fused to all five classical NLSs. These combinations of NLSs in the context of a single fusion protein conferred nuclear accumulation of GFP. NLS4 and NLS5 together weakly conferred nuclear accumulation, whereas NLS1, NLS2, and NLS3 fused to NLS4 and/or NLS5 conferred strong nuclear accumulation. Taken together, these results strongly indicate that the five classical NLSs and the bipartite NLS can cooperatively target AreA to the nucleus.

**DISCUSSION**

The presence of sequences capable of interacting with nuclear transport machinery is a vital component for nuclear entry of most transcription factors. We have now shown that AreA has multiple functional NLSs. The RRX33RXR bipartite NLS is conserved in filamentous fungal AreA orthologs, in the A. nidulans GATA factors AreB and SreA, *S. cerevisiae* negative-acting GATA factors Dal80p and Gzf3p, and in mammalian GATA factors. *S. cerevisiae* Gat1p has a conservative substitution of one of the bipartite NLS arginines for lysine, and it is conceivable that this sequence may act as an NLS. In *S. cerevisiae* Gln3p, however, there is a nonconservative substitution in one of the key arginine residues, and evidence that this region does not serve as a functional NLS (20). The five classical NLSs found in AreA are conserved across most of the filamentous fungi but not in the *S. cerevisiae* nitrogen GATA factors. These NLSs appear to work together in various combinations to mediate nuclear import, and the bipartite NLS is independently able to localize AreA to the nucleus. The four arginine residues in the bipartite NLSs are critical for AreA-dependent gene expression as seen in growth tests on a range of nitrogen nutrients and in *fmd5-lacZ* reporter gene assays. This is likely due to the fact that they are DNA contact residues in the AreA zinc finger, and the arginine-to-alanine mutations likely disrupt AreA DNA binding (89). Mutation of these four arginine residues simultaneously in the GATA-4 bipartite NLS abolishes nuclear localization, and mutation of any of the four residues abolishes or severely inhibits DNA binding and transcriptional activation (10). Although mutation of the bipartite NLS abolishes AreA function, the bipartite NLS mutant AreA protein accumulates in the nucleus during nitrogen starvation. However, when we deleted all five of the classical NLSs and mutated the bipartite NLS simultaneously, AreA was not functional and did not accumulate in the nucleus.

There is a stark mechanistic difference in the localization of *A. nidulans* AreA compared with its *S. cerevisiae* homolog Gln3p. In *S. cerevisiae*, nuclear import is the regulated step, as Gln3p is held in the cytoplasm by a cytoplasmic anchor Ure2p during nitrogen-sufficient conditions (28). During nitrogen limitation, dephosphorylation of Gln3p and Ure2p leads to release of Gln3p, and Gln3p is imported into the nucleus (20, 28). Gln3p has only one functional classical NLS that is inactivated by cytoplasmic anchoring (20). A second potential NLS in Gln3p was found to be dispensable for nuclear import (20). In contrast, AreA nuclear localization is regulated primarily by nuclear export via CrmA, as the export in response to the addition of nitrogen nutrients of AreA is...
rapid (46). The kinetics of AreA nuclear accumulation, however, are slow, and we have found no evidence for differential regulation of AreA nuclear import.

AreA is unusual in the large number of NLSs it contains. Nuclear localization signals in other transcription factors are quite variable in both type and number. For many transcription factors, a single NLS mediates nuclear import. For example, A. nidulans PrnA, the constitutively nuclear transcriptional activator for proline utilization pathway genes, has a tripartite NLS located in its N-terminal region (15). A single NLS is also found in other A. nidulans transcription factors: AlcR, NirA and AmyR each have a tripartite NLS (16, 17, 31), and VeA and PacC have a classical bipartite NLS (32, 90, 91). There are many examples of nuclear proteins containing multiple NLSs; however, there are usually no more than three (24, 26). A. nidulans HapB has two monopartite NLSs located in the C-terminal domain (18, 19). One of these NLSs is conserved in fungal, yeast, and human HapB orthologs, and is functional in A. nidulans HapB, S. cerevisiae Hap2p, and human NF-YA proteins expressed in S. cerevisiae (19). The other NLS is found only in the aspergilli, but it is required for nuclear localization of HapB in A. nidulans (19). Both NLSs are functional in Aspergillus oryzae HapB (92). The AreA NLSs show apparent redundancy in their ability to promote localization of AreA and GFP to the nucleus. If these sequences share truly redundant functions, we might expect them to be lost over time in different lineages. However, all of the NLSs are conserved across most fungal species, suggesting that each NLS has an important and unique function. One possibility is that AreA may use alternative importins for nuclear import under different growth conditions due to differential expression of importins. A. nidulans has 17 nuclear importins, but the expression of these across different growth conditions has not been determined (12). Alternatively, multiple NLSs could allow for more-efficient nuclear import. The cooperativity observed for the AreA classical NLS suggests low binding affinities of individual NLSs to nuclear importin(s). α-Importin binds to different NLSs, including classical NLSs, via either of two NLS-binding grooves (93). Binding of multiple AreA NLSs to different binding grooves of importin(s) may confer stronger binding affinity and more-efficient nuclear import. The RXX3RXR bipartite NLS of GATA-4 interacts with β-importin, but not α-importin (10). If the interaction with β-importin is conserved for AreA, the AreA NLSs may mediate interaction with both importins of the α-importin–β-importin complex.

The presence of multiple NLSs has been proposed to allow for multiple regulatory steps for import to mediate a gradation of nuclear protein levels under different conditions compared with having only a single strong NLS, which could function more like an on/off switch (24). None of our observations suggest that nuclear import of AreA is differentially regulated or that the six AreA NLSs allow varied levels of nuclear import depending on nitrogen conditions. We have demonstrated a high degree of redundancy of the NLSs in AreA. Either the bipartite NLS alone or the classical NLSs together can strongly promote protein accumulation into the nucleus. What is unclear is why this functional redundancy has not been curtailed by evolution. This hints to the possibility of various importins recognizing AreA to ensure import during constantly changing environmental conditions and nitrogen nutrient availability.

ACKNOWLEDGMENTS

This work was supported by the Australian Research Council’s Discovery Projects funding scheme project DP0558802 (M.A.D. and R.B.T.), a K-INBRE ARRA Scholarship supported by award P20RR16475 from the National Center for Research Resources (NCRR) (C.C.H.), Kansas NSF EPSCoR First Award EPS-0903806 (R.B.T.), Kansas State University Plant Biotechnology Center (R.B.T.), and the Johnson Center for Basic Cancer Research (R.B.T.).

The content of this article is solely the responsibility of the authors and does not necessarily represent the official views of the NCRR or the National Institutes of Health.

We acknowledge the expert technical assistance of K. Nguyen, M. Wallis, M. Ghosn, K. Smith, J. Bowne, S. Murray, G. Y. Busot, and B. T. Pfannenstiel and preliminary GFP fusion analysis by M. C. Zanker.

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