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Analysis of Promoter Function in *Aspergillus fumigatus*

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The filamentous fungus *Aspergillus fumigatus* is an important opportunistic pathogen that can cause high mortality levels in susceptible patient populations. The increasing dependence on antifungal drugs to control *A. fumigatus* has led to the inevitable acquisition of drug-resistant forms of this pathogen. In other fungal pathogens, drug resistance is often associated with an increase in transcription of genes such as ATP-binding cassette (ABC) transporters that directly lead to tolerance to commonly employed antifungal drugs. In *A. fumigatus*, tolerance to azole drugs (the major class of antifungal) is often associated with changes in the sequence of the azole target enzyme as well as changes in the transcription level of this gene. The target gene for azole drugs in *A. fumigatus* is referred to as *cyp51A*. In order to dissect transcription of *cyp51A* transcription and other genes of interest, we constructed a set of firefly luciferase reporter genes designed for use in *A. fumigatus*. These reporter genes can either replicate autonomously or be targeted to the *pyrG* locus, generating an easily assayable uracil auxotrophy. We fused eight different *A. fumigatus* promoters to luciferase. Faithful behaviors of these reporter gene fusions compared to their chromosomal equivalents were evaluated by 5′ rapid amplification of cDNA ends (RACE) and quantitative reverse transcription-PCR (qRT-PCR) analysis. We used this reporter gene system to study stress-regulated transcription of a Hsp70-encoding gene, map an important promoter element in the *cyp51A* gene, and correct an annotation error in the actin gene. We anticipate that this luciferase reporter gene system will be broadly applicable in analyses of gene expression in *A. fumigatus*.

Aspergillus fumigatus is an important filamentous fungal pathogen that causes severe invasive infection and pneumonia in immunocompromised hosts, resulting in high mortality rates. The increase in immunocompromised patient population as well as an increase in the incidence of drug resistance among *A. fumigatus* clinical isolates has aggravated the problem (2, 6, 7, 9, 12, 19, 20, 29, 30, 36, 51). Hence there is an urgent need to identify the molecules and mechanisms that contribute to pathogenesis and discover drug targets that will lead to a better treatment regimen of this important pathogen.

The sequencing and annotation of the *A. fumigatus* genome (40) have facilitated the process of studying *A. fumigatus* genes and their role in pathogenesis. However, *A. fumigatus* is still not genetically very tractable, and limited progress has been made in the understanding of how this saprophytic fungus turns into a prominent opportunistic pathogen. Most of the molecular genetic tools currently in use in *A. fumigatus* have been borrowed from the *A. nidulans* system.

The role of promoters in regulating expression of genes at the transcriptional level is important in the mechanism of action of processes across fungal species and beyond. To study this regulatory feature, reporter gene fusions have been employed in many systems as important genetic tools for analyzing the expression of particular genes under various conditions. They have also been used to identify DNA sequences upstream of a gene that function as potential regulatory elements as well as in ultimately determining proteins that bind to these elements. There are no comprehensive gene reporter systems or reagents in place for *A. fumigatus*. Most of the gene reporter fusions in *A. fumigatus* so far have been made to characterize individual genes. There has been a study using enhanced green fluorescent protein (eGFP) to analyze differential expression of the *A. fumigatus* cyclic AMP-dependent protein kinase A gene, *pksP*, *in vitro* and *in vivo* (26). The β-galactosidase-encoding *Escherichia coli lacZ* gene has been used to characterize individual promoters such as *pksP* (27) and a putative laccase-encoding gene, *abr2* (56). There have also been studies using *Gaussia princeps* luciferase to study infection models involving the *gpdA2* (AFUA_5G01970) promoter (5, 11, 13).

This study describes the generation of autonomous as well as integrating vectors with firefly luciferase as the reporter gene for use in *A. fumigatus*. The reporter gene system described here was authenticated by comparison with transcript levels measured by quantitative reverse transcription-PCR (qRT-PCR), coupled with 5′ rapid amplification of cDNA ends (RACE) to map the transcription start site of the fusion gene with respect to the native gene. Quantitative analysis of the activity of several promoters fused to the luciferase reporter gene targeted to the *pyrG* locus led to several insights into the functioning of *A. fumigatus* promoters. Use of a luciferase reporter gene fused to the promoter of the Hsp70-encoding $hspA^{SSA1}$ uncovered the induction of this gene in the presence of ethanol. Additionally, *cyp51A*-luciferase fusions provided evidence that the promoter of this gene increased in activity upon introduction of a mutation commonly associated with drug resistance. Finally, analysis of the *A. fumigatus* actin promoter (from the *actA* gene) demonstrated that an error was present in the current annotation for this gene and that the ActA promoter drove high levels of luciferase expression. Our hope is that the reporter system and reagents described here will serve as valuable tools for the *A. fumigatus* research community to study gene function and its regulation in this important opportunistic filamentous fungal pathogen.

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TABLE 1 *A. fumigatus* strains used in this study

Strain	Parent	Description	Source or reference
Af293		Wild type	43
SPF1	Af293	*hph gpdA-luc*::*pyrG*	This study
SPF4	Af293	*hph luc*::*pyrG*	This study
SPF5	Af293	*hph hspA*^{SSA1}*-luc*::*pyrG*	This study
SPF9	Af293	*hph gpdA2-luc*::*pyrG*	This study
AfS35		*loxP*::*akuA*	FGSC (24)
SPF40	AfS35	*hph luc*::*pyrG*	This study
SPF42	AfS35	*hph hspA*^{SSA1}*-luc*::*pyrG*	This study
SPF44	AfS35	*hph abcA-luc*::*pyrG*	This study
SPF46	AfS35	*hph abcB-luc*::*pyrG*	This study
SPF48	AfS35	*hph gpdA2-luc*::*pyrG*	This study
SPF50	AfS35	*hph* F1 *cyp51A -luc*::pyrG	This study
SPF52	AfS35	*hph cyp51B-luc*::*pyrG*	This study
SPF54	AfS35	*hph* F2 *cyp51A-luc*::pyrG	This study
SPF65	AfS35	*hph* C *actA-luc*::*pyrG*	This study
SPF67	AfS35	*hph* A *actA-luc*::*pyrG*	This study
SPF69	AfS35	*hph* B *actA-luc*::*pyrG*	This study
SPF71	AfS35	*hph* T0 *cyp51A-luc*::*pyrG*	This study
SPF73	AfS35	*hph* T1 *cyp51A-luc*::*pyrG*	This study
SPF75	AfS35	*hph* F0 *cyp51A-luc*::*pyrG*	This study
SPF77	AfS35	*hph* T2 *cyp51A-luc*::*pyrG*	This study

