Functional Analysis of DNA Sequences Required for Conidium-Specific Expression of the SpoC1-C1C Gene of Aspergillus nidulans

Kimberly E. Stephens, Karen Y. Miller, and Bruce L. Miller

Department of Microbiology, Molecular Biology and Biochemistry, University of Idaho, Moscow, Idaho 83844

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Stephens, K. E., Miller, K. Y., and Miller, B. L. 1999. Functional analysis of DNA sequences required for conidium-specific expression of the SpoC1-C1C gene of Aspergillus nidulans. Fungal Genetics and Biology 27, 231-242. The SpoC1-C1C gene is centrally located within the A. nidulans conidium-specific SpoC1 gene cluster. With one exception, the 14 genes within the cluster are coordinately regulated. C1C transcript is first detected late in conidiation, coincidental with the appearance of mature conidia, and accumulates ∼1000-fold in conidia. We show that C1C expression is restricted to conidia, with mRNA abundance decreasing immediately after induction of germination. C1C transcription and translation are not temporally separated and, similar to C1C RNA abundance, a C1C::β-galactosidase fusion protein is first detected with the appearance of mature conidia and decreases after induction of germination. Cell-specific C1C expression requires both a position-dependent mechanism of regulation, responsible for repression in hyphae, and a position-independent mechanism of regulation, responsible for developmental expression. We show by functional analysis of upstream DNA sequences that a 10-bp sequence and two adjacent 6-bp direct repeats are necessary for position-independent, conidium-specific expression of both the intact C1C gene and the reporter gene. At least one repeat (CAACAT) is required for normal levels of expression. We find that the C1C gene is not a direct target of the BrlAp and AbaAp developmental regulators, but of a yet unidentified conidium-specific transcriptional activator.

Asexual reproduction (conidiation) in the filamentous Ascomycete Aspergillus nidulans (Emericella nidulans) has provided an excellent system for understanding molecular genetic mechanisms that control developmental gene expression and the organization of multicellular structures in fungi (for reviews see Miller, 1990; Timberlake and Clutterbuck, 1994; Adams et al., 1998). Conidiation involves the differentiation of uninucleate cell types, including conidia, from multinucleate vegetative hyphae. Therefore recent studies have extended this analysis to include processes that modulate and coordinate cell cycle regulation with cell morphogenesis and developmental gene regulation (Harris, 1997; Marabito and Osmani, 1994; Dutton et al., 1997).

Conidia are formed on a multicellular reproductive structure, or conidiophore (Boylan et al., 1987; Mims et al., 1988). Conidiophore development begins with the differentiation of a hyphal element into a foot cell. The latter cell can be distinguished by the presence of thickened cell walls. The foot cell gives rise to an aerial hyphal stalk that grows by apical extension to a predetermined height. The stalk apex switches to an apolar growth pattern and enlarges to form a multinucleate vesicle. Buds appear synchronously over the surface of this vesicle and a single nucleus migrates into each bud. These cells differentiate
into uninucleate metulae, each of which subsequently buds two or three times to form phialide cells. Each phialide bud to form a daughter cell that eventually differentiates into a conidium. Continuing mitotic divisions of a phialide result in chains of conidia with each new conidium displacing previously formed conidia. Mature conidia are dormant (G1 arrested) and capable of surviving adverse environmental conditions.

Developmental induction leads to activation of a core regulatory pathway that directs terminal differentiation of conidia. Bristle (brlA), Abacus (abaA), and Wet-White Conidia (wetA) are the central components of a genetic network (brlA > abaA > wetA) that controls the expression of numerous conidiation-specific genes and drives terminal differentiation (Boylan et al., 1987; Adams et al., 1988; M irabito et al., 1989). Target genes have been placed into categories depending upon the temporal expression and combination of core regulators needed for their expression. These include Class A (early), Class B (spore/conidia-specific), Class C and D (phialide-specific), and Class D1 (WetA-induced phialide-specific) (M irabito et al., 1989; M arshall and Timberlake, 1991). Normal differentiation of the multicellular conidiophore required at least two additional developmental regulators, Stunted (stuA) and Medusa (medA). Stunted and Medusa modulate spatiotemporal expression of brlA, abaA, and a subclass of conidiation-specific target genes (M iller et al., 1992; B 1996; D 1997). The cis-acting response elements have been identified for BrLaP, a C1H2/Zn+2 finger protein; AbA, an ATTSE/TEA protein; and StuA, an APSES protein (Adams et al., 1987; A ndrianopoulos and Timberlake, 1991, 1994; C hang and Timberlake, 1993; D 1997). DNA-binding activities have not been demonstrated for WetA or MedA; however, ectopic expression activates transcription of some classes of conidiation target genes and brlA, respectively (M arshall and Timberlake, 1991; K . Y. M iller and B.L. M iller, unpublished).

