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Light and Developmental Regulation of the Gene *con-10* of *Neurospora crassa*[1]

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The gene *con-10* of *Neurospora crassa* is expressed preferentially during conidiation and following illumination of vegetative mycelia with blue light. In this study we have examined the segmental locations of the genetic elements associated with *con-10* that are responsible for light and developmental expression. A translational fusion was prepared between the initial segment of *con-10* and *Escherichia coli lacZ*. Deletions were then introduced into the *con-10* upstream region associated with this translational fusion. Each construct was integrated at the *his-3* locus of *N. crassa* by transformation and homologous recombination. Photoinduction of mycelia containing the translational fusion with the intact upstream region revealed a two phase stimulus–response curve. Exposure to light for as little as 5 sec induced a transcriptional response. Following this initial induction, a period of 15 min in the dark or light was required for appearance of a second phase response. Only a brief light treatment was necessary for induction of the second phase response. Deletions within the upstream region altered normal light and developmental expression of constructs containing the *con-10–lacZ* translational fusion. The deleted segments appear to contain a mycelial repression site, two conidiation activation sites, and two dark repression sites. A repeated 17-bp sequence acted as a transcriptional enhancer. One copy of this enhancer is in the upstream region. The second copy, with the opposite orientation, is located in the first *con-10* intron. The enhancer was required for proper mycelial and conidial expression of the *con-10–lacZ* fusion. The initial 10 bp of this enhancer sequence were sufficient to restore conidial expression to a deletion construct lacking both copies of the 17-bp repeat. Proteins were detected in extracts of mycelia and conidia that specifically bound to the enhancer sequence *in vitro*. Our findings suggest that conidiation-specific and mycelial-specific expression of *con-10* requires the action of several factors acting independently and/or in concert at distinct sites located in the regulatory regions for *con-10*.

INTRODUCTION

During the vegetative phase of its life cycle the filamentous fungus *Neurospora crassa* grows as a mycelium consisting of branched, interconnected hyphal strands. *N. crassa* forms three different types of haploid spores: ascospores, the product of the sexual cycle; uninucleate microconidia; and multinucleate macroconidia (called conidia throughout). Of the developmental pathways leading to formation of the three types of spores, synthesis of conidia has been studied in greatest detail. Appreciable morphological, genetic, and molecular information has been gathered in analyses of conidiation (reviewed in Springer, 1993). The developmental program that is responsible for conidia production is influenced by blue light (Turian, 1977) and the endogenous clock of the fungus (reviewed in Dunlap, 1993).

Molecular investigations of the process of conidiation in *N. crassa* began about a decade ago. Analyses of protein and mRNA species present in mycelia, aerial hyphae, and conidia established that specific changes in gene expression occurred throughout conidiation (Berlin and Yanofsky, 1985a). On the basis of these observations a differential screening procedure was used to identify and clone a set of genes that were highly expressed preferentially during conidiation (Berlin and Yanofsky, 1985b). One of these conidiation-specific genes is *con-10*. *con-10* is not expressed in vegetative mycelium but is highly expressed 8–10 hr following induction of conidiation (Berlin and Yanofsky, 1985b; Sachs and Yanofsky, 1991; Springer *et al.*, 1992). Both *con-10* mRNA and protein are present in conidia, and both disappear rapidly upon germination of conidia. The CON10 protein is localized in the conidiophores of conidiating cultures and is distributed uniformly throughout the cytoplasm of mature conidia (Springer *et al.*, 1992). Like the process of conidiation itself, *con-10* mRNA accumu-