TABLE 2 Plasmids used in this study

Plasmid	Description	Source or reference
pPRTII	*ptrA AMA1 lacZ bla*	25
pSP5	pPTRII Sc*CEN6 ARSH4* Sc*URA3 gpdA2-Fluc*	This study
pSP7	pPTRII Sc*CEN6 ARSH4* Sc*URA3 gpdA2-Rluc*	This study
pSP15	pSP5 *hph*	This study
pSP17	pSP7 *hph*	This study
pDHT	*A. tumifaciens* vector *kan*	38
pSP19	pDHT Sc*CEN6 ARSH4* Sc*URA3*	This study
pSP21	pSP19 *pyrG*::*hph gpdA2-Rluc*::*pyrG*	This study
pSP23	pSP19 *pyrG hph gpdA2-luc*::*pyrG*	This study
pSP25	pSP23 *pyrG hph luc*::*pyrG*	This study
pSP27	pSP25 *pyrG hph* A *actA-luc*::*pyrG*	This study
pSP28	pSP25 *pyrG hph hspA*^{SSA1}*-luc*::*pyrG*	This study
pSP29	pSP25 *pyrG hph abcA-luc*::*pyrG*	This study
pSP30	pSP25 *pyrG hph abcB-luc*::*pyrG*	This study
pSP31	pSP25 *pyrG hph gpdA2-luc*::*pyrG*	This study
pSP33	pSP25 *pyrG hph* F1 *cyp51A -luc*::*pyrG*	This study
pSP34	pSP25 *pyrG hph cyp51B-luc*::*pyrG*	This study
pSP35	pSP25 *pyrG hph* B *actA -luc*::*pyrG*	This study
pSP51	pSP25 *pyrG hph* C *actA -luc*::*pyrG*	This study
pSP52	pSP25 *pyrG hph* T0 *cyp51A -luc*::*pyrG*	This study
pSP53	pSP25 *pyrG hph* T1 *cyp51A -luc*::*pyrG*	This study
pSP56	pSP25 *pyrG hph* F0 *cyp51A -luc*::*pyrG*	This study
pSP57	pSP25 *pyrG hph* T2 *cyp51A -luc*::*pyrG*	This study

MATERIALS AND METHODS

***A. fumigatus* strains, growth conditions, and transformation.** Two strains were used in this study: the genome-sequenced *A. fumigatus* Af293 strain (43) and the *akuA* (AfS35) strain (24), which was used to target *A. fumigatus* promoter reporter gene fusions to the *pyrG* locus. These strains were typically grown at 37°C in either rich medium (YG [0.5% yeast extract, 2% glucose]) or in minimal medium (MM) (1% glucose, nitrate salts, trace elements, 2% agar, pH 6.5; trace elements, vitamins, and nitrate salts are as described in the appendix of reference 23). For solid medium, 1.5% agar was added. Media for the growth of uracil auxotrophs were supplemented with 1.2 g/liter each of uracil and uridine. All *A. fumigatus* strains used in this study were transformed by generating protoplasts as described in reference 42. For regeneration of protoplasts upon transformation, 182 g/liter of sorbitol was added, along with 200 mg/liter of Hygromycin Gold (Invivogen) to select for transformants. Strains with the fusion promoters integrated at the *pyrG* locus, derived from the AfS35 strain, are listed in Table 1.

Plasmids. DNA manipulations were done according to the method of Sambrook et al. (48) or according to reagent manufacturer instructions. The autonomous plasmid containing the *AMA1* origin of replication to generate promoter-reporter gene fusions for propagation in *A. fumigatus* was derived from pPRTII (25). To generate a *gpdA2*-luciferase fusion construct in pPTRII, the following fragments were PCR amplified: *CEN6-ARSH* and Sc*URA3* genes from pRS316 (49), the *gpdA2* promoter from *A. fumigatus* strain Af293, either *Renilla reniformis* luciferase from pH12.7 (55) or firefly luciferase from pLG3 (Promega), and the *gpdA2* transcription terminator region from the Af293 genome. These PCR fragments were targeted to SmaI/KpnI-digested, gapped pPTRII, using recombinational cloning in *Saccharomyces cerevisiae* (41), with Sc*URA3* as a selection marker. The primers were designed such that each PCR fragment had an overlapping 40-bp sequence with the adjoining DNA fragment while the terminal DNA fragments had a 40-bp overlapping sequence with the termini of gapped pPTRII to enable simultaneous cloning of the DNA pieces using homologous recombination in *S. cerevisiae*. The vector harboring firefly luciferase as a reporter gene was named pSP5, and the plasmid having *Renilla* luciferase was named pSP7. The pyrithiamine resistance marker from pSP5 and pSP7 was swapped with the hygromycin resistance marker cassette (Hph) using recombinational cloning in *S. cerevisiae* to form plasmids pSP15 and pSP17, respectively.

The integrating vector for targeting the *A. fumigatus* promoter-firefly luciferase fusion constructs to *pyrG* was generated in the pDHT/sk vector (38) amenable to *Agrobacterium tumefaciens*-mediated transformation (57). Initially, Sc*CEN6-ARSH* and Sc*URA3* genes were PCR amplified from pRS316 (49) and cloned in pDHT gapped by PmlI and SphI digestion, by homologous recombination in *S. cerevisiae*, to form pSP19. To generate a *gpdA2*-luciferase fusion construct in pSP19, the following fragments were PCR amplified: 1 kb DNA upstream of *pyrG* from the Af293 genome, the Hph selection cassette from pHPH3, the *gpdA2* promoter from *A. fumigatus* strain Af293, either *Renilla* luciferase from pH12.7 or firefly luciferase from pLG3, and 1 kb DNA downstream of *pyrG* from the Af293 genome that also served as the transcription terminator. These PCR fragments were targeted to pSP19 termini gapped with SacI and KpnI digestion, using recombinational cloning in *S. cerevisiae*, with Sc*URA3* as a selection marker. The primers were designed such that each PCR fragment had an overlapping 40-bp sequence with the adjoining DNA fragment while the terminal DNA fragments had a 40-bp overlapping sequence with the termini of SacI- and KpnI-digested, gapped pSP19, to enable simultaneous cloning of the DNA pieces using homologous recombination. The vector harboring *Renilla* luciferase as a reporter gene was named pSP21, and the plasmid having firefly luciferase was named pSP23. The *gpdA2* promoter was removed from pSP23 using SmaI/SnaB1 digestion, followed by blunt-end ligation using T4 DNA ligase. The resulting firefly luciferase integrating vector without any *A. fumigatus* promoter was named pSP25. Different *A. fumigatus* promoters were cloned in pSP25 by targeting 1 kb DNA upstream of annotated genes, PCR amplified from the Af293 genome, to PmeI/NotI-digested, gapped pSP25. The primers for the above PCR was designed such that it contained 40 bp overlapping with the termini of the gapped plasmid such that the *A. fumigatus* promoter was placed directly upstream of the ATG start codon of the firefly luciferase gene. The list of the plasmids derived from pSP25, with different Afu promoters upstream of the reporter gene, used in this study is given in Table 2.