Approximately 1200 different poly(A)+ RNAs are associated with conidiophore development and conidia formation ( Timberlake, 1980). Approximately 200 of these specifically accumulate in mature conidia. The products of these conidia-specific RNAs probably function in either conidia maturation or germination. Approximately 80% of these conidium-specific genes are organized into clusters, a feature that may be related to the regulation of the genes and/or to their evolution (Orr and Timberlake, 1982). The organization and regulated expression of one of these clusters, SpoC1, has been studied in detail (Timberlake and Barnard, 1981; Gwynne et al., 1984). The SpoC1 gene cluster extends over 38 kb and is flanked at either end by 1.1-kb direct repeats. (The 14 genes within the cluster, with one exception (L8B), are coordinately regulated with RNAs being present at 1 to 50 copies per conidium and either absent or present at low levels in hyphal and conidiophore tissue (Timberlake and Barnard, 1981; Gwynne et al., 1984.)) Both the levels of expression and the developmental specificity are greatest for genes, such as C1C, that are located in the central region of the cluster. Regulated expression progressively decreases for genes located away from this center. The expression of SpoC1 cluster genes is controlled, in part, by a regional (position-dependent) regulatory mechanism that represses expression in undifferentiated hyphae and may involve developmentally altered changes in chromatin conformation within the SpoC1 domain (M iller et al., 1987). Thus, the C1C gene is expressed at variably elevated levels in undifferentiated hyphae when it is repositioned outside the cluster. By contrast, C1C mRNA abundance always increased to normal levels in conidia regardless of the chromosomal position of the C1C gene. Therefore, a position-independent mechanism controls conidium-specific expression. This cell-specific expression requires 290 bp of C1C upstream sequences (M iller et al., 1987).

Specific information regarding upstream regulatory sequences of conidiation-specific target genes is limited. In two reported cases, rodA (a hydrophobin) and yA (conidial laccase) possess BrlA and AbaA response elements in 5’ sequences and require BrlA and/or AbaA for regulated expression (Stringer et al., 1991; C hang and Timberlake, 1993; A ramayo and Timberlake, 1993). However, the rodA hydrophobin is found in the conidiophore and conidial walls, while yA is apparently expressed in the phialide. This report focuses on a functional analysis of cis-acting sequences necessary and sufficient for the developmental regulation of an A. nidulans gene that is truly cell-specific and a member of the B class of genes as defined by M irabito et al. (1989). Therefore, the cis-elements described herein represent components of the genetic circuit regulating late, or terminal, phases of development. The functions of C1C and the other genes of the SpoC1 gene cluster are unknown (M iller et al., 1987; A ramayo et al., 1989). To provide additional insights into possible functions for C1C, and perhaps other genes of the SpoC1 cluster, we used a translational fusion gene to show that C1C transcription and translation are not temporally separated and that the C1C protein is probably a conidium-
specific intracellular protein. While the exact biological function for the C1C gene remains unknown, it has been possible to use the expression of this gene as a tool to understand mechanisms controlling cell-specific gene expression in A. nidulans.

**MATERIALS AND METHODS**

**Fungal and Bacterial Strains**

A. nidulans strains used in this study were FGSC 237 (pabaA1, yA2; trpC801; ΔC1C) and UCD 3 (pabaA1, yA2; trpC801; ΔC1C). Plasmids were propagated in Escherichia coli HB101 [F-, recA13, ara-14, proA2, lacY1, galK2, rpsL20 (Smr), xyl-5, mtl-1, supE44] and JM101 [Δlacpro, supE, thi, F’traD36, proAB, lacZM15]. E. coli strain TG1 [K12, Δ(lac-pro), supE, thi, hsdS/D5/F’traD36, proA+B+, lacI, lacZAM15] was transformed by recombinant M13 RF DNA molecules used for in vitro mutagenesis.

**A. nidulans Methods**

Cultures were grown in liquid or solid rich medium (YG-MTV) as previously described (Miller et al., 1987, 1992). A. nidulans strains were transformed as described by Yelton et al. (1984) and Miller et al. (1987). FGSC 237 was transformed with pC1ClacZ and UCD 3 with the C1C promoter deletion plasmids. Synchronous conidiation was induced and tissue harvested as described by Miller et al. (1987, 1992). Purified conidia were harvested as described by Miller et al. (1987).

**Nucleic Acid Manipulations**

A. nidulans DNA was isolated as described by Yelton et al. (1984). Total RNA was isolated as described by Miller et al. (1987). RNA and digested DNA were transferred to Hybond-N (Amersham Life Science), as recommended by the manufacturer. Blots were probed with random primed probes (Amersham Random Priming kit).

