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Molecular Analysis of a *Neurospora crassa* Gene Expressed during Conidiation

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The asexual developmental pathway in the life cycle of the filamentous fungus *Neurospora crassa* culminates in the formation of spores called conidia. Several clones of genomic *Neurospora* DNA have been isolated that correspond to mRNA species expressed during conidiation and not during mycelial growth (V. Berlin and C. Yanofsky, Mol. Cell. Biol. 5:849–855, 1985). In this paper we describe the characterization of one of these clones, named pCon-10a. This clone contains two genes, *con-10* and *con-13*, which are induced coordinately during the later stages of conidiation. The two genes are separated by 1.4 kilobases of DNA; they are located on linkage group IV and are transcribed from the same strand of DNA. The molecular organization and sequence of one of these genes, *con-10*, and its flanking regions are presented. Full-length cDNA clones for *con-10* also were isolated and sequenced, and transcription-initiation and polyadenylation sites were defined. The *con-10* gene contains an open reading frame interrupted by two small introns and encodes an 86-amino-acid residue polypeptide that is both hydrophilic and weakly acidic. Expression of the *con-10* gene in various mutants defective at different stages of conidiation indicates that it plays a role after aerial hyphal development. Possible functions, organization, and regulation of conidiation-specific genes are discussed.

Under vegetative conditions, *Neurospora crassa* grows as a mycelium composed of branching hyphae. During the asexual phase of its life cycle, aerial hyphae develop perpendicularly from the mycelium. Asexual spores called macroconidia develop at the distal ends of branches of these aerial hyphae (49; macroconidia will be called conidia throughout this paper). The environmental and physiological factors that trigger this process are unclear but are thought to involve dessication and nutrient deprivation (40). Microscopic studies have shown that developmental stages in the conidial pathway are fairly ordered (37). Since major changes in gene expression occur during the asexual cycle of *N. crassa*, as shown by alterations in steady-state levels of many proteins and mRNAs (7, 55), it is likely that conidiation involves selective expression of subsets of this organism's genes. Some changes associated with conidial development appear to involve shifts in metabolic functions (12), whereas others lead to the synthesis of novel structural components (4). Genetic studies with *N. crassa* have identified many loci involved in conidiation (37, 44, 50). Mutations in these genes result in an altered morphology of the culture attempting conidiation or prevent conidiation, but little is known about the lesions responsible for these phenotypes. In the related filamentous fungus *Aspergillus nidulans*, three genes that cause defective conidial development have recently been cloned (10, 27). Initial characterization has shown that these genes are transcribed only during conidiation.

We would like to determine the regulatory events that mediate the process of conidiation. Our approach has involved the isolation of clones corresponding to genes that are differentially expressed during the asexual cycle. This was accomplished by screening an *N. crassa* genomic library with a cDNA probe enriched in sequences expressed in conidiating cultures (8). The clones obtained were defined by the time of appearance of their mRNAs. This report presents an analysis of one of these clones, pCon-10a. The series of genomic clones Con-1 to Con-12 described by Berlin and Yanofsky (8) have been renamed pCon-1 to pCon-12 to distinguish them from the genes they encode. Consequently Con-10a will be referred to as pCon-10a throughout this paper. pCon-10a was initially selected for analysis since it hybridizes to two transcripts, 0.66 and 1.35 kilobases (kb) in length, which first appear at approximately the same time, 4 to 8 h after induction of conidiation. These transcripts were not detected in mRNA isolated from mycelia. The genes corresponding to the 0.66 and 1.35-kb transcripts have been named *con-10* and *con-13*, respectively.

In this paper we present a physical characterization of the pCon-10a clone. The transcribed regions were defined by Northern analyses, and the DNA sequence of *con-10* and its flanking regions was determined. Full-length cDNA clones for *con-10* were isolated from a conidial cDNA library prepared in λgt10. The cDNA clones helped to identify the open reading frame of *con-10* and defined the positions of two small introns. The *con-10* gene encodes a polypeptide predicted to be 86 amino acid residues in length. Expression of *con-10* is greatly reduced or absent in the aconidial mutants *fl*, *acon-2*, and *acon-3*, indicating that transcription of *con-10* is dependent on prior development events that cannot occur in these mutants. Our findings will facilitate studies aimed at determining the factors and sites responsible for differential expression of conidiation-specific genes and elucidating the functions of the proteins these genes encode.

MATERIALS AND METHODS

Strains and vectors. The strains of *N. crassa* employed in this study are shown in Table 1. Cultures were grown as described by Berlin and Yanofsky (7). Subclones for sequencing were constructed in the vectors M13mp10, M13mp11 (41), M13mp18, and M13mp19 (59) and pUC118

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TABLE 1. *N. crassa* strains used in this study

Strain[a]	Mutation (allele)	Description
74-OR23-1*A*		Wild type
RS91	Aconidiate 2 (*acon-2 A*)	Macroconidiation blocked at 34°C, some conidia formed at 25°C
RS503	Aconidiate 3 (*acon-3 A*)	Macroconidiation blocked
UCLA37	Conidial separation 1 (*csp-1 A*)	Conidia fail to separate
UCLA101	Conidial separation 2 (*csp-2 a*)	Conidia fail to separate
UCLA191	Easily wettable (*eas a*)	Rodlets lacking from surface of conidia
p	Fluffy (*fl A*)	Macroconidiation blocked

[a] All strains have been described by Perkins et al. (44).

and pUC119 (56). Propagation of the clones was in *Escherichia coli* TG1 [K-12; Δ(*lac-pro*) *supE thi hsd*Δ*5*/F′*traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*Δ*M15*].