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lation was found to be influenced by light (Lauter and Russo, 1991) and to be controlled by the endogenous clock of the organism (Lauter and Yanofsky, 1993). Light, clock, and developmental regulation of *con-10* expression is shared by two other cloned conidiation-specific *N. crassa* genes, *con-6* (Lauter and Yanofsky, 1993; White and Yanofsky, 1993) and *eas* (Lauter *et al.*, 1992; Bell-Pedersen *et al.*, 1992). *eas* encodes the major rodlet protein that coats the surface of the conidium. The functions of the *con-6* and *con-10* products are not known. *con-10* has a homolog located immediately following the *trp* operon in *Escherichia coli* (Corrochano and Yanofsky, unpublished), a gene of unknown function designated *orf1* (Stoltzfus *et al.*, 1988). *con-10* was mapped to the right arm of linkage group IV; it was isolated in a clone that also contains a conidiation-specific upstream gene, *con-13* (Roberts *et al.*, 1988). The *con-10* open reading frame, encoding an 86-residue polypeptide, is interrupted by two small introns (Roberts *et al.*, 1988). The transcription initiation site of *con-10* was mapped approximately 1.5 bp downstream of *con-13*, just before the *con-10* start codon (Roberts *et al.*, 1988). In view of the relative locations of the *con-13* and *con-10* coding regions it is reasonable to assume that the regulatory sites that control *con-10* expression are mostly located in the 1.5-bp region between *con-13* and *con-10*.

The objective of the present study was to analyze the effects of different environmental conditions on expression of *con-10* and to identify the genetic segments that contain elements that are responsible for light and developmental control. To quantify *con-10* expression conveniently, we constructed a translational fusion of *con-10* with *E. coli lacZ*. We integrated this fusion at the *his-3* locus of *N. crassa* and assayed the effects of light and development (conidiation) on its expression. Fusions containing the complete upstream region of *con-10*, or deletions thereof, were examined. These constructs permitted us to identify genetic segments that are required for normal light and developmental expression of *con-10*. We also identified two copies of a 17-bp enhancer sequence that is necessary for maximal *con-10* expression. Proteins were detected in extracts of vegetative mycelia and conidia that bind to this enhancer sequence. We note several sequences in the *con-10* promoter that are present in the promoters of other light- and conidiation-inducible genes of *N. crassa*.

MATERIALS AND METHODS

Strains

The *N. crassa* strains used as transformation recipients had either of the following *his-3* alleles: Y155M261 (*his-3 A*, FGSC 462) or 1-234-1438 (*his-3 a*, FGSC 6524). Transformation was performed with plasmids containing a 5′-truncated segment of wild-type *his-3*. We selected His^+ recombinants; these arose by recombinational integration of the plasmid at the *his-3* locus. Integration resulted in duplicated *his-3* genetic material, including one functional copy of *his-3*, flanking the plasmid sequences containing the *con-10–lacZ* fusion (Sachs and Ebbole, 1990). Southern analyses were performed on transformants to verify that a single copy of the *con-10–lacZ* fusion had been integrated at the *his-3* locus. Independent isolates transformed with the same plasmid produced similar amounts of β-galactosidase in control experiments (results not shown). All DNA manipulations were performed by standard procedures (Sambrook *et al.*, 1989).

Construction of con-10–lacZ Fusions

All the *con-10–lacZ* translational fusions contained the first 40 codons of *con-10* fused to the eighth codon of *E. coli lacZ*. Plasmids and strains bearing various deletions were designated by the 5′ end of the *con-10* sequence relative to the major transcription start site, e.g., pDE1559 contains the segment from base pair −1559 to codon number 40 of *con-10*. The sequence of each fusion junction and relevant DNA segment was verified by sequencing. The parental vector for pDE1559, pDE1559ΔI, pDE353, pDE353ΔI, pDE191, and pDE191ΔI was pH303 (Sachs and Ebbole, 1990), a plasmid that contains a 5′-truncated *N. crassa his-3* gene. The *Bam*HI–*Hin*dIII DNA fragment containing the 3′ flanking region of *trpC* of *Aspergillus nidulans* from pDH25 (Cullen *et al.*, 1987) was incorporated into pCON101 (a *Kpn*I–*Bam*HI subclone of pCon10a (Berlin and Yanofsky, 1985b) constructed by Dr. Karl Hager (Stanford University). The *Bam*HI *lacZ* cartridge from pMC1871 (Shapira *et al.*, 1983) was inserted to create fusions to *con-10*. The *con-10/lacZ/trpC* fusions were excised from this plasmid and inserted into *Bam*HI (blunt ended by treatment with Klenow fragment and dNTPs)–*Hin*dIII-cut pH303. Plasmids pDE353ΔI10F and pDE353ΔI17F were constructed by inserting double-stranded synthetic DNA (Table 1) into the *Spe*I site of pDE353ΔI. Plasmids pDE778, pDE265, pDE236, and pDE778Δ45 (internal deletion removing base pairs −236 to −192) were constructed by inserting the appropriate *con-10* fragments into vector pDE3 (Ebbole, 1990).