Luciferase assays. *Renilla* and firefly luciferase assays were done using the *Renilla* and firefly luciferase assay kits, respectively (Biotium, Inc.). To prepare cell lysates for luciferase assays, *A. fumigatus* spores were grown in

5 ml YG medium at 37°C for 12 to 14 h, and the mycelia were collected with an ashless Whatman 40 filter paper using a vacuum pump and washed in deionized water. This mycelium was then transferred to a 1.5-ml microcentrifuge tube containing 100 μl of 0.5-mm glass beads (Biospec, Inc.). Then, 50 μl of the cell lysis buffer (Biotium) was added to wet the mycelium. Using a battery-operated pestle grinder (catalog no. 03-392-106; Fisherbrand), the mycelium was ground for 1 min. Then, 200 μl of cell lysis buffer was added to the mycelium-glass bead slurry and vortexed at 4°C for 5 min. The mycelium was then centrifuged at 16,000 × *g* for 5 min, and the supernatant was used as the cell lysate for the luciferase assay. A 2-μl sample of the cell lysate was added to half-area flat-bottom 96-well plates in triplicate. For the *Renilla* luciferase assay, 5 μl of *Renilla* luciferase assay enhancer (Biotium) and 5 μl of *Renilla* luciferase assay solution (2× coelenterazine solution; Biotium) were added to the cell lysate. For the firefly luciferase assay, 10 μl of firefly luciferase assay solution (containing 0.2 mg/ml luciferin; Biotium) was added to the cell lysate. The 96-well plate was then mixed on a Titramax platform for 15 s. Luminescence was immediately measured using the IVIS 100 imaging system (Caliper Life Sciences). The number of photons emitted per second (luminescence) was then normalized to the amount of total protein present in the cell lysate per ml using a Bradford assay. The luciferase assay data depicted in this paper is quantitative, and the values shown are the average of luminescence from at least two independent experiments done in triplicates, with standard deviations between experiments shown in the form of error bars.

Real-time PCR. Quantitative reverse transcription (qRT)-PCR was performed as described in reference 44 with the following modifications. Mycelium (100 mg) collected by filtration was ground into fine powder in liquid nitrogen using a mortar and pestle. Total RNA was then isolated from the ground mycelium using the RNeasy plant minikit (Qiagen). Synthesis of cDNA was performed using an iScript cDNA synthesis kit (Bio-Rad) with 1 μg of RNA as the template. For the quantitative PCR, primers were designed using the Primer Select program from the DNAStar software package. Primer concentrations were optimized for each gene, and annealing profiles were analyzed to evaluate nonspecific amplification by primer dimers. Control reaction mixtures including RNA instead of cDNA were made for each gene. The threshold cycle (C_T) values were determined in the logarithmic phase of amplification for all genes, and the average C_T value for each sample was calculated from three replicates. The C_T value of the gene coding for actin (*actA*) was used for normalization of variable cDNA levels, and the fold difference in transcript levels was determined with respect to *abcA*. Amplification was carried out in the iCycler apparatus from Bio-Rad in a two-step process as follows: a denaturation step of 3 min at 95°C and 40 cycles of 95°C for 10 s each, with annealing and extension at 60°C for 45 s each. The reporter signals were analyzed using the iCycler iQ software (Bio-Rad).

Southern blot analysis. Genomic DNA was isolated from *A. fumigatus* as described in reference 39. Genomic DNA (1 mg) was subjected to overnight digestion with BclI. Southern blotting was performed as described in reference 48. The probe used corresponded to the firefly luciferase gene and was labeled using [α-^{32}P]dCTP (Perkin Elmer) by random primer labeling using the Klenow fragment of DNA polymerase I.

5′ RACE. 5′ rapid amplification of cDNA ends (RACE) was carried out using the ExactSTART eukaryotic mRNA 5′-RACE kit (Epicentre) according to the manufacturer's instructions. Briefly, total RNA was extracted from Afu mycelium ground with a mortar and pestle in the presence of liquid nitrogen using an RNeasy plant minikit (Qiagen). Total RNA (10 mg) was subjected to APex heat-labile alkaline phosphatase to convert uncapped RNA into a nonligatable 5′-hydroxyl RNA. The reaction product was cleaned up using an RNeasy Minelute kit (Qiagen). The 5′ cap was then removed from 5′ intact mRNAs using tobacco acid pyrophosphatase. The 5′-monophosphate poly(A) RNA thus generated was ligated to 5′-RACE acceptor oligonucleotide (with PCR priming site) using T4 RNA ligase. First-strand cDNA was synthesized from the above template using Moloney murine leukemia virus (MMLV)-reverse transcriptase using a primer that bound to the 5′-RACE acceptor oligonucleotide. RNA from this reaction was removed using RNase. Using the above-described reaction product as the template, second-strand cDNA was synthesized and amplified by PCR using a forward primer that bound to the 5′-RACE acceptor oligonucleotide and a gene-specific reverse primer (corresponding to either the firefly luciferase or the gene of *A. fumigatus* promoter under study). This PCR product was purified using a DNA Minelute kit (Qiagen) and used as the template for the sequencing reaction using a nested gene-specific primer.

RESULTS

Development of episomal/integrating vectors for promoter-luciferase reporter gene assays in *A. fumigatus*. One of the key regulatory steps in any organism is control of gene expression at the level of promoter function. In the case of the filamentous fungal pathogen *Aspergillus fumigatus*, very little information exists concerning transcriptional control regions and the factors that exert regulatory effects there. Our goal was to develop reporter plasmids that would facilitate analysis of promoter function in *A. fumigatus*.

To accomplish this goal, we tested the utility of two different genes encoding luciferase enzymes, from either *Renilla reniformis* or *Photinus pyralis* (firefly). These enzymes use different substrates to generate light but are both very sensitive indicators of gene expression when used as reporter cassettes. Each of these two different luciferase genes was inserted into two different plasmid constructs to allow introduction into *A. fumigatus* strains. We generated both an episomally replicating plasmid and an integrative vector for delivery of reporter genes. The episomal vector used was derived from pPTRII (25) with the *AMA1* origin of replication and was modified to contain a hygromycin resistance cassette for selection in *A. fumigatus* (Fig. 1A). Similarly, a pDHT-based Ti plasmid was constructed to deliver luciferase gene fusions to the *pyrG* locus by *Agrobacterium tumefaciens*-mediated transformation (ATMT) of *A. fumigatus* (57). Both of these plasmids were modified to contain a Sc*CEN6-ARSH4* fragment and Sc*URA3* selection marker to enable facile recombinational cloning in *S. cerevisiae*. The episomal plasmid was introduced into *A. fumigatus* by protoplast transformation (42). To target the fusion promoter constructs to the *pyrG* chromosomal locus by ATMT, the promoter-reporter gene fusion constructs flanked by 1 kb DNA upstream and downstream of the *pyrG* open reading frame were introduced between the T-DNA repeats of the pDHT integrative plasmid (Fig. 1B). Alternatively, the promoter-reporter gene fusion constructs were targeted to the *pyrG* locus by releasing it from the integrative vector by restriction enzyme digestion and transforming it into protoplasts of *A. fumigatus* strain AfS35 (*akuA*Δ) (24).

To test the efficacy of these reporter genes in *A. fumigatus*, the *gpdA2* (AFUA_5G01030) promoter was used to provide presumably strong and constitutive transcription. A PCR fragment corresponding to DNA immediately 5′ of the annotated ATG for this locus to 1,000 bp upstream was prepared and inserted adjacent to the luciferase coding sequences. The resulting plasmids were then introduced into episomal or integrated contexts as appropriate. Hygromycin-resistant transformants were selected, and properly integrated reporter genes were detected by their simultaneous acquisition of uracil auxotrophy. Representative transformants were grown in liquid media and assayed for the level of luciferase activity produced (Fig. 1C).