Isolation of nucleic acids. Plasmid DNA was isolated by alkaline lysis (9). Large-scale preparations were banded in cesium chloride-ethidium bromide gradients. Total RNA and poly(A)$^+$ RNA were purified as described by Berlin and Yanofsky (8).

Preparation of DNA probes. Radiolabeled probes were prepared by hexamer priming (18). ^{32}P-labeled cDNA was synthesized as described by Berlin and Yanofsky (8).

Filter blot hybridizations. Northern analyses were carried out as described previously (8). Southern analyses were performed with the hybridization and washing conditions described previously (51).

DNA sequencing and computer analysis. DNA was sequenced by the M13 dideoxy chain-termination method of Sanger et al. (47) as described by Bankier and Barrell (2). The entire sequence was determined on both strands by using overlapping clones. Templates were prepared by subcloning the appropriate restriction fragments or by the exonuclease III-S1 nuclease method of Henikoff (24). In several instances, additional sequence data were obtained from a template by priming at an internal site with a custom-synthesized oligonucleotide. Using similar strategies the sequence of the cDNA clone for *con-10* was determined on both strands.

Sequences were entered, assembled, and edited by using the GELIN, DBAUTO and DBUTIL programs of Staden (52, 53) run on a Digital Equipment Vax computer. The sequence was analyzed using the programs of the University of Wisconsin Genetics Computer Group (15).

Synthesis of cDNA library. The RNA used for the cDNA library construction was isolated from a 24-h-old conidiating culture of *N. crassa*, 74-OR23-1*A*, grown as described by Berlin and Yanofsky (7). Cultures containing conidia, aerial hyphae, and mycelial mats were disrupted by passage twice through a French press under a pressure of 20,000 lb/in^2. RNA was isolated by the guanidinium thiocyanate method (13). A cDNA library was prepared in phage λgt10 by a modification of a previously described procedure (20). As starting material, total RNA was used instead of poly(A)$^+$ RNA. Synthesis of the first-strand cDNA was carried out with two samples of 25 μg of total RNA, each contained in a reaction volume of 40 μl. Second-strand synthesis was performed with 30 U of *E. coli* RNase H per ml and 460 U of DNA polymerase I per ml. Then standard methods were used to obtain blunt-ended molecules, protect internal *Eco*RI sites with *Eco*RI methylase, and ligate phosphorylated *Eco*RI linkers. After cleavage with *Eco*RI to generate *Eco*RI sites at both ends of each cDNA molecule, the cDNA was separated from the cut linker molecules by electrophoresis on a 1% agarose gel. The cDNA molecules larger than 214 base pairs (bp) were electroeluted onto DEAE membranes (NA45; Schleicher & Schuell Co.). After the bound material was washed in water and low salt, approximately 70% could be eluted in 1.5 M NaCl–5 mM EDTA–20 mM Tris hydrochloride (pH 8.0). The cDNA molecules were ligated into *Eco*RI-cut λgt10, packaged in vitro, and screened. From 50 μg of total RNA as starting material, 2.5 × 10^5 independent clones were isolated.

Screening and hybridization. The library was screened at a density of 5,000 PFU/150-mm plate as described by Huynh et al. (26). The DNA from λ plaques was transferred to nitrocellulose as described by Maniatis et al. (36). All filters were hybridized in 5× SSPE (1× SSPE is 0.15 M NaCl, 0.01 M NaH_2PO_4, 0.001 M EDTA [pH 7.4])–0.3% sodium dodecyl sulfate–50% formamide–100 μg of denatured salmon sperm DNA per ml at 42°C as described by Beatty and Cohen (3) and then washed three times for 10 min each at room temperature with 2× SSPE–0.1% sodium dodecyl sulfate and once for 30 min in 1× SSPE–0.1% sodium dodecyl sulfate at 50°C.

Primer extension. A synthetic oligonucleotide complementary to nucleotides 1750 to 1774 in the *con-10* sequence (see Fig. 3) was used for primer extension to locate the 5′ end of the *con-10* transcript; 1.5 ng of this oligonucleotide and 5 μg of poly(A)$^+$ RNA isolated from conidiating cultures 16 h after induction were precipitated and suspended in 10 μl of hybridization buffer (0.1 M NaCl, 20 mM Tris hydrochloride [pH 7.9]). The sample was heated to 100°C for 2 min, transferred to 42°C, and incubated at this temperature for 3 h. Extension reactions contained 5 μl of the annealed primer-RNA solution, 50 mM Tris hydrochloride (pH 8.3), 0.14 M KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 4 mM sodium pyrophosphate, 400 μM each dATP, dGTP, and TTP, 20 μCi of [α-^{32}P]dCTP, and 20 U of avian myeloblastosis virus reverse transcriptase in a total volume of 50 μl. The samples were incubated for 30 min at 42°C, 5 μl of a solution containing 5 mM each dATP, dGTP, dCTP, and TTP was added, and the reactions were continued for 15 min at 42°C. Reactions were stopped by the addition of EDTA to 10 mM, precipitated with ethanol, and analyzed on a standard sequencing gel.