Site-Directed Mutagenesis

Single-stranded DNA of pCON101 was isolated from the *dut− ung−* strain BW3113 by rescue with the M13 helper phage MK07. Site-directed mutagenesis was performed (Kunkel *et al.*, 1987) using the oligonucleotides in Table 1 to generate deletions. The deletions were transferred to *lacZ* fusion plasmids by replacement of

the *Nru*I-*Bam*HI fragment of the *con-10* region (pDE1559ΔI) or by introducing the *Eco*RI-*Bam*HI *con-10* fragment into the *Sma*I-*Bam*HI sites of pDE3, to generate pDE778Δ45.

Light Induction Experiments

Cultures were grown and mycelia were illuminated for the times indicated, as described (Lauter and Russo, 1991), using Sylvania Energy Saver lamps (6 W/m^2 in the blue light region). Cultures were prepared by inoculating 10^6 conidia into 75 ml of Vogel's liquid minimal medium (Davis and de Serres, 1970) containing 2% sucrose as carbon source and 0.2% Tween 80 as wetting agent in 250-ml Erlenmeyer flasks. Log phase cultures were grown for 24 hr at 34°C, with agitation (200 rpm). The mycelia in each flask were collected by filtration through filter paper in a Büchner funnel and the resulting mycelial pads were cut in half. The dry weight of mycelia per flask was typically 200–250 mg. One half pad was illuminated while the other was kept in the dark as a control. All mycelial pads were wetted with 1 ml of prewarmed (25°C) growth medium initially and at 30-min intervals thereafter. This treatment prevented aerial hyphae formation and arrested growth in the vegetative phase. All manipulations were performed in the dark under a red safelight, at room temperature. After illumination, mycelial pads were incubated in the dark for 30 min (with the exception of the experiments described in Fig. 5) and were then frozen in liquid nitrogen and stored at −80°C. The values obtained with mycelium incubated for 30 min in the dark were essentially identical to values obtained with mycelium freshly harvested in the dark.

Conidial Stocks

Erlenmeyer flasks (125 or 250 ml) containing 50 ml of Vogel's minimal medium with 1.5% sucrose and 1.5% agar were inoculated with conidia and grown for 3–4 days at 34°C. The flasks were then incubated at room temperature under fluorescent light for 7 to 10 days. Conidia were harvested on 2.4-cm-diameter glass fiber disks in a filtration manifold and rinsed with water. Protein extracts were prepared immediately or the conidia were wrapped in aluminium foil, frozen in liquid nitrogen, and stored at −80°C.

Protein Extracts and β-Galactosidase Assays

Protein extracts were prepared by adding 10–100 mg of cell material to 1.5 ml of β-galactosidase assay buffer (Z buffer) (Miller, 1972) in 1.9-ml screw-cap tubes with 1.5 g of zirconium beads (0.5 mm diameter). Cells were disrupted by two 1-min pulses, separated by 4 min cooling on ice, in a mini-beadbeater (Biospec products) at 4°C. The extracts were clarified by successive centrifugations, first in a microfuge (13,000 rpm) for 5 min and then in a TLA45 rotor in a Beckman ultracentrifuge at 40,000 rpm for 15 min. The protein content of final supernatants was determined using the Bio-Rad protein assay calibrated with known amounts of bovine serum albumin. β-Galactosidase-specific activity was determined using the following formula: β-galactoside units = $(A_{420} \times 380)/(\text{min} \times \text{mg protein})$, as described (Miller, 1972). Two units of β-galactosidase activity were subtracted from all values to correct for the β-galactosidase activity of extracts of wild-type *N. crassa*.