**Quantification of SpoC1-C1C Expression in Promoter Deletion Strains**

RNA was isolated from conidia and hyphal tissue of each promoter deletion strain and the control strain FGSC 237. Ten micrograms of total RNA from each sample was run on a gel and blotted onto Hybond-N nylon membrane. Replicate blots were probed with an argB-specific probe as an internal standard. Identical sets of blots were probed with the C1C internal 0.9-kb HindIII fragment (Fig. 1). Expression was quantitated using standard curves generated by blotting varying amounts (0–11 µg) of FGSC 237 conidial RNA and hybridizing with either an argB- or a C1C-specific probe. An LKB laser densitometer was used to quantitate signal intensities. The signal observed for 10 µg of FGSC 237 conidial RNA, relative to the argB signal from the same sample, was taken as 100% (argB expression does not change significantly during conidiation and therefore variations among samples were corrected using the argB signal as an internal control.) Quantification of C1C-probed conidial RNA required only a 5-h exposure to X-ray film, whereas the signal intensities of the C1C-probed hyphal RNA blots were only measurable after 5 days of exposure. Therefore, the argB signals from 5-day exposures were compared to the 5-h exposures for each RNA sample. This information was then used to estimate the signal intensities of C1C-probes hyphal RNA blots that would have been observed after a 5-h exposure. Replicate blots and measurements for each signal measured were within 10%.

**DNA Sequence Analysis**

Double-stranded and single-stranded sequencing was performed using Sequenase as described by the supplier (Amersham Life Science).

**S1 Analysis**

A kinased oligomer, 5’-AAATAGAGTGATACCACGTC-3’, complementary to the +68 to +97 region of C1C, was annealed to the single-stranded mp19BSII derived by cloning the BamHI/SstI fragment.
of C1C into M13mp19. The single-stranded DNA was isolated according to Messing (1983). Following annealing, the oligomer was extended with the Klenow fragment of DNA polymerase I and then cut with PstI. This produced a 224-bp labeled fragment which was cut from a denaturing agarose gel and electro-eluted (Maniatis et al., 1982). A total of 50,000 cpm of this probe was annealed to 25 µg of total RNA overnight at 60°C. The mixture was then treated with S1 nuclear according to Yelton et al. (1984) and Miller et al. (1992). The DNA/RNA hybrids were then precipitated, dissolved in TE and DNA sequencing stop dye, and loaded onto an 8% acrylamide–urea sequencing gel. The single-stranded mp19BSII template was sequenced with the same primer used in the S1 reactions and run alongside the S1 analysis lanes for reference.

**Construction of Plasmids Containing 5', 3', and Internal Deletions of the C1C Promoter Region**

A genomic 3.0-kb BamHI/EcoRI fragment containing the SpoC1C-C1C gene and essential 5' flanking sequences was cloned into pBR329 to create the plasmid pBE3 (Fig. 1). The 290 bp upstream of the C1C transcriptional start sites are sufficient for position-independent, conidium-specific expression (Miller et al., 1987). The sequence encompassing this region and the first 20 codons of the C1C open reading frame are shown in Fig. 2. The 5' deletions were derived from the plasmid pCDS5', which was constructed by subcloning the NruI/BglII fragment of pBE3 into the Smal site of the Bluescript M13 phagemid and then blunt ending the BglII overhang with Smal nuclease. The deletions were then generated by restricting pCDS5' with BstXI and BamHI, resecting with Exonuclease III, blunting with Mung bean nuclease, ligating. The internal deletions, designated the pL series, were constructed by combining selected 5' and 3' deletions. The EcoRI/SstI fragment of the desired pCDS5' deletion plasmid was subcloned into the EcoRI/SstI site of each 5',3', and internal deletion.

**In Vitro Mutagenesis**

The RAPI-like binding region (−97 to −87) of C1C was altered by in vitro mutagenesis using the Amerham oligonucleotide-directed mutagenesis kit, according to the manufacturer's instructions. A 31-mer oligonucleotide was used to replace 5 of 10 nucleotides within the wild-type core sequence, 5'-ACCCAAACATC-3', with an

```plaintext
-290
totaagtctt agaactaat cgaacctctg gcaactctg tctggtttna tttgggttcn ataatettt gataactgta gcaatcctgt tcaatcttga
-190
Psbl
totcctttt agactagctt gatagctct gtttggttt aacctctact ctgcagacta gttatggag aagactatgg ccaccaaca
-90  HindIII  -60  -50  -40  SmaI  -20  -10  tcaactgac cagacacttc atagccgata ggtactctgtc gctgctctg caaccaatgtacacgatcttct
20   10  30  50  70  80  90 (20)
tgcaacctt  asacctatc cagc
ATG  GAT  ACT  CGG  CTT  GAA  ATA  CTC  CCT  GCT  GAG  CTT  GAC  CTT  GCT  ATT  ACT  ACT  CTA
met  asp  thr  pro  pro  glu  ile  leu  pro  ala  cys  glu  leu  gln  arg  gli  ile  thr  leu
```