S1 nuclease mapping. The 3′ end of the *con-10* transcript was mapped by S1 nuclease protection studies. Hybridizations and digestions were essentially as described by Berk and Sharp (6) with 10 μg of poly(A)$^+$ RNA and 50,000 Cerenkov cpm of end-labeled probe at 52°C. The probe used extended between the *Bgl*I and *Eco*RI sites shown on the right-hand side of Fig. 2 and was uniquely labeled on the 3′ terminus at the *Bgl*I site.

RESULTS AND DISCUSSION

Localization of the pCon-10a transcripts. The genomic clone pCon-10a was described previously (8). This clone hybridizes with two mRNAs from conidiating *N. crassa* cultures, 0.66 and 1.35 kb in length. These transcripts are not found in RNA isolated from mycelial cultures or young conidiating cultures (2 and 4 h after induction of conidiation) but are present at low levels in RNA cultures that are 8 h old and at high levels by 12 h. Synthesis of these two messages is therefore likely to be induced between 4 and 8 h into conidiation (8).

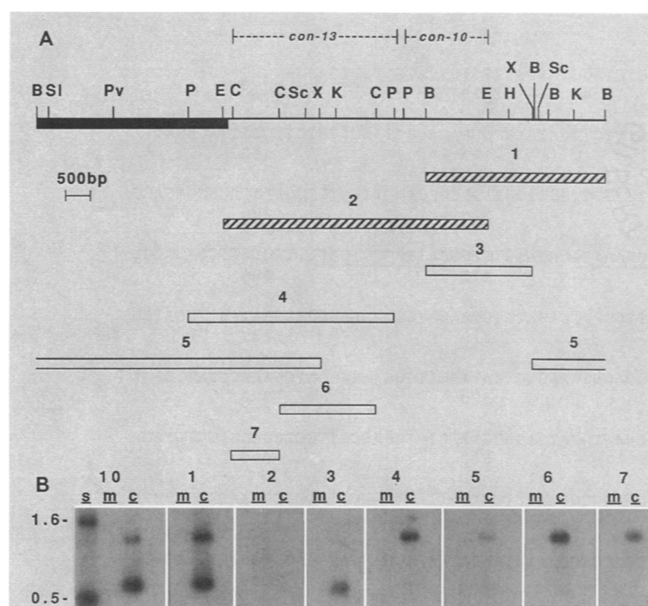

FIG. 1. Localization of pCon-10a transcripts. The top portion of the figure contains a restriction site map of pCon-10a. The single line represents *N. crassa* cloned DNA, and the filled bar identifies the vector pRK9 (48). (A) Fragments 1 and 2 (▨) were deleted from pCon-10a, and the remaining DNA was used as a probe. Fragments 3 through 7 (▭) were gel-purified restriction fragments. (B) The isolated fragments or deletion derivatives of pCon-10a were radioactively labeled and used to probe Northern blots of poly(A)$^+$ RNA isolated from mycelia (m) or conidiating cultures (c). The number above each set of lanes indicates which fragment or deletion derivative was used as a probe. Panel 10 was probed with the intact parental plasmid, pCon-10a. DNA markers together with their sizes (kilobases) are indicated in the left-hand lane (s). Restriction sites: B, *Bam*HI; Sl, *Sal*I; Pv, *Pvu*II; P, *Pst*I; E, *Eco*RI; C, *Cla*I; Sc, *Sac*I; K, *Kpn*I; H, *Hin*dIII; X, *Xho*I. The exact order of the *Bam*HI and *Xho*I sites in close proximity on the right-hand side is not known.

A restriction map of pCon-10a is shown in Fig. 1. Northern analyses localized the regions of pCon-10a that specify the 0.66- and 1.35-kb transcripts. Two deletion derivatives of pCon-10a were prepared by removal of either the *Bam*HI fragment labeled 1 or the *Eco*RI fragment labeled 2. Northern blots were performed with radioactive probes prepared from these two derivatives. The DNA remaining after removal of fragment 1 hybridized with the two conidial RNA species (Fig. 1B, panel 1). In contrast, the DNA remaining after removal of fragment 2 did not detect these RNA species (Fig. 1B, panel 2). These two experiments indicated that most of clone pCon-10a corresponding to the genes of interest lay in the central portion of the restriction map. Northern blots probed with the radioactively labeled restriction fragments designated 3, 4, 5, 6, and 7 located the positions of the two genes more precisely (Fig. 1). The region between the *Pst*I and *Eco*RI sites labeled *con-10* hybridized mainly to the 0.66-kb transcript. Likewise, the region between the *Pst*I site and *Cla*I site near the junction of *N. crassa* and vector DNA labeled *con-13* corresponded mainly to the 1.35-kb transcript.