Several important findings arose from these experiments. First, the *R. reniformis* luciferase-expressing transformants failed to

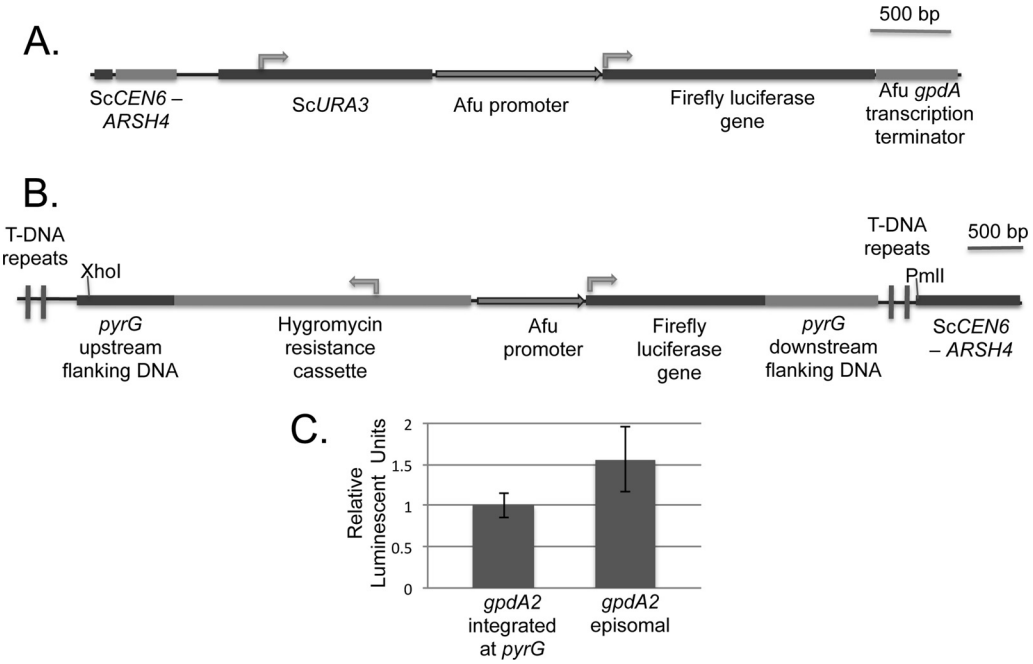

FIG 1 Map of promoter-reporter fusion gene constructs. (A) Diagram of a region of the pPTRII (25)-based episomal plasmid with the *A. fumigatus AMA1* origin of replication and the hygromycin resistance cassette as a selection marker in *A. fumigatus* shown. *S. cerevisiae CEN6-ARSH4* and *URA3* were incorporated to propagate the plasmid in *S. cerevisiae*. The episomal promoter-reporter gene construct consisted of the *A. fumigatus* promoter of choice, the luciferase reporter gene, followed by the *A. fumigatus gpdA2* 3′ untranslated region (3′ UTR) containing the likely transcription terminator. Arrows indicate the direction of gene transcription. (B) A partial map of the pDHT-based (38) plasmid for integrating promoter-reporter gene fusion constructs at the *pyrG* locus is presented. The integrative promoter-reporter gene construct, comprised of the *A. fumigatus* promoter of choice, the luciferase reporter gene, followed by the *pyrG* 3′ UTR, was cloned along with the hygromycin cassette and *pyrG* 5′ UTR flanking DNA within the T-DNA repeats (required by the *Agrobacterium tumefaciens* transformation system) using recombinational cloning in *S. cerevisiae*. Alternately, the plasmid can be digested by XhoI and PmlI and transformed into *A. fumigatus* protoplasts. (C) Comparison of a *gpdA2*-firefly luciferase fusion gene expressed in *A. fumigatus* from the *AMA1*-containing episomal plasmid versus one integrated into the chromosome at the *pyrG* locus, as measured by *in vitro* luminescent assay. Enzyme activities were normalized to those produced from the integrated luciferase fusion gene.

produce any detectable luciferase activity. We also tested these same constructs in the heterologous *S. cerevisiae* host and found readily assayable luciferase activity (data not shown). Second, the firefly luciferase-expressing plasmids produced readily detectable luciferase enzyme and focused our experiments on the use of these constructs. Finally, we compared the firefly luciferase levels in transformants corresponding to the episomally maintained or the integrated *gpdA2*-luciferase fusion genes. There was a modest but reproducible increase in the luciferase levels driven by the episomally carried *gpdA2*-luciferase fusion gene (Fig. 1C). However, owing to the uncertain replication status of the *AMA1*-based episomal plasmid, we restricted our studies to integrated firefly luciferase fusions targeted to the *pyrG* locus.

Analysis of different *A. fumigatus* promoters by *in vitro* luciferase assay. To validate the ability of this reporter gene system to faithfully reproduce *A. fumigatus* promoter function, we compared the ability of a number of different promoters to drive luciferase activity. As described above for *gpdA2*, DNA fragments corresponding to approximately 1,000 bp immediately upstream of the annotated ATG were prepared from each gene and inserted into the integrating firefly luciferase reporter gene vector. The AfS35 strain of *A. fumigatus* was employed, as this mutant strain lacks the *akuA* gene and exhibits enhanced homologous recombination (24). Transformants containing integrated reporter gene fusions were selected on hygromycin-containing medium and counterscreened for their uracil auxotrophy. Representative transformants were grown to mid-log phase, cell-free protein extracts prepared, and luciferase activities measured. All activities were normalized to a fusion gene lacking any inserted *A. fumigatus* promoter fragment (Fig. 2A). Correct genomic integration was confirmed by Southern blotting (Fig. 2C) and PCR analyses (data not shown).

Along with *gpdA2*, we also selected two other promoters from genes that could be expected to exhibit strong transcription of the luciferase reporter gene. These promoters corresponded to the actin-encoding gene (*actA*) and a homologue of the *S. cerevisiae SSA1* gene called *hspA*^{SSA1} (AFUA_1G07440). Sc*SSA1* is robustly expressed and regulated (10), attributes we anticipated might be conserved with its *A. fumigatus* counterpart. The *hspA*^{SSA1} promoter placed upstream of the luciferase gene produced the highest levels of enzyme activity among the initial promoter fusions, while, to our surprise, the *actA*-luciferase fusion gene produced no detectable luciferase activity. The reason for the unexpected behavior of the *actA*-luciferase fusion is presented below.