FIG. 2. Upstream sequences of the Aspergillus nidulans SpoC1-C1C gene. 5' sequences sufficient for conidial-specific SpoC1-C1C expression are illustrated (Miller et al., 1987). The complete sequence can be found under Accession No. M 83571. DNA sequence analysis predicts that the SpoC1-C1C gene encodes a 49,330-Da protein. Amino acids are numbered within parentheses to the right. A potential TATA-like sequence at −63 and a pyrimidine-rich tract between −25 and −10 are indicated by dotted underlining. A major (large arrow) and two minor (small arrows) transcriptional start sites are shown.
NRul and a Saci site. Mutated bases are indicated by underlining.

C1C-108 to −78 5′AAGAAAATGCCACCCCAACATACAACAT

GACCG3′

31-mer 3′TTCTTTAAACGGGCTCGAGTGTAGA

CTGGC5′

mutant C1C 5′AAGAAAATGCTCGGAGGTCAACAT

GACCG3′

The 31-mer oligonucleotide was annealed to the single-stranded template generated from an M13 recombinant, mp19BSII, and mutagenesis was performed according to manufacturer’s recommendations (Amersham Life Science). The RF form from several plaques was isolated and digested with NRul and SacI to determine if they contained mutated sequences. The clone found to contain the mutated sequence was sequenced. The 0.6-kb BglII/SstI fragment of the mutant RF was then cloned into the BamHI/SstI site of pCD S5′ to generated pM98. The 4.1-kb Xhol/Xhol fragment of trpC was then dropped into the Sall site of this plasmid. The final plasmid is designated pMT98.

Construction of the pC1ClacZ Reporter Plasmid

pL5 contains a 5′ deletion of C1C sequences upstream of −169, but maintains wild-type levels of C1C expression (Figs. 4 and 5). pL5 was derived from the plasmid pCDS5′ as described above. The 3.0-kb lacZ BamHI fragment of pM C1871 (Casadaban et al., 1983) was subcloned into the single NRul site of pL5 to which BamHI linkers (CG-GATCCG) had been added. The resulting construct created a translational fusion between amino acid 178 of C1C and amino acid 9 of lacZ (Casadaban et al., 1983; Kalnins et al., 1983). The C1C::lacZ junction was confirmed by DNA sequencing. In addition, pC1ClacZ (11.5 kb) contains a copy of the A. nidulans trpC gene as selectable marker.

β-Galactosidase Assays

Protein extracts from hyphal and developmental cultures lacking conidia were prepared and β-galactosidase assays performed as previously described (Miller et al., 1992). Endogenous β-galactosidase activity in A. nidulans was repressed by growing strains in medium containing 2% glucose (Fantes and Roberts, 1973; van Gorcom et al., 1985). Protein extracts from purified conidia and developmental cultures with conidia were prepared slightly differently. Conidia were collected from plates in 0.01% Tween 80, 0.05 M Tris–HCl, pH 8.5, washed one time with cold sterile distilled, deionized water, and then suspended in 3 ml of extraction buffer. An equal volume of Z buffer (0.06 M Na2HPO4, 0.04 M NaH2PO4, 0.01 M KCl, 0.001 M MgSO4, 0.05 M β-mercaptoethanol, pH 7.0) and 6 g of acid-washed 250- to 300-µm glass beads were added. The conidia were vortexed for 30 s followed by 30 s on ice, and this was repeated for a total vortexing time of 4 min. The conidial debris was pelleted at 15,000 g for 15 min at 4°C. The supernatant was collected and assayed in the same manner as the hyphal extracts. Protein extracts from conidiating cultures were prepared as for conidia except the tissue was first ground in liquid nitrogen. The protein concentration of extracts was determined by the method of Bradford (1976).