Both genes were transcribed from the same strand of DNA (left to right in Fig. 2). The direction of transcription of *con-10* was determined by probing dot blots of single-stranded M13 clones containing fragments of the *con-10* gene with ^{32}P-labeled cDNA synthesized in vitro from conidial poly(A)$^+$ RNA. The direction of transcription of *con-13* was

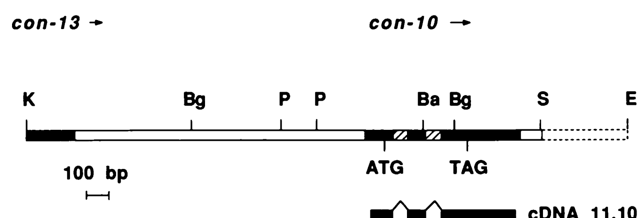

FIG. 2. Organization of *con-10* and its relationship to *con-13*. The sequenced DNA extends from the *Kpn*I site to the *Sau*3A site indicated. Symbols: (▬) maximum extent of the transcribed DNA, (▨) introns. The longest *con-10* cDNA clone (cDNA11.10) is displayed underneath. The translation initiation and termination codons for *con-10* and relevant restriction sites are shown. Only the *Sau*3A site used to create exonuclease III deletions is shown. This site is not unique. The region from the *Sau*3A site to *Eco*RI site (dotted) was not sequenced. Restriction sites: K, *Kpn*I; Bg, *Bgl*I; P, *Pst*I; Ba, *Bam*HI; S, *Sau*3A; E, *Eco*RI.

determined by hybridizing Northern blots of conidial RNA with gel-purified single-stranded probes prepared from M13 templates. In both cases, the results (not shown) were consistent with transcription proceeding from left to right.

Isolation of cDNA clones for *con-10*. Approximately 50,000 plaques from the conidial cDNA library were screened with a radioactively labeled probe prepared from a gel-purified preparation of the *Pst*I-*Bam*HI fragment that covers the 5′ end of the *con-10* gene (see above). Between 70 and 80 positive plaques were identified, of which 20 were purified. A Southern blot of *Eco*RI-digested DNA prepared from these clones probed with the same *Pst*I-*Bam*HI fragment revealed that the clones contained DNA inserts ranging in size from 0.5 to 0.75 kb that hybridized with the probe (data not shown). Northern blot analysis had shown that the *con-10* transcript was 0.66 kb (8) (Fig. 1). The *Eco*RI fragments from five λgt10 clones were subcloned into the *Eco*RI site of pUC119 in both orientations, and the single-stranded M13 form was induced and sequenced. Four of the clones, named cDNA4.10, cDNA11.10, cDNA18.10, and cDNA20.10, were found to be identical, although of different lengths. The DNA of the fifth clone appeared to be internally rearranged and was not pursued further. The 5′ ends of the cDNAs corresponded to positions 1674, 1691, and 1713 of the genomic sequence, and their homology with the genomic DNA ended at bp 2377 or within the cluster of A residues between bp 2379 and 2384 (Fig. 3). Beyond this point, bp 2379 to 2784, A residues were present in the cDNA corresponding to the mRNA poly(A) tail. From this information the open reading frame corresponding to *con-10* was located within the genomic sequence. The sequence of the longest clone, cDNA11 · 10, was determined on both strands.

Isolation of cDNA clones for this gene precludes the possibility that *con-10* might have represented a developmentally regulated nonpolyadenylated RNA species.

DNA sequence of the conidiation-specific gene *con-10* and its flanking regions. The structure of the *con-10* gene and flanking regions is shown in Fig. 2, and its DNA sequence and corresponding amino acid sequence are shown in Fig. 3. The sequence of the cDNA clones located the position of the open reading frame for the *con-10* gene and defined intron and exon boundaries (Fig. 2). In addition, the presence of poly(A)$^+$ tails at the end of each cDNA confirmed the direction of transcription of *con-10* (*Pst*I to *Bam*HI to *Eco*RI). Since *con-13* is transcribed in the same orientation as *con-10*, the sequence shown in Fig. 3 therefore represents the 3′ end of *con-13*, approximately 1.4 kb of intergenic

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KpnI           con-13
GGTACCAAATTCAGGGTCTGAAGCGGGAACTATGATCGATTCCAGGTCCTGGGCTCTAGCTGTGAGTTCAGTCAGGGTGTTGAGGAAGTTGCGAGGCCTC
    10                  30                  50                  70                  90

AGTTGTGAGCGACGTCATCAAACCGTCTCCTTTTGGGATAATGATAACCTTTTATTTCTGGATAACTGGGACAGGTTAGGCTGTCTTTGTCGATAGACTA
   110                 130                 150                 170                 190

GGTACGTAAGAATTGATTTGATGCTTGTTCGATGCTTTTAAGTTGTTGTCGCTTGTGGTTGCGAGGTAGTCGGCAGGTTTGTTTGGATAGACGGGAGACG
   210                 230                 250                 270                 290

CCCACTCGCACCCAGGGCGATGAATAACGAAGGCCGATGGCTCTTTCCATGTGGGAAATACACAAGTCTGGCATTGTCCACTTGTTTGTCTTCGAGCGGG
   310                 330                 350                 370                 390

GTTACGATTTCTGTCAAGCCCTTTGCTCCTTTCTTCCGAGAACAAAGGAAGTTTTCGATCCAGATCGCCAACATCCGAAAAGGGAGGAATAGTTCGATCG
   410                 430                 450                 470                 490

ATGTACCTTGACGGCTCGGCCATCGATCTGATCTGCATTTCCCACTCTGGATTCCAGGGGAAGGGTCATATGATGGAAACGAGATCGAAACCCATTGAGT
   510                 530                 550                 570                 590