Prompted by our interest in drug resistance in *A. fumigatus*, we prepared five other fusion gene constructs. Two promoters were taken from *A. fumigatus* genes encoding ATP-binding cassette transporter proteins that exhibited the highest degree of sequence conservation with a major ABC transporter from *S. cerevisiae* called *PDR5* (reviewed in references 17, 22, and 45). These genes were designated *abcA* (AFUA_2G15130) and *abcB* (AFUA_1G14330), respectively. Additionally, fusion genes

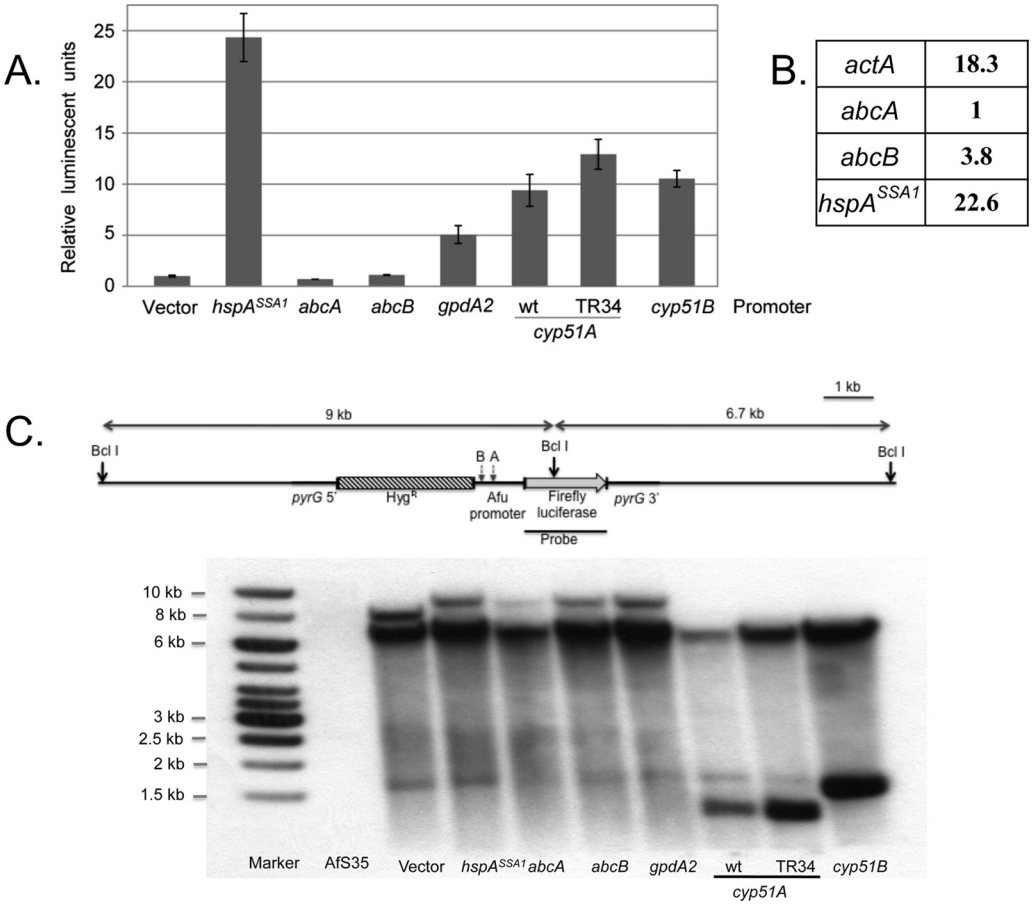

FIG 2 Characterization and validation of luciferase fusion genes. (A) All luciferase fusion genes were integrated at the *pyrG* locus in the AfS35 strain (*akuA*Δ). Transformants were grown to mid-log phase, and protein extracts were prepared and assayed for levels of luciferase activity as described above. Enzyme activities were normalized to the level produced in the absence of an inserted *A. fumigatus* promoter (Vector). Inserted promoters included *hspA*^{SSA1}, corresponding to the *A. fumigatus* homologue of the Sc*SSA1* Hsp70-encoding gene; *abcA* and *abcB* are two *A. fumigatus* homologues of ABC transporter Sc*PDR5*; *gpdA2* encodes an *A. fumigatus* glyceraldehyde-3-dehydrogenase isoform, *cyp51A* and *cyp51B* are the genes encoding the lanosterol 14α demethylase, while TR34 indicates the presence of a 34-bp duplication in the *cyp51A* promoter associated with azole resistance. (B) Quantitative RT-PCR analysis of transcripts from the Af293 strain was carried out using total RNA and the indicated gene-specific primers. Transcript levels were normalized to those from the *abcA* gene. (C) Southern blot analysis to confirm single-copy targeted integration of the fusion promoter constructs at the *pyrG* locus. Genomic DNA was digested with BclI, which cuts once within the firefly luciferase gene, and also within the *cyp51A* and *cyp51B* promoters. The relative positions of the BclI restriction sites present in either *cyp51A* (A) or *cyp51B* (B) are indicated. The probe was prepared from the indicated segment of the luciferase gene. The expected DNA fragment sizes upon single integration at *pyrG* are indicated along the lanes. A nonspecific, cross-hybridizing DNA fragment is also seen around 1.5 kb.

were constructed between luciferase and the two genes encoding the lanosterol α14 demethylase enzyme: *cyp51A* and *cyp51B*. A fusion gene containing an allele of *cyp51A* commonly associated with elevated azole resistance was also prepared. This form of *cyp51A* contains a duplication of a 34-bp element in the promoter that is believed to lead to enhanced expression of the relevant transcript and is referred to as *cyp51A*^{TR34}.

Both *cyp51A* and *cyp51B* produced readily detectable and similar levels of luciferase activity. Introduction of the *cyp51A*^{TR34} fusion promoter led to increased expression of luciferase enzyme, consistent with enhanced promoter function in this mutant. Surprisingly, levels of expression from either the *abcA* or *abcB* promoter fusions were very low: not significantly greater than enzyme activity produced by a construct lacking an inserted Afu promoter. We attribute the latter to the presence of the divergently placed but strong constitutive *Aspergillus nidulans* promoter (46) that drives expression of the adjacent hygromycin resistance gene.

Confirmation that the luciferase reporter gene system was faithfully reflecting transcription of the endogenous Afu genes came from a comparison of the various luciferase activities and mRNA levels measured by quantitative reverse transcription-PCR (qRT-PCR). Total RNA was prepared from wild-type cells, and levels of four different transcripts were compared by qRT-PCR. Transcripts were normalized to *actA* (AFUA_6G04740), and fold differences in mRNA transcript levels were compared with respect to AbcA (Fig. 2B).

The relative expression of these different mRNA species is reflected by the luciferase activities produced by each reporter gene. The best-expressed mRNA under these conditions corresponded to the *hspA*^{SSA1} gene that was 23-fold higher than the *abcA* transcript; a difference entirely consistent with that predicted by comparison of the luciferase activities produced from the two different reporter genes. Expression of *actA* was 18-fold elevated compared to that of *abcA*. This differential exhibited good agreement once

FIG 3 5′ RACE analysis to map transcription start sites of *A. fumigatus* promoters. Total RNA derived from relevant *A. fumigatus* promoter-reporter gene fusion strains targeted to the *pyrG* locus was used to generate cDNA. These cDNAs were sequenced using gene-specific primers directing sequencing toward the 5′ end of the transcript. Primers were generated that corresponded to either the native *A. fumigatus* gene or the firefly luciferase coding sequence. The ATG codons present in the 5′ UTRs from *abcA* and *abcB* are shown in bold. The length of each 5′ UTR is indicated at the right. The 5′ untranslated sequences from both the authentic and the fusion genes were identical in all cases tested, except for the *gpdA2* promoter. In the case of *gpdA2*, there were seven additional nucleotides (shown in lower case) found in the transcript produced from the native gene compared to the fusion gene.

appropriate *actA* reporter constructs were prepared (see below). To ensure that authentic *A. fumigatus* transcription start sites were being used when these relatively small promoter fragments were transferred to the heterologous *pyrG* locus, we compared 5′ endpoints of several genes that were produced both from the endogenous locus and when present in the fusion context by 5′ RACE (5′ rapid analysis of cDNA ends).