DNA Binding Assays and DNase I Footprinting

Extracts were prepared from mycelial cultures grown in the light in Vogel's liquid minimal medium or from liquid cultures induced to conidiate by growing them in the light on low levels of nitrogen (Guignard *et al.*, 1984; Ton That and Turian, 1978). Cultures were harvested onto filter paper as described and resuspended in 2× binding buffer (1× binding buffer is 50 m*M* KCl, 10 m*M* Hepes, pH 7.9, 2 m*M* dithiothreitol, and 10% glycerol). Cells were disrupted by two passes through a French press cell at 16,000 psi. After centrifugation of extracts at 10,000 rpm in an Sorval SS-34 rotor for 10 min, supernatants were frozen in dry ice and stored at −80°C. Extracts prepared by sonication or by breakage with beads yielded similar results in band shift experiments. DNA binding assays and DNase I footprinting were performed as described (Ebbole *et al.*, 1991). Poly(dI·dC) was used as nonspecific competitor in all DNA binding assays (Ebbole *et al.*, 1991). The DNA binding experiments were performed with oligonucleotides (Table 1) and the 193-bp *con-10* intron DNA fragment flanked by *Hin*cII and *Bam*HI sites at positions +87 to +280. The isolated *con-10* DNA fragment and oligonucleotides were end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. In band shift analyses electrophoresis was carried out in a 4% polyacrylamide (80:1 acrylamide:bisacrylamide) gel containing 5% glycerol. In the footprint analysis, cleavage products were visualized by electrophoresis on a 6% sequencing gel, with a sequencing ladder used as size standard.

RESULTS

Light Induction of con-10 Expression

To examine the locations of elements responsible for light and developmental expression of *con-10*, a series of plasmids were constructed, each of which contained the first 40 codons of *con-10* fused in phase to codon 8 of *E.*

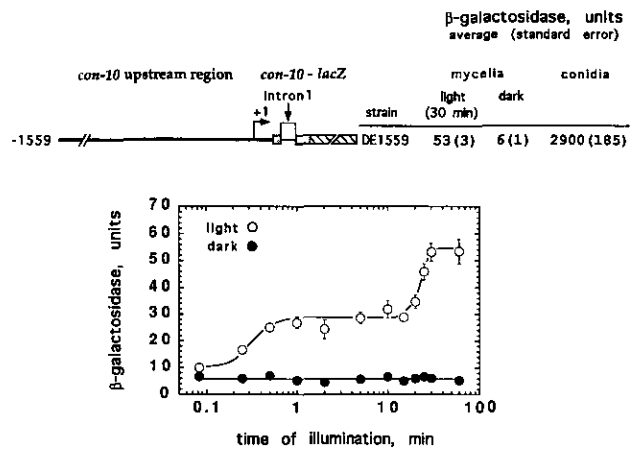

FIG. 1. Photoinduction of *con-10–lacZ* expression in vegetative mycelium. Top left: the "parental" *con-10* construct (DE1559) that was analyzed is diagrammed. DE1559 consists of the intact *con-10* upstream region and the *con-10–lacZ* translational fusion. The transcription initiation site (+1) and the coding region are indicated. The *con-10–lacZ* translational fusion contains the first 40 codons of *con-10* (stippled) fused in phase to the eighth codon of *E. coli lacZ* (diagonal lines). Top right: β-galactosidase values obtained with samples from strain DE1559 prepared as follows: mycelial samples were grown for 24 hr in the dark at 34°C, they were then harvested by filtration, and each filter pad was cut in half. One half pad was illuminated at 25°C for 30 min and then incubated in the dark for 30 min at 25°C. The other half pad was incubated in the dark for 60 min at 25°C, as a control. Light and dark exposures were terminated by quick-freezing half pads in liquid nitrogen. Protein was extracted from each sample and assayed for β-galactosidase activity. Conidial β-galactosidase activity was determined with protein extracts of conidia of strain DE1559 grown for 3–4 days in the dark at 34°C, followed by 7–10 days of incubation in the light at 25°C. β-Galactosidase units refer to specific activity in nmole/min/mg protein. Each point in this and subsequent figures represents the average value obtained in at least three independent experiments; the standard error of the mean is given. Bottom graph: to obtain the values presented in this graph the same procedure was followed except that one half pad was illuminated for 5 sec to 60 min (open circles) prior to 30 min incubation in the dark at 25°C. The other half pad was incubated in the dark for 30 to 90 min at 25°C (filled circles).