ATTTGAGCTTGTCGTCTATCCCGCCTCATATATTGTAGTTGTAGCCATATGGACAAGTGCATGAGACGTTGTTCTGGAGCATTGCTCGACCAGCTGTAGA
   610                 630                 650                 670                 690

CTAACCGCCTGGTATATGCTGAACGAGGAATCTATCGAGTCCGGCCGATGATTCGAGCCGCAACCTCATCATCACTTGGCTTGTCAGGAAATTTCACAAC
   710                 730                 750                 770                 790

ACCAAACCCGCCGCGTGGGCCTTTGCTGGTTTCGCACGTCGATGTGCCTAAATCGACCTCCAAAGTCAGATCAATCGATTACGAAGTGGCTGAGCGGTTG
   810                 830                 850                 870                 890

TGGAAGCTCCCAAACAAACAAACAAACAAACAAACAAACATTAGGTATGAACCGACAAAGTGATGCGACTCGCCGTGCCATGAAGCACTTTTTGTCATTA
   910                 930                 950                 970                 990

TGTTACAAATCGAATGAAAGAATGCAAACCAAAGACGAAGTGAAATGGTGACGAGCTGATTATGAAATTTACACTAGCTGGTTGAAAGATTTGGGGCTGA
  1010                1030                1050                1070                1090

CAGGCACGGCAATGCCGCTGTGGGGTTAGTGTGGGGCCCAGGGAGCTTATTCCCCGCGTGATGACGCTTACACAGGAGACTAGCGCAGGGCCGTAGGTCA
  1110                1130                1150                1170                1190
                                              PstI
CCCCGACCACTCACAAACGACGCTTCTTGCCTTGACCCCGCTGCAGCCTGGAAGCTGCCGAAAGCAAAGGGTCTTGGCACCTCCAAATCGATCCGAATTG
  1210                1230                1250                1270                1290

TTGACATTTCCAAATGCGGCTGCACCAGATGGACAGGTTGGGATCGACGAGATGGATCGAACCCGATTTAGGTCGCTGTCAGAATCTCAGCTGTTGGTAT
  1310                1330                1350                1370                1390
               PstI
ACCGAATCCGATCTCTGCAGTTGGCATCACGTTGACATCACCATCTCTCATTCTGAACCGATCGCGAGATGCAAAGGGCTACTGTACTATGCGGATGAGA
  1410                1430                1450                1470                1490

CGAAAACGAACATTCAGTGGCAGATAGCATCTCATCATACAAACTGACATCACAAATTCACAATAGTCAGGTTGCTGGTTTACATCAGTTGTTGTTTTTG
  1510                1530                1550                1570                1590
                                                                  con-10
TGTATATAAGCATCTCTTATCCTCCTCCCAATCGAACAATATCTTCTCCTCCATCAAGCAACTCATCAACAGTCAACAGCATCCACTCAACAACTTCAAC
  1610                1630                1650                1670                1690

                                          M   A   G   T   G   N   D   N   P   G   N   F   A   N
GCACAACTACTACAAATCACATTTTCCTAAACAACATCGTCAAC ATG GCT GGC ACT GGT AAC GAC AAC CCC GGC AAC TTC GCC AAC
  1710                1730                1750                1770

R                      intron 1                            P   K   E   E   V   Q
CG GTATGTTTCCCATCATTATCTCCTCAACCACACTGCCAAGACACTTTGCTAACATAATCCATTTCTCCAG C CCC AAG GAA GAG GTT CAG
1790                1810                1830                1850                1870

A   I   A   S   K   G   G   Q   A   S   H   S   G   G   F   A   S   M   D   P   E   K   Q
GCC ATC GCC TCC AAG GGT GGT CAG GCC AGC CAC AGC GGA GGC TTC GCC AGC ATG GAT CCC GAG AAA CAG GTATGTTT
            1890                1910                1930                1950

                  intron 2                                    R   E   I   A   S   K   G   G   K
TTCATTTCCACACCTCTTTGGAGTGAACCATGACTGAGCGTTGACCAACACATGAACCCAAAACAG CGC GAA ATT GCT TCC AAG GGC GGC AAG
      1970                1990                2010                2030

A   S   S   G   S   F   E   P   G   S   E   K   A   R   E   A   G   R   K   G   G   K   A   S   G
GCC TCC AGC GGC AGC TTT GAG CCT GGC TCC GAG AAG GCC CGC GAG GCC GGT CGC AAG GGC GGC AAG GCT TCC GGT
2050                2070                2090                2110

G   T   G   A   D   D   D   E   *
GGC ACT GGT GCC GAC GAC GAT GAG TAG AGGTCATCAACCTTTTACTACGACTACCATCATCTGATTTTGACCTGGCGCACGGGATACGAAT
    2130                2150                2170                2190                2210

TAATGACTTTTGTCTTTGGATGACGGAACTGTTACAACAGCACGGGATATGACACGGGGTTGCAATTGGGATTTATTTGGCTGGCGTGAGAAGGCGCACT
      2230                2250                2270                2290                2310