5′ RACE analysis to map transcription start sites of the fusion promoters. To confirm that the fusion gene promoters were still utilizing the authentic *A. fumigatus* 5′ start sites of transcription, even when moved to a different chromosomal location, we carried out 5′ rapid analysis of cDNA ends, or RACE, analysis. Reverse transcription primers specific for *cyp51A*, *hspA*^{SSA1}, *abcA*, *abcB*, *gpdA2*, and *actA* were used along with a primer specific for luciferase sequences. Use of these two types of primers allowed us to directly compare transcription start sites used by the native mRNA and the chimeric luciferase fusion gene. In every case, the primary PCR product was sequenced, in order to avoid any artifacts that could be introduced by cloning a rare cDNA product. Total RNA was annealed to either the native primers or luciferase-specific primers, and cDNAs were prepared and amplified using 5′ RACE (Fig. 3).

This analysis demonstrated that for every gene examined, except *gpdA2*, a unique transcription start site was identified. Importantly this same transcription start site was used by both the mRNA produced from the normal chromosomal locus as well as from the luciferase fusion promoter integrated at *pyrG*. Only the *gpdA2* transcript exhibited two different 5′ endpoints, although these were separated by only 7 bp. Comparison of the wild-type *cyp51A* and *cyp51A*^{TR34} transcripts determined that the presence of the duplicated region in the TR34 allele did not alter the start point of transcription. In the cases of both *abcA* and *abcB*, out-of-frame ATG sequences were detected in the relatively long 5′ untranslated regions (UTRs) of these two genes. The significance of these ATG codons is unclear, but their position in the transcript has been associated with translational control elements in other, better-characterized mRNAs (reviewed in reference 53).

The Afu *hspA*^{SSA1} promoter is induced by ethanol. Having established that good general agreement existed between mRNA and luciferase levels driven by the reporter fusions, we wanted to confirm that this reporter gene system would be responsive to acute changes in environmental conditions. Since Hsp70-encoding genes have often been observed to respond to changes in the environment, we tested the *hspA*^{SSA1}-luciferase fusion gene for its response to several different stress agents. Transformants were grown to mid-log phase and challenged with several different stress conditions for 2 h, and then luciferase activities were determined as described previously (Fig. 4A).

Treatment of *hspA*^{SSA1}-luciferase-containing transformants with 2.5 or 5% ethanol led to a roughly 3-fold increase in luciferase activity. Using this induction regimen, we did not observe significant elevation in luciferase expression when transformants were subjected to heat-, H_2O_2- (not shown), or dithiothreitol (DTT)-induced stress.

Next, the kinetics of ethanol induction were evaluated. Transformants containing luciferase fusions to either *hspA*^{SSA1} or *gpdA2*, along with the base luciferase fusion plasmid lacking any inserted Afu promoter, were grown to mid-log phase and challenged with 5% ethanol for 0, 30, or 60 min. At each time point, luciferase activity assayed was normalized to the total protein extracted from the cell lysate (Fig. 4B).

Rapid induction of *hspA*^{SSA1}-luciferase was detected as full activation of the fusion gene and was observed within 30 min of ethanol exposure. Neither the *gpdA2* nor the promoter-less luciferase fusion genes showed any response to the ethanol challenge. These data indicate that this Hsp70-encoding gene is selectively responsive to stress regimens likely to be experienced by *A. fumigatus*.

Deletion analysis of the *cyp51A* promoter. Use of a reporter gene system facilitates the functional analysis of promoters (8). The *cyp51A* promoter plays an important role in azole tolerance in *A. fumigatus*, as most resistant isolates contain both a change in the coding sequence for the lanosterol 14α demethylase enzyme and a duplication of a 34-bp element present in the promoter region (31, 33–35, 37, 52). The presence of this duplication in the *cyp51A* promoter is referred to as the TR34 promoter. To evaluate the contribution of this 34-bp region in *cyp51A* to expression of the gene, several different constructs were prepared containing different segments of the *cyp51A* promoter fused to luciferase (Fig. 5A). The 34-bp region in *cyp51A* was duplicated to form *cyp51A*^{TR34} in two constructs (F2 and T2) as well as omitted from two others. All these fusion genes were integrated at the *pyrG*

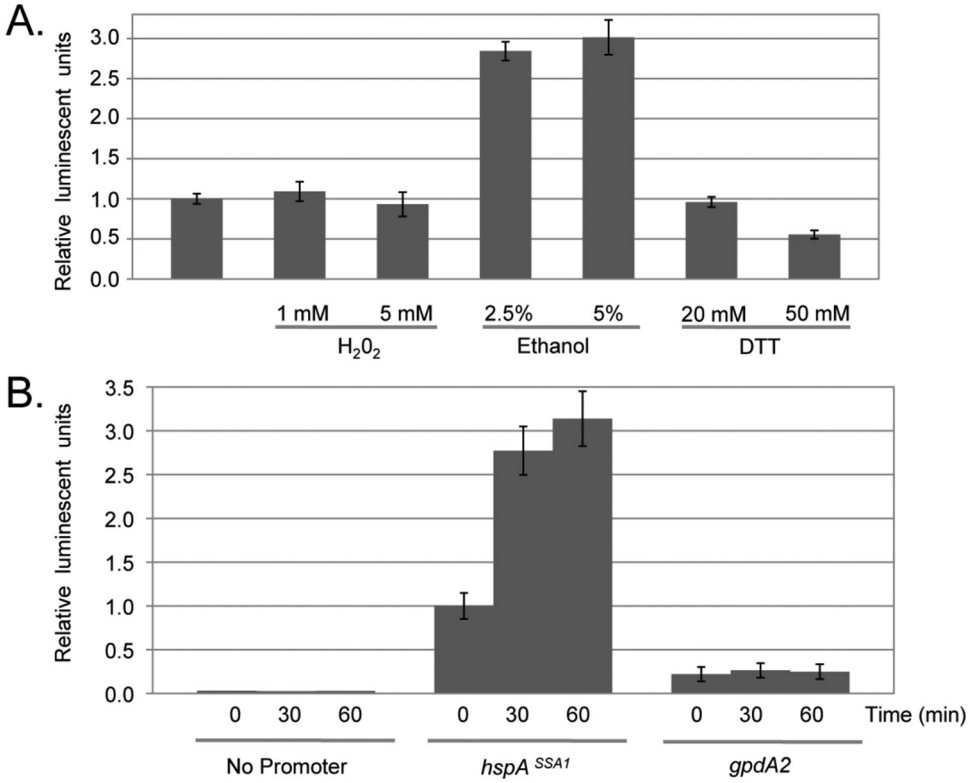

FIG 4 Analysis of the *hspA*^{SSA1} promoter. (A) A representative transformant containing the *hspA*^{SSA1}-luciferase fusion gene promoter targeted to the *pyrG* locus in AfS35 was grown in the presence of different stress conditions. In each case, the stress was given for 2 h before mycelia were harvested for luciferase assay. Luciferase activities were normalized to those produced by the *hspA*^{SSA1}-luciferase fusion gene in the absence of stress. (B) Mid-log-phase transformants containing the indicated luciferase fusions were stressed by the addition of 5% ethanol. Aliquots were removed at the indicated time points to assess the kinetics of *hspA*^{SSA1} promoter induction. As controls, the luciferase levels driven in the absence of an inserted promoter (No Promoter) or by the *gpdA2* promoter were also determined.

locus. Representative transformants were grown to mid-log phase, and their luciferase activities were determined. Relative luciferase values were obtained by normalizing to the activity produced by the wild-type *cyp51A* promoter containing 1.2 kb of 5′ noncoding DNA.