RESULTS

Transcription and Translation of C1C are Both Conidium Specific

The C1C gene has previously been shown to produce a highly prevalent mRNA expressed specifically in conidia during asexual reproduction (Timberlake and Barnard, 1981; Gwynne et al., 1984). However, a function for this gene during conidiation has not yet been identified. We wished to determine if the C1C mRNA was not actually translated, but served as storage RNA in dormant conidia and as a source of nucleic acids during early germination. Alternatively, transcription and translation of C1C might be temporally unlinked with translation occurring in germings. In this case, the C1C protein might play a role in the early stages of germination rather than during conidium differentiation. Therefore, pC1ClacZ was used to transform FGSC 237. This construct has 169 bp of upstream C1C sequences that are necessary to drive conidium-specific expression (see below and Figs. 4 and 5). The transformant, LC-9, has a single copy of the C1C::lacZ reporter gene integrated at the trpC locus and therefore outside of the SpoC1 cluster. LC-9 was selected because our objective was to look at position-independent regulation of C1C.

C1C::lacZ mRNA abundance followed the same pattern of conidium-specific expression as the native C1C transcript. Both RNAs were virtually absent in hyphae and
present at greatly elevated levels in conidia (Fig. 3). A sharp decline in C1C and C1C::lacZ mRNA abundance was observed immediately after induction of germination. Quantitative laser densitometry showed a 10-fold drop in C1C transcript levels and a 6-fold drop in C1C::lacZ transcript levels within the first 15 min (Fig. 3). No transcripts could be detected after 30–60 min.

β-Galactosidase (β-GAL) activity was measured to determine the temporal relationship between transcription and translation and to attempt to determine the cellular localization of the C1C::β-GAL fusion protein. The β-GAL activity of the conidia could not be extracted with standard extraction buffer (50 mM sodium phosphate, pH 7.0, 1 mM EDTA, 20 µM PMSF), high salt extraction buffer (0.5 M NaCl), or detergents. β-GAL activity could only be detected after breaking open the conidia by vortexing them with 250-30-µm glass beads. Therefore, it appears that the fusion protein is intracellular and not targeted to the cell membrane or conidial wall by the 178 N-terminal amino acids of the C1C protein. The intact C1C protein is also most likely to be intracellular because its 446-amino-acid sequence does not predict the presence of membrane spanning domains or features, suggesting that it is not a cell wall-associated protein (Accession No. M83571).

The temporal pattern of β-GAL activity during conidiation reflected C1C::lacZ mRNA abundance (Table 1). Vesicles and metulae were present by 10 h. At 17 h postinduction one to two conidia/chain were present. By 24 h, long chains of mature conidia were observed. β-GAL activity in conidiating LC-9 cultures was similar to hyphal values up to 10 h postinduction. At 17 and 24 h β-GAL activity increased 2.3- and 4.6-fold, respectively. These levels of β-GAL activity are low relative to isolated conidia due to the large amounts of developmental tissue in these samples that are not expressing the C1C::lacZ reporter gene. This distortion is apparent when the values from total 24-h tissue (2.78 units) and from conidia isolated from a 24-h time point (17.0 units) are compared. The β-GAL activity of isolated LC-9 conidia was 26-fold that of LC-9 hyphal tissue (Table 1) when compared on a per milligram protein basis and 23-fold when compared on a per gram tissue basis. An apparent 9-fold difference between FGSC 237 conidia and hyphal values in Table 1 appears to be due to an artifact. It was observed that the addition of 1 M Na2CO3 (used to stop the β-GAL assay) to conidial debris suspended in assay buffer without ONPG resulted in extraction of the yellow pigment of the FGSC 237 conidia. No yellow pigment was evident when FGSC 4, having the wild-type yA gene and green-pigmented conidia, was treated similarly. To minimize this artifact, conidial debris was pelleted and removed before the assays were performed.

The inability to separate cell types makes it impossible to determine precisely whether β-GAL activity is present in immature conidia or other cell types. However, it can be concluded that β-GAL activity, like the C1C and C1C::lacZ

### FIG. 3
Expression of the SpoC1-C1C and SpoC1-C1C(p)::lacZ genes during germination. Fresh LC-9 conidia were collected from plates in 0.01% Tween and, within 5 min, were used to inoculate a flask containing liquid medium. (Conidia representing t = 0 were not inoculated into medium.) Conidia were shaken at 250 rpm at 37°C. At the desired time point, the conidia were pelleted and washed in cold, sterile distilled, deionized water. These last two manipulations required approximately 30 min. Total RNA was subsequently isolated from each sample and 10 µg loaded per lane. The blot was probed with the 32P-labeled 0.9-kb internal HindIII fragment of C1C. Numbers above each line indicate minutes postinoculation when sample was taken. hyp, hyphae.