CAGATAATTTTGCTTTGACTCGGTTAGCACCTTTCTCTAGGCAACTGAATGCTTTGACAATTTGCTAAAAAGACTTGTTCACTGTTCACGTCGTGAAATA
      2330                2350                2370                2390                2410
                                                                                            Sau3A
CAAAACTCCTGTGACATCGGTTATCTTAAACGTGTAATCCTTGGTCTGGGATGCTGCACTTGGTTCGTGGTGGGCAAAGGAGTTGGTTTGTCGATCA
      2430                2450                2470                2490                2510
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Intron	Size (nt)	5' splice site	internal consensus	Distance between internal consensus and 3' splice site	3' splice site
1	70	GTATGT	GCTAACA	13	CAG
2	74	GTATGT	ACCAACA	13	CAG
N. crassa consensus	53-323	GTA(A/C)GT	(A/G)CT(A/G)ACX	6-20	(C/T)AG
Yeast consensus		GTATGT	TACTAACA	5-53	(C/T)AG

FIG. 4. Intron splice signals of *con-10*. The *N. crassa* consensus was compiled from the following genes: *am* (28), ADP-ATP carrier (1), copper metallothionein (39), iron-sulfur subunit of ubiqinal-cytochrome *c* reductase (23), plasma membrane ATPase (22), *his-3* (34), histones H3 and H4 (58), *qa*-1S (25), and *tub-2* (42). The yeast consensus was described by Langford et al. (33).

DNA, and the complete *con-10* gene. We were interested in obtaining the sequence of the region upstream of *con-10* since it would contain the *con-13* transcription-termination region and would be valuable for the construction of transcriptional and translational fusion derivatives of *con-10*. Since transcription of *con-10* and *con-13* is induced at approximately the same time in conidiation (8), it is conceivable that these two genes are regulated in a similar manner and share upstream control features.

The *con-10* coding region is divided by two introns, 70 and 74 nucleotides in length. Both introns possess highly conserved 5' and 3' splice signals in addition to an internal element that is probably involved in lariat formation (Fig. 4) (46). The 3' end of each intron occurs at the first CAG sequence after the internal conserved element. Similar intron splicing signals are found in *Saccharomyces cerevisiae* (33).

The coding sequence of the *con-10* gene was comprised of three exons, 115, 88, and 358 bp in length. The G+C content of the segment of DNA sequenced was 49%, slightly lower that the published value of 54% for the whole *N. crassa* genome (57). However, the G+C content of the exons was 61 to 67%, significantly higher than the 41 to 44% found in the introns and 39 to 45% found in the 200 bp of DNA flanking the exons. Translation of the cDNA sequence for *con-10* in the three reading frames showed that each was open for at least 50 codons starting at approximately 80 to 100 bp into the cDNA sequence. However, the open reading frame selected was the only one with an ATG initiation codon near its beginning. This ATG was in a proper sequence context (see below). The open reading frame showed a codon usage profile typical for an *N. crassa* gene (data not shown). These characteristics include the finding of pyrimidines in the third position in preference to purines in amino acid families where a choice existed; additionally, codons terminating with a G or C residue were found more often than those terminating with an A or T residue. The codon usage of the proposed open reading frame for *con-10* followed these guidelines; notably only 1 codon out of 52 had a purine in the third position rather than a pyrimidine in the relevant four- and six-codon families. Because the *con-10* open reading frame only encoded an 86-residue polypeptide, a statistical analysis of its codon usage was not possible. Therefore we could not determine whether *con-10* showed the polarized codon usage of abundantly expressed genes such as *am* or *tub-2* (28, 42) or the less selective usage characteristic of weakly expressed genes, for example, *his-3* or *trp-1* (34, 48).

The selected open reading frame of the *con-10* gene encoded a polypeptide of M_r 8,568 composed of 86 amino acid residues. This polypeptide was weakly acidic in nature. Surprisingly, the following related peptide motifs were present in the predicted *con-10* polypeptide; IASKGG, IASKGGKAS, and KGGKAS. The method of Kyte and Doolittle (32) was used to examine the hydropathy index of the protein. The results indicated that the predicted *con-10* polypeptide was hydrophilic throughout, a characteristic not observed in typical globular or membrane-bound proteins. The algorithm of Chou and Fasman (14) predicts that the conformation of the *con-10* polypeptide is mainly α-helical in character. No significant homologies were found when the amino acid sequence of the *con-10* polypeptide was compared with the protein sequences contained in the National Biomedical Research Foundation Protein Identification Resource (19) by the program FASTP (35).

The entire genomic sequence was surveyed in both orientations for other open reading frames. Procedures that detected the three exons of *con-10* did not locate any additional significant open reading frames. Examination of the sequence for other interesting features located a seven-copy repeat of the motif CAAA upstream of the *con-10* transcript (bp 911, Fig. 3). There are a number of potential stem-loop structures at the 3' ends of both the *con-10* and *con-13* genes (K. Hager, A. Roberts, and C. Yanofsky, unpublished observations). Four examples are marked in Fig. 3. The functional significance, if any, of these sequences and structures awaits further study.

Transcriptional analysis of the 5' end of the *con-10* gene. The positions of the 5' ends of the cDNA clones gave an indication of where the *con-10* transcriptional start might lie

FIG. 3. Nucleotide sequence of *con-10* and flanking regions. The mRNA-equivalent strand is shown with the predicted amino acid sequence in the one-letter code above. Introns are labeled. Transcription-initiation sites of *con-10* determined by primer extension analysis are indicated (●); polyadenylation sites of *con-10* determined by S1 mapping analysis are indicated (↓). The 3' end of the mature *con-13* transcript determined by S1 mapping analysis and sequencing of cDNA clones (unpublished data) is marked (↓) at bp 234. The *con-10* translation stop codon (*) and relevant restriction sites are indicated. Boxes identify various noteworthy sequence elements: the Goldberg-Hogness box (TATAA) and CCAAT sequence (CAAAT). Other interesting features are underlined: a capping sequence at bp 1651, a common 3' sequence at bp 2389, and the CAAA repeated motif at bp 911. Four examples of potential stem-loop structures are indicated by horizontal arrows.