The effect of the TR34 element could be seen in the context of the 1.2-kb cyp51A promoter fragment, but the increased expression represented a modest change (Fig. 5). Interestingly, removal of the wild-type 34-bp region from the cyp51A promoter (F0) led to a 90% reduction in expression compared to the wild-type pro-

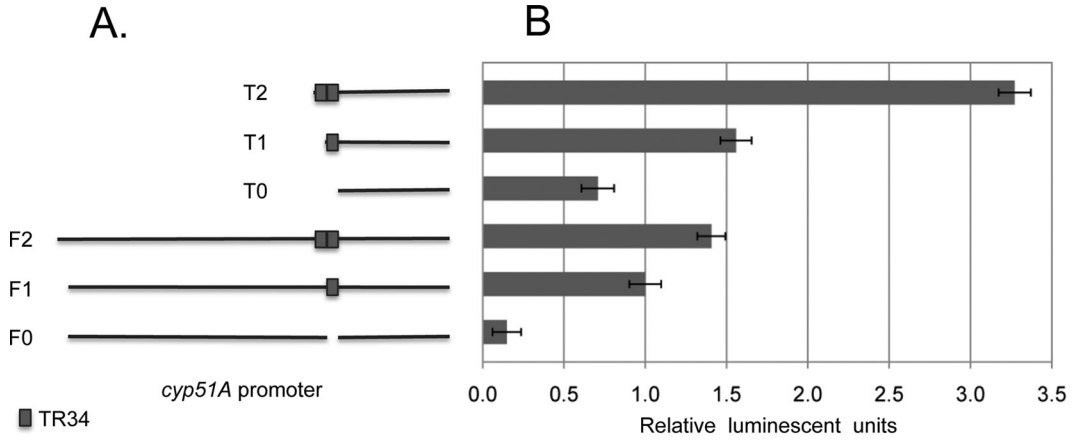

FIG 5 Deletion mapping the *cyp51A* promoter. (A) Maps of the various *cyp51A* promoter fusions constructed and tested for luciferase activity are shown. F0, F1, and F2 represent the presumably full-length *cyp51A* promoter with 0, 1, and 2 34-bp (TR) elements, respectively. T0, T1, and T2 represent the truncated *cyp51A* promoter with 0, 1, and 2 34-bp (TR) elements, respectively. (B) The luciferase activities produced by transformants containing integrated copies of the *cyp51A* promoter derivatives shown to the left are presented. Luciferase activities are normalized to those produced by the wild-type full-length *cyp51A* fusion gene.

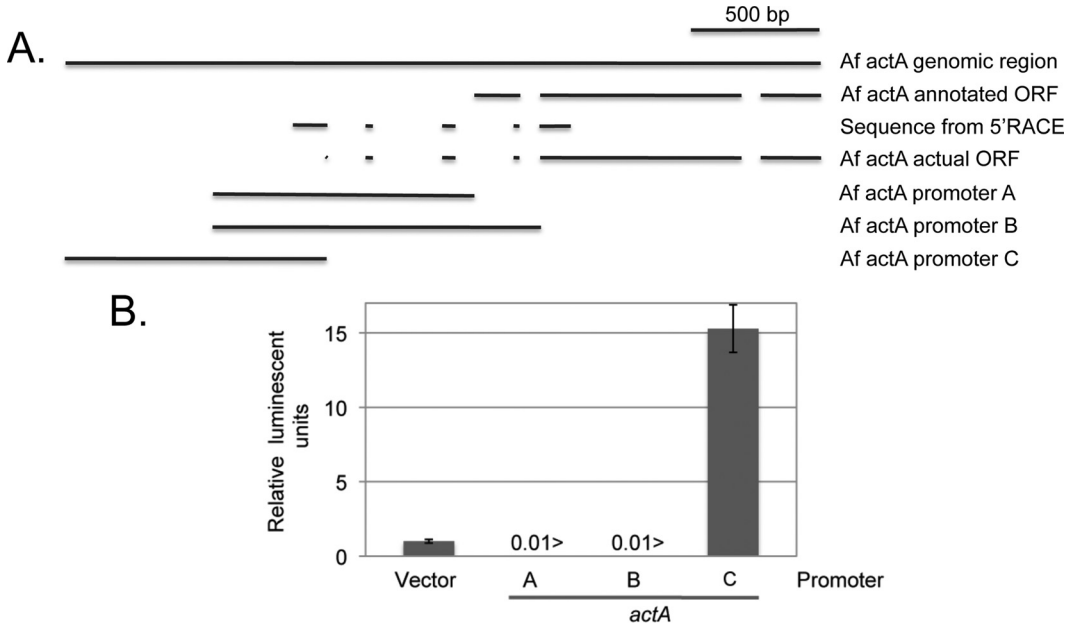

FIG 6 Analysis of the *actA* transcript and promoter. (A) Diagram of the theoretical and empirical 5′ region of the *actA* transcript is shown. Af actA annotated ORF refers to the open reading frame (ORF) expected from the current *A. fumigatus* annotation. Gaps represent predicted introns. Sequence from 5′ RACE denotes regions of the *actA* locus found in the cDNA product from this gene. actA actual ORF represents the location of the coding sequences for the actin derived from the cDNA sequence (and by comparison with *A. nidulans*). The bounds of the DNA fragments used to test for *actA* promoter activity as fusion genes to luciferase are indicated as A, B, and C. (B) The luciferase activities produced from transformants containing a single copy of the indicated *actA*-luciferase reporter genes. Vector denotes a transformant with no inserted *A. fumigatus* promoter. Enzyme activities were normalized to those produced in the absence of any inserted *A. fumigatus* promoter.

moter (F1). The 34-bp repeat plays a major role in function of the wild-type cyp51A promoter.

To examine the contribution of DNA regions upstream of the 34-bp element in *cyp51A* promoter activity, a series of 5′ truncation mutants were generated. A construct containing only 239 bp upstream of the *cyp51A* transcription start site (T0) still produced 70% of the luciferase activity driven by the full 1.2-kb promoter fragment. This −239 construct lacked the 34-bp element. Returning 1 copy (T1) or the duplicated copy (T2) of the 34-bp element led to a greater than 2-fold or nearly 5-fold increase in luciferase expression, again illustrating the stimulatory nature of this 34-bp repeat on expression.