coli lacZ (Fig. 1). The 40-codon segment from *con-10* contained its natural first intron. Various deletions were introduced into the *con-10* upstream region, and the corresponding plasmid constructs were integrated at the *his-3* locus of *N. crassa* by homologous recombination. The integrants obtained were used to quantify the effects of imposing different inducing conditions on *con-10* expression. Southern analyses established that each transformant selected had a single integrated copy of the *con-10–lacZ* fusion (data not shown). β-Galactosidase activity was measured with extracts prepared from mycelia kept in the dark, from mycelia illuminated for various periods, and from mature conidia.

Before examining the effects of various deletions on the mycelial response to light and development, expression was examined in vegetative mycelia from strain DE1559, which we believe contains the entire *con-10* upstream regulatory region (1559 bp). Expression of the *con-10–lacZ* fusion was inducible by conidiation, as shown by the accumulation of 2900 units of β-galactosidase in conidia, compared to 6 units in dark-grown mycelia (Fig. 1). Additionally, expression was observed to be photoinducible, leading to about a 10-fold increase in β-galactosidase units after 30 min of illumination (Fig. 1). Thus, this *con-10–lacZ* fusion behaves much like the endogenous, intact *con-10* gene (Roberts *et al.*, 1988; Sachs and Yanofsky, 1991; Lauter and Russo, 1991; Lauter and Yanofsky, 1993). To examine the light response in detail, the period of illumination was varied. Figure 1 shows that there is a two-phase response. Following exposures of 1–15 min, a finite level of β-galactosidase activity was detected (Fig. 1). Only upon further illumination was an additional response observed; this response doubled the β-galactosidase level. Illumination for as little as 5 sec elicited a first stage response (Fig. 1). A second stage response was observed only 25–30 min after initiation of the first stage response, i.e., following the initial exposure to light a period of incubation in the dark or light was required to obtain the second stage response. The second stage response occurred if mycelia were illuminated for more than 30 min, or if mycelia were exposed for two 1-min periods separated by 28 min in the dark (Fig. 2). It appears that vegetative mycelia contain components that allow an instantaneous response to light; however, to obtain the maximum response to light, an additional 15–20 min of incubation is required during, or prior to, the second exposure.

Regulatory Elements Involved in Light and Developmental Expression of con-10

Strains bearing deletions of segments of the *con-10* upstream region were assayed for β-galactosidase activ-

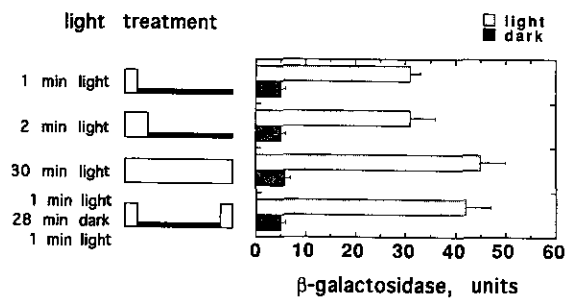

FIG. 2. The effect of the time and period of illumination on *con-10* expression. Mycelia of DE1559 were harvested as described in the legend to Fig. 1. Half pads were illuminated according to the light regime given at the left or kept in the dark as controls. Extracts from each sample were assayed for β-galactosidase activity. The bar represents the average activity and the line, the standard deviation.