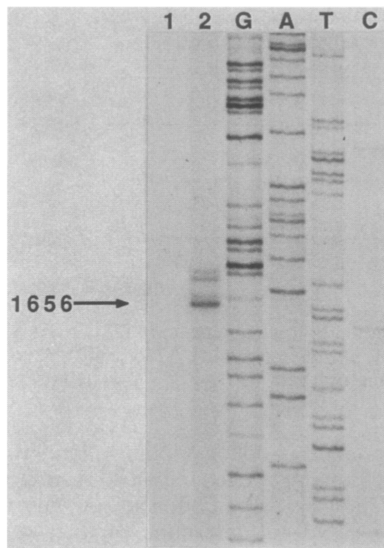

FIG. 5. Primer extension mapping of the 5′ end of *con-10* mRNA. Primer extension products complementary to poly(A)$^+$ RNA isolated from mycelial and conidial cultures are shown in lanes 1 and 2, respectively. Next to the extension (lanes GATC) are shown the dideoxy sequencing ladders for the *Bam*HI-*Pst*I fragment that contains the 5′ end of *con-10* (Fig. 2) primed with the same oligonucleotide used in lanes 1 and 2. The major transcription start site located at bp 1656 (Fig. 3) is indicated by an arrow.

(bp 1674 to 1713; Fig. 3). Precise mapping of the 5′ end of the *con-10* transcript by primer extension detected six start sites between bp 1652 and 1660 (Fig. 3), of which the start at bp 1656 was most prominent (Fig. 5). No primer extension products were detected when mycelial poly(A)$^+$ RNA was used as the template for reverse transcriptase (Fig. 5), consistent with previous Northern analyses (8). These results were confirmed by dideoxy sequencing of poly(A)$^+$ RNA with the same primer as that used for primer extension (data not shown). From these results it is apparent that the isolated cDNA clones were almost full length. The transcriptional starts lie 85 to 93 bp upstream of the ATG initiation codon.

One of our main interests is the regulation of conidial gene expression. Examination of the 5′-flanking DNA of *con-10* has identified a number of sequences that may be important for transcription. Sequence elements that are involved in transcription in higher eucaryotes include the Goldberg-Hogness box of TATAAATA (11) and the CCAAT box (16, 17). Near the 5′ end of translated transcripts the capping sequence of PyCATTCPur is found (54). In general, simple comparisons have revealed that these sequences are present in some *N. crassa* genes but not others, although as yet too few *Neurospora* genes have been sequenced for consensus elements to be clearly defined. We identified a potential Goldberg-Hogness box sequence for *con-10* of TATAA, located 51 bp from the major RNA initiation site (Fig. 3). In addition, a potential candidate for a CCAAT element (CAAAT) was located 104 bp from the major transcription initiation site of *con-10* (Fig. 3). Several sequences resembling the capping consensus were found near the transcription initiation site; the one nearest the 5′ end of the mRNA (PyCATCAPur) is underlined in Fig. 3 (bp 1651 to 1657). The function of potential *N. crassa* expression sequences will require experimental validation.

Mapping the 3′ end of the *con-10* transcript. The 3′ end of the 0.66-kb *con-10* transcript was mapped by using RNA

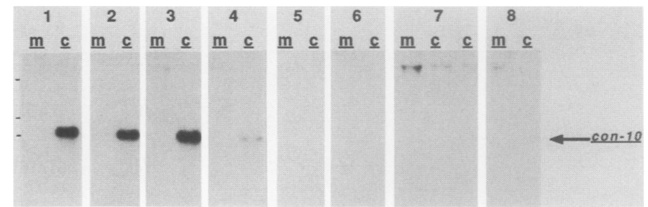

FIG. 6. Northern blots of RNA from *N. crassa* conidial mutants probed with *con-10* cDNA. RNA was extracted from mycelial (m) and conidial (c) cultures of each mutant and run in adjacent lanes. The time in parentheses refers to the time during conidiation at which RNA was prepared. Panels 1, wild type (24 h,; 2, *csp-1* (16 h); 3, *csp-2* (16 h); 4, *eas* (16 h); 5, *fl* (16 h); 6, *acon-3* (16 h); 7, *acon-2* at 34°C (16 and 28 h); 8 *acon-2* at 25°C (30 h). The small lines on the left-hand side refer to DNA size markers of 1875, 910, and 657 nucleotides.

prepared from conidiating cultures. A cluster of major bands, spanning a region of about 32 bp from positions 2374 to 2405 (Fig. 3), was protected from S1 nuclease digestion (data not shown). This region was located 225 to 256 bp beyond the TGA stop codon. No bands were protected from S1 nuclease digestion by RNA isolated from mycelial cultures (data not shown). All four cDNAs terminated between bp 2377 and 2384 (Fig. 3), beyond which poly(A)$^+$ tails were visible. These positions agree with those determined by S1 mapping analysis.