Definition of the *actA* promoter. An initially puzzling finding came from attempts to use the *actA* promoter as a representative of a strong, constitutive promoter, as it often is in other fungi (32). Analysis of the *actA* mRNA by qRT-PCR indicated that this transcript was quite abundant (Fig. 2B). However, when 1 kb upstream of the presumptive *actA* ATG was inserted into the luciferase fusion plasmid, no detectable luciferase activity was produced (Fig. 6B). This first *actA*-luciferase fusion construct is referred to as the promoter A fusion. The failure of the promoter A fusion to drive luciferase prompted us to revisit the annotation of *actA* that indicated the first 50 amino acids of this fungal actin were unique to *A. fumigatus*. A second luciferase fusion gene construct (promoter B) was produced, forming a translational fusion between the predicted *actA* coding sequence and luciferase. The fusion joint within *actA* corresponded to a position that was highly conserved between actin proteins, although the 5′ end of this clone was identical to the promoter A fusion. This new chimera also failed to produce luciferase activity.

We next carried out 5′ RACE analysis of the 5′ end of the endogenous *actA* mRNA and found a very different splicing pattern than that predicted from the genomic annotation (Fig. 6A). Based on this new information, a third *actA*-luciferase fusion gene was constructed (promoter C). This luciferase reporter gene produced readily detectable enzyme activity and represented one of the strongest fusion genes produced here.

DISCUSSION

The work described here provides an important new tool for analysis of gene expression in the filamentous fungal pathogen *Aspergillus fumigatus*. Development of reporter gene systems was extremely valuable in the progression of the analysis of promoter function in *S. cerevisiae* (16) as well as *Candida albicans* (54). Previous studies in *A. fumigatus* have described the use of *lacZ*-based reporter systems (4, 27, 28, 50, 56) in addition to expression of firefly luciferase as an indicator of the presence of *A. fumigatus* in infection models (4, 5, 11, 13). Here we detail the generation of both integrating and autonomously replicating luciferase reporter vectors that will allow analysis and dissection of promoter regions from this fungus. Given the limited understanding of the mechanisms underlying control of gene expression in *A. fumigatus*, progress in this area is a crucial need. Comparison of the levels of the reporter gene fusions described here with their relative transcription levels assessed recently by RNA-seq (15) also supports the view that these constructs faithfully reflect native expression levels.

By the reporter gene analysis from this study, we provide new insights about the regulation and function of several *A. fumigatus* promoters. Here we demonstrate that the *hspA*^{SSA1} promoter re-

sponds to exposure to ethanol. These data also argue that the promoter region alone is sufficient to drive this ethanol induction of gene expression. We were surprised to find that this promoter was not activated by the presence of DTT, H_2O_2, or heat (data not shown). One possible explanation for the relatively limited range of induction of the *hspA*^{SSA1} promoter is the presence of multiple other hsp70 homologues in the *A. fumigatus* genome. This is common in eukaryotic organisms and suggests the increased specialization of Hsp70 function in this fungus as seen in other fungal species. DTT, for example, is often associated with an increase in the unfolded protein response and as such may signal directly to the endoplasmic reticulum (ER)-associated Hsp70 protein in *A. fumigatus*. This organism expresses an Hsp70 with a high level of sequence similarity with an ER-localized Hsp70 from *S. cerevisiae*: Kar2 (47) (designated AFUA_2G04620). This *A. fumigatus* gene may be the DTT-responsive Hsp70 in this fungus. The reporter gene system here will allow the comparison of these different Hsp70-encoding gene promoters.

One of the few examples of an *A. fumigatus* gene in which transcription has been implicated as an integral part of its function is the *cyp51A* gene. Changes in both the *cyp51A* promoter and its coding sequence are required to express a lanosterol 14α demethylase protein that confers resistance to azole drugs (34). Experiments from several groups have established that the mRNA levels of *cyp51A* are elevated in these azole-resistant strains (1). More recent data have implicated the hypoxic regulator SrbA in stimulation of *cyp51A* transcription upon depletion of oxygen (1, 3). The binding site for SrbA lies in the upstream region of *cyp51A* but has not been precisely defined. We believe that SrbA binds an element distinct from the 34-bp element, as previous experiments determined that a cyp51A promoter fragment lacking the 34-bp element was bound by recombinant SrbA (3). Further experiments are required to confirm this suggestion.

The preliminary analysis of the *cyp51A* promoter suggests the presence of multiple, interacting regulator sites in this transcriptional control region. Consideration of the effects of varying the 34-bp element from 0 to 2 (TR34) copies in the context of either 1.2 kb of 5′ flanking region from *cyp51A* or only 288 of upstream DNA demonstrated very different expression patterns. The 34-bp element was critically important in maintaining wild-type expression, while introduction of the TR34 repeat caused a modest increase in expression. Strikingly, the fusion construct containing 288 bp of 5′ flanking DNA drove 7-fold-higher levels of luciferase expression than a much larger construct lacking the 34-bp element. This suggests the possibility that negative regulatory elements upstream of the 34-bp region act to inhibit the function of the promoter. This inhibition is prevented in the presence of either 1 or 2 copies of the 34-bp element.

There also appear to be DNA sequences downstream of the 34-bp element that are significant contributors to *cyp51A* expression. These positive elements are also responsive to the presence of the 34-bp element, either in single or double copy. Taken together, these data support the view that upstream of the 34-bp element, negative contributors to *cyp51A* expression reside while downstream of this element, positive effectors will be found. This suggestion can now be readily tested by mutagenesis of the *cyp51A* promoter using the reporter gene system developed here.

The construction of the *actA*-luciferase genes also led to correction of the current annotation for this gene in the *A. fumigatus* genome. All reporter gene fusions were designed based on the annotation in the GenBank database and in every other case, these fusions behaved as expected. We selected *actA* for this analysis, as this gene is typically well expressed in other fungi. The finding that two different reporter constructs failed to produce any detectable luciferase activity prompted a more detailed consideration of the presumptive N terminus of *A. fumigatus actA*. Along with our 5′ RACE analysis, we also examined the known splicing pattern of *A. nidulans actA* (14) and found this to contain a very different transcript structure from that predicted by the *A. fumigatus* genomic annotation. This analysis also points out the utility of this reporter system to confirm the likely gene structure of *A. fumigatus* loci, irrespective of the presence of homologues in other fungi.

The 5′ RACE analysis provided several important findings related to *A. fumigatus* transcription. Most apparent was that the majority of transcripts exhibited a unique transcription start site, at least in this small sample of genes. This is in marked contrast to *S. cerevisiae*, in which global transcription start site mapping indicates that most gene contain multiple transcription start sites (58). The 5′ leader regions detected in the 5′ RACE analysis were also generally fewer than 70 nucleotides, with no AUG codons in this region. The two notable exceptions were the ABC transporter-encoding genes, in which both long leaders and an AUG codon were found. Preliminary data suggest that this leader is important for expression as deletion mutations removing the 3′ segment of the leader failed to express luciferase activity (data not shown), but more analyses are required to confirm this suggestion.

Increased availability of molecular biological tools for use in *A. fumigatus* is a necessary step toward accelerating the understanding of the biology of this important fungal pathogen. Even in the more extensively studied filamentous fungus *A. nidulans*, analysis of the molecular basis of transcriptional regulation remains at an early stage compared to research on organisms like *S. cerevisiae* (recently reviewed in reference 18). The combination of reagents aimed at the study of single genes, like the reporter system described here, coupled with genomic approaches such as RNA-seq and construction of a library of heterologously regulated genes (21), will help to refine our understanding of transcriptional mechanisms in this filamentous fungus.

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