### TABLE 1
β-Galactosidase Activities of LC-9 and FGSC 237 during Conidiation

<table>
<thead>
<tr>
<th>Hours postinduction</th>
<th>β-Galactosidase activitya</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LC-9</td>
<td>FGSC 237</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.61</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.53</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>1.43</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>2.78</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>Isolated conidia</td>
<td>17.00</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td>Hyphae</td>
<td>0.66</td>
<td>0.06</td>
<td></td>
</tr>
</tbody>
</table>

a Hyphal cultures of LC-9 and FGSC 237 were grown for 17 h in YEG and plated to induce conidiation as described under Materials and Methods. The LC-9 transformant has 5’ C1C sequences −169 to −1 driving lacZ expression.

b nmol of o-nitrophenol/min/mg of protein. Replicate assays and treatments were within 10%.
transcripts, is detected at 17 h postinduction, a time corresponding to the appearance of mature conidia.

A rapid decline in β-GAL activity reflected the loss of C1C and C1C::lacZ mRNA abundance during germination of LC-9 conidia (Table 2). After 1 h 59% of the activity remained, 30% after 2 h, and only 9% after 3 h. To determine that this decrease in β-GAL activity was not due to a rapid increase in the total protein during germination, β-GAL activity based on a per gram tissue basis was also calculated. Similar results were observed. Therefore, transcription and translation of C1C::lacZ, and by analogy C1C, are temporally linked throughout the A. nidulans life cycle.

**Novel Response Elements Are Required for Conidium-Specific C1C Expression**

The 290 bp upstream of the C1C transcriptional start sites are sufficient to drive position-independent, conidium-specific expression (Miller et al., 1987). This region has a potential TATA box at −62 and a pyrimidine-rich tract from −11 to −26 (Fig. 2). Although developmentally regulated expression of C1C is dependent upon functional BrlAp and AbaAp, these 5’ flanking sequences lack response elements for either transcriptional regulator (BrApRE = M RAGGGR; AbaApRE = CATT CY). Plasmid constructs with 5’, 3’, and internal deletions of the 5’ flanking sequences were generated to identify novel regulatory elements controlling conidium-specific expression. These constructs were used to transform the A. nidulans strain UCD 3, in which the internal 0.9-kb HindIII fragment of the resident C1C gene had been deleted. Potential position effects were avoided by selecting only transformants in which integration had occurred by homology at the trpC locus. RNA was isolated from undifferentiated hyphae and from isolated conidia of each selected transformant and used for Northern blot analysis (Fig. 4). Signal intensities were quantitated using laser densitometry. The average peak area values are represented on a relative percentage basis, with wild-type conidial values expressed as 100% (Fig. 5).

Sequences upstream of −115 can be deleted without affecting regulated expression of the C1C transcript (Figs. 5 and 6; deletions L2, L5, L9). By contrast, transcript abundance in conidia is only 7% of wild-type levels when sequences upstream of −72 are deleted (L7). Deletion of nucleotides upstream of −42 results in complete elimination of C1C expression in conidia (L10). This deletion eliminates the TATA-like at position −62. The L14 transformant was used as a control because it lacks the transcriptional start sites and, as expected, did not express the C1C transcript. An internal deletion from −121 to −73 (LST2) also reduced conidial expression to 7% of wild-type levels and confirmed the importance of sequences between −115 and −73 for conidium-specific regulation. Transcript levels in conidia were reduced to 29% of the wild type when nucleotides −83 to −73 were deleted (LST1). Thus sequences between −115 and −83 allow intermediate levels of conidium-specific expression, although, sequences between −83 and −73 are also required for wild-type levels.

The C1C transcript is undetectable in wild-type hyphae. However, when the C1C gene is integrated at the trpC locus, expression is low, but detectable in hyphae (0.7% of wild-type conidia). Deletion strains L2, L5, and L9 expressed this same low level of C1C transcript. Abundance of the C1C mRNA in the hyphae of the LST1 strain was only slightly reduced, whereas hyphal expression in the L7 and LST2 strains was reduced to ∼10% of wild-type levels. Extended exposures of Northern blots show apparent C1C transcripts in hyphae of deletion strains L10 and L12. These strains both retain the pyrimidine tract upstream of the +1 site. However, this expression may be an artifact with transcription starting within the upstream pUC13 vector sequences. In fact, one blot (not shown) revealed that the bands appearing in hyphae of L10 and L12 were actually larger than predicted for the C1C mRNA. Therefore these signals were considered as background. The transcriptional start sites for each of the C1C promoter deletion strains was mapped using S1 nuclease (data not shown). In each case, where C1C transcript was present in conidia (L9, L7, LST1, LST2, MT98) the transcriptional start sites were the same as in wild type. S1 products using

### TABLE 2

β-Galactosidase Activity of LC-9 Conidia during Early Stages of Germination

<table>
<thead>
<tr>
<th>Hours postgermination</th>
<th>β-Galactosidase activity^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>17.0 (100%)</td>
</tr>
<tr>
<td>1</td>
<td>10.1 (59%)</td>
</tr>
<tr>
<td>2</td>
<td>5.1 (30%)</td>
</tr>
<tr>
<td>3</td>
<td>1.6 (9%)</td>
</tr>
</tbody>
</table>

^a nmol of o-nitrophenol/min/mg of protein. Replicate assays and treatments were within 10%. The LC-9 transformant has 5’ C1C sequences −169 to −1 driving lacZ expression.
RNA from L10 and L12 transformants were either absent or indicated aberrant initiation.