The *con-10* gene does not contain a sequence resembling the poly(A)$^+$ recognition element of AATAAA in its 3′-flanking DNA (45). However, a sequence similar to another conserved element at the 3′ end of many transcripts (TTTT CACTGC) (5) was found near the polyadenylation sites of *con-10* between bp 2389 and 2998 (Fig. 3).

Translational signals. The nucleotide sequence immediately adjacent to the initiation codon is important for ensuring correct translation (29). This sequence (GCC$^{A}_{G}$CCATGG) is conserved in higher eucaryotes (30) and *N. crassa* ($^{A}_{G}$T CA$^{A}_{C}$AATGG; compiled for 20 genes). The sequence around the *con-10* initiation codon, GTCAACATGG, matches the *N. crassa* consensus well.

Expression of *con-10* in conidiation-defective mutants. In experiments designed to assess the role of *con-10* in conidiation, expression of this gene was examined in certain *N. crassa* mutants with lesions that prevent normal conidial development. *con-10* was not expressed in RNA prepared from *fl* or *acon-3* mutants (Fig. 6, panels 5 and 6) and only very weakly expressed in the *acon-2* mutant grown at the nonpermissive temperature, 34°C (Fig. 6, panel 7). Expression in *csp-1* (panel 2), *csp-2* (panel 3), and *eas* (panel 4) was at a level similar to that of the wild type (panel 1), with the *eas* sample showing a slight reduction.

To show that equal amounts of RNA were loaded in each lane of the gel used for Northern analysis of *con-10*, we stripped the blot and reprobed it with a cDNA clone for the *N. crassa* gene (*crp-1*) encoding a cytoplasmic ribosomal protein (31). The results of this blot (not shown) revealed that similar relative levels of this RNA species were present in each conidiating sample and each mycelial sample when mutants and the wild type were compared. Thus the absence of the *con-10* transcript in the mutants *fl*, *acon-2*, and *acon-3* reflects the deficiency of this species from the cellular RNA pool.

The *csp-1* and *csp-2* mutants conidiate in a normal manner, except that individual conidia do not separate from the proconidial chains, possibly due to a defective autolytic

activity (40, 50). Likewise, *eas* cultures lack rodlets from the conidial surface but in other respects conidiate relatively normally (4). Since these mutations are confined to a discrete late stage in conidiation, not unexpectedly *con-10* is expressed in these strains. In contrast, the *fl*, *acon-2*, and *acon-3* lesions introduce early blocks in conidial development, and are relatively pleiotropic; aerial hyphae are formed to various extents, but conidia are not produced (37, 44). The observation that *con-10* is expressed only weakly or not at all in these strains suggests that this gene is involved in a process that occurs after aerial hyphal growth and is likely to be involved in events that are specific to the conidia themselves. (Although *acon-2* is temperature sensitive at 34°C on agar slants, a small number of empty-looking conidia were produced at 34°C under our conidial induction conditions, which may account for the low levels of RNA detected in this strain; at 25°C, *acon-2* conidiated very slowly.)

Function of *con-10* and *con-13* during conidiation. In this paper we characterized a region of the *N. crassa* genome that contained two adjacent genes, *con-10* and *con-13*, that were expressed during the late stages of conidiation. The mRNA for both genes accumulated at about the same time during conidiation, and transcription proceeded from the same strand of DNA. Since *con-10* and *con-13* shared a number of features, it is probable that their expression is regulated by a common mechanism. However, much more *con-10* mRNA was generally seen than *con-13* mRNA.

In *A. nidulans* there is considerable evidence that conidiation-specific genes are clustered (21, 43). Several of the conidiation-specific clones of *N. crassa* we have isolated hybridize to more than one differentially regulated RNA species, demonstrating that some clustering does exist (8). However, not enough developmentally regulated *Neurospora con* genes have been mapped to determine whether the clustering is as extensive as in *Aspergillus* genes. It is possible that *con-10* and *con-13* belong to a larger complex of developmentally expressed genes. Evidence in *A. nidulans* implicates the action of a silencer in the regulation of conidiation-specific genes in normal hyphal growth; apparently, action of the silencer is relieved by a *trans*-acting factor during conidiation (38). We have no evidence for such a function in *N. crassa*, although genetic manipulation of the upstream region of *con-10* in fusion constructs may address this possibility.

It is interesting to consider what role the *con-10* gene product might serve. The level of mRNA for *con-10* is particularly abundant, which presumably reflects a high rate of synthesis for the corresponding polypeptide. The highly hydrophilic nature of the *con-10* polypeptide is unusual, and the repeated amino acid motif is intriguing, but as yet a function for this polypeptide remains elusive. The absence of expression of this gene in the aconidial mutants which all produce aerial hyphae to various extents indicates that it is expressed in the wild-type strain at a later stage. Functions with which *con-10* might be involved include changes induced by nutrient deprivation, events associated with the conversion of mycelial polymers into conidial components, and the synthesis of novel conidiation-specific structures. The adjacent gene, *con-13*, which is expressed at the same time as *con-10* in wild-type cultures, may serve a related function; however, as mentioned above, expression of *con-13* appears to be weaker. Mutational analyses with *con-10* and *con-13* and cellular localization studies with their polypeptide products should provide greater insight into the biological roles of these genes.

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