**A RAP1-Like Response Element Enhances C1C Expression in Hyphae**

In addition to the unique 11-bp sequence between −83 and −72, the region from −115 to −83 contains several sequences of interest (Fig. 6). These include two 6-bp direct repeats (−95 to −84) and a RAP1-like response element (−99 to −89). Saccharomyces cerevisiae RAP1 (Repressor/Activator Protein) is a sequence-specific DNA-binding protein that binds to [C(1–3)A]n tracts at telomeres, elements at the silent mating type loci, and promoter elements of numerous genes (reviewed in Shore, 1994). A comparison of the upstream C1C sequence and known RAP1 binding sites is shown in Fig. 7. Site-directed mutagenesis was used to eliminate the RAP1-like element, while maintaining proper spacing of other potential promoter sequences (Fig. 6, MT98). Conidium-specific C1C expression in mutant strain MT98 was similar to wild type. However, hyphal abundance of C1C RNA was reduced to approximately 10% of wild-type levels (Figs. 4 and 5). Mutants L7 and LST2 also lack the RAP1-like sequence and have the same reduced levels of hyphal expression as MT98. By contrast, the RAP1-like sequence is still present in LST1 and this strain shows normal C1C expression in hyphae. It should be noted that the LST1 linker, which replaces the deleted region, is 10 bp long. Therefore, the spacing and/or the orientation of potentially interacting proteins should not be affected.

**DISCUSSION**

The analysis of differentiation in filamentous fungi has largely focused upon global regulatory networks involved in the initiation of asexual or sexual reproduction and the elaboration of multicellular structures and tissues that make up the reproductive apparatus. However, there have been only limited studies describing cis or trans components regulating cell-specific gene expression. For conidiation in *A. nidulans*, this has been problematic because only vegetative hyphae and conidia can be isolated in pure form. We initiated a study of SpoC1-C1C gene expression because it is a conidium-specific gene. Furthermore, little information is available about late, or terminal, components of the developmental regulatory network when C1C transcription occurs. Previous studies have shown that the C1C RNA is present at vanishingly low abundance in vegetative hyphae. During conidiation, C1C RNA abundance begins to increase with the appearance of conidia and accumulates ~1000-fold in mature conidia. The

![Northern blot analysis of SpoC1-C1C expression in promoter mutants.](image-url)
function of the C1C gene appears to be highly cell specific. C1C RNA abundance decreased 10-fold within the first 15 min after inducing germination and was negligible 30–60 min after germination. A C1C::lacZ reporter gene mimicked this pattern of expression at both the transcriptional and translational levels. β-GAL activity was reduced by approximately 90% by 3 h after germination and was probably negligible by the time of germ tube emergence at 5–6 h. No phenotype has been associated with a C1C loss of function mutation and the sequence has been uninformative. Furthermore, Aramayo et al. (1989) did not observe an obvious phenotype when the entire SpoC1 cluster was deleted. The authors attributed this to the existence of redundant functions or to an unidentified and highly specialized role for the SpoC1 genes. Our results provide some insight into possible functions for the C1C gene.

**FIG. 5.** Relative expression of the SpoC1-C1C promoter mutants. Each C1C promoter mutation is diagramed. The signal intensities from Northern blots similar to those in Fig. 5 were quantitated by densitometry and the values represented on a relative percent of wild-type conidial C1C transcript, the latter being assigned as 100%. The start of transcription is indicated by an arrow. A TATA-like sequence and the pyrimidine tract (−25 to −10) are indicated. Con, conidia. Hy, hyphae. The asterisk indicates that no C1C transcript is detected in hyphae of the wild type where the C1C gene is at its normal chromosomal position.

**FIG. 6.** Nucleotide sequence of the putative SpoC1-C1C UAS. The nucleotide sequence of the putative C1C UAS along with the C1C promoter mutations used to define this region. The putative UAS exists between −115 and −73. This region contains two direct repeats indicated by solid arrows and a potential RAP1-like binding site indicated by the dashed arrow. Altered nucleotides in the MT98 mutant are indicated above the line.
product. Transcription of the C1C gene and mRNA translation are not significantly separated temporally. This suggests that the C1C mRNA probably is not a stored transcript that is translated during early germination. Nor is the sole purpose of the C1C mRNA to function as a source of ribonucleic acids during early germination. Rather, the C1C protein may be important either during conidial maturation or the earliest stages of conidium germination. It is also possible that the C1C protein represents a class of soluble storage proteins used during germination. The C1C gene, or other SpoC1 cluster genes, might be expendable under laboratory conditions, accounting for the apparent lack of phenotype in mutants.

The C1C gene has provided a valuable tool for investigating mechanisms controlling late development and cell-specific gene expression. Both position-dependent and independent mechanism control C1C expression. Position-dependent mechanisms repress expression of C1C and the other SpoC1 cluster genes in undifferentiated hyphae. However, one or more transcriptional activators are required for conidium-specific expression. We identified three regions between −115 and −72 that are required for normal position-independent expression in undifferentiated hyphae or regulated expression in conidia. These cis-regulatory elements are necessary and sufficient to drive conidium-specific expression of a heterologous gene.

Nucleotides −99 to −83 resemble a S. cerevisiae RAP1 binding site. In addition to binding telomere sequences, the yeast RAP1 protein binds numerous sites that include the yeast mating-type silencers, the UAS of MATα mating type locus, the UAS's of yeast ribosomal protein genes (TEF1, TEF2, RP51A), and UAS's of the yeast glycolytic enzyme genes PYK (pyruvate kinase), ADH1 (alcohol dehydrogenase 1), and PGK (phosphoglycerate kinase) (Ogden et al., 1986; Shore and Nasmyth, 1987; Shore et al., 1987; Nishizawa et al., 1989). In yeast, RAP1 plays the role of a general transcription factor and functions to repress or activate transcription based upon the context of adjacent transcription factors with which it physically interacts (Shore, 1994). Site-directed mutagenesis or deletion of the RAP1-like sequence in strains MT98, L7, and LST2 caused a 90% reduction in hyphal expression. A comparison of strains MT98, LST1, LST2, and L7 demonstrates that the putative RAP1-like protein affects only hyphal expression and that conidium-specific expression is regulated independently.

Nucleotides −83 to −72 are the most critical for cell-specific expression, although two direct repeats (CAACAT) directly upstream (−95 to −83) contribute to overall expression in conidia. One of these repeats is eliminated in the MT98 strain, indicating that only one repeat is essential for wild-type levels of expression in conidia. We cannot formally eliminate a role for nucleotides −115 to −99; however, the simplest model would be one in which a single transcriptional activator binds to the 6-bp repeat and one to nucleotides between −83 and −72. In the case of strain LST1, the former factor might bind weakly in the absence of the second factor to provide reduced levels of conidial expression. In either case, the factor(s) binding at sequences −95 to −73 enhance conidial expression. It is noteworthy that although deleting sequences upstream of −72 reduces C1C expression to 7% of wild-type levels, RNA abundance in conidia of strain L2 remains 10-fold greater than in undifferentiated hyphae. This suggests the −98 to −73 region may be an upstream activating sequence, or enhancer, but that additional factors at sites downstream, including the TATA box at −62, may contribute to conidium-specific regulation. Such factors might include development-specific TATA-binding factor associated factors (TAFs) or components of the transcriptional pre-initiation complex (PIC) similar to those observed in other systems (Hahn, 1998; Ranish et al., 1999).

Further experiments are required to define clearly the
nature of the conidia-specific regulation. However, we can conclude that conidium-specific C1C gene expression is the result of transcriptional activation in conidia and not repression in hyphae. C1C expression required BrlAp, AbaAp, and WetAp (Timberlake and Barnard, 1981; Marshall and Timberlake, 1991). However, regulation by BrlAp and AbaAp must be indirect. Neither BrlAp nor AbaAp response elements are present upstream of the C1C transcriptional start site. WetAp is a putative transcriptional activator, but potential response elements and DNA-binding activity have not been reported. It is possible that the conidium-specific cis-element(s) we have identified includes a WetAp binding site. We compared the 5′ flanking sequences of three other coregulated genes of the SpoC1 gene cluster to important upstream SpoC1-C1C sequences that we have identified. We found that the SpoC1-C1D, SpoC1-C1A, and SpoC1-C1E do not share any of the regulatory elements identified in our study, nor were any other homologous sequences evident (K. Y. Miller and B. L. Miller, unpublished). Therefore, we can also conclude that while these four SpoC1 genes are coordinately expressed during conidiation, they must be direct targets of other factors that represent branch points in the regulatory network controlling late development and conidial differentiation.

REFERENCES