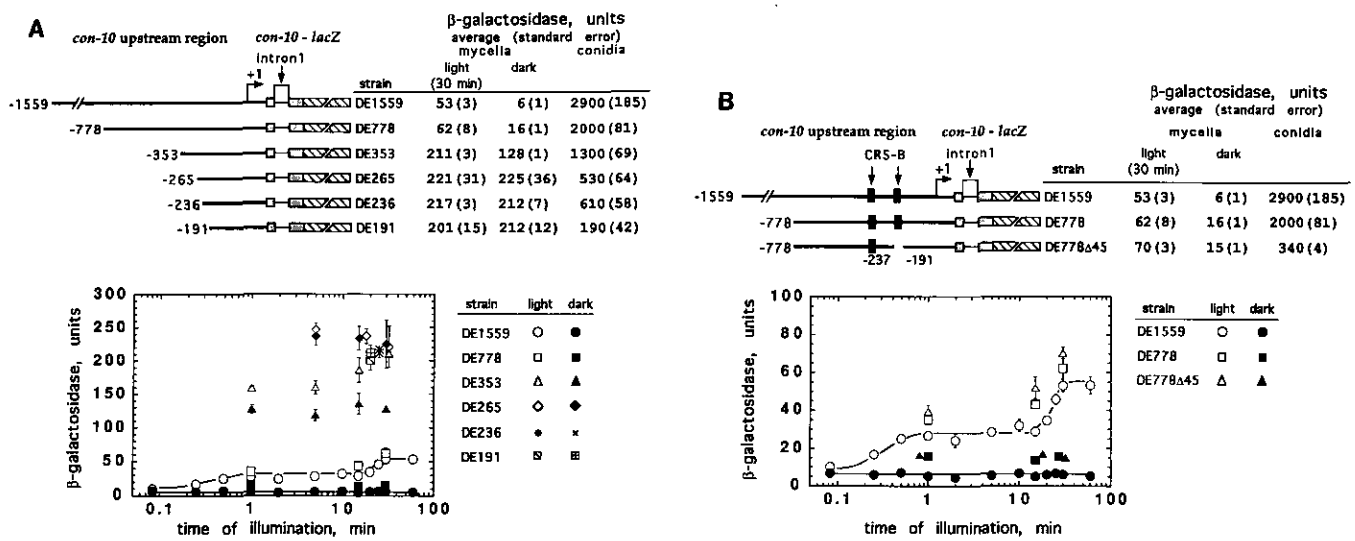

FIG. 3. Induction of *con-10* expression by light and development. (A) Expression of constructs containing different 5′ promoter deletions. (B) Expression of a construct with an internal deletion removing one copy of CRS-B. Top left, A and B: Schematic representations indicating the promoter segment retained in each deletion construct. Top right, A and B: β-galactosidase values for mycelial samples illuminated for 30 min and then incubated for 30 min in the dark, or maintained in the dark for 60 min. β-Galactosidase values are also shown for extracts of conidia prepared and assayed as described. Bottom, A and B: Graphs showing β-galactosidase units formed by each deletion strain as a function of the period of illumination. Mycelial pads of strains carrying the different deletions were illuminated for the times shown on the abscissa (as described in Fig. 1) (open symbols) or kept in the dark as controls (filled symbols). Protein was extracted from mycelial samples and conidia and assayed for β-galactosidase activity, as described in legend to Fig. 1. Note that the scale of β-galactosidase units differs in A and B. The same DE1599 and DE778 values are presented in A and B. The ends of the internal deletion in DE778Δ45 are indicated. This internal deletion, −236 to −192, is designated by the 5′ and 3′ base pairs that are joined by the deletion. The positions of some overlapping symbols were slightly shifted horizontally for clarity.

ity following incubation in the dark or in the light, or following conidiation (Fig. 3). A deletion removing the segment from −1559 to −778 (strain DE778) increased the dark mycelial level about threefold (Fig. 3A). This deletion had little or no effect on the light value and only slightly reduced the conidial value. The shape of the stimulus-response curve to light was similar to that of DE1559. These findings suggest that a weak dark repression site may be located in the segment from −1559 to −778. Deletion to nucleotide (nt) −353 (DE353) appreciably increased both the light and dark β-galactosidase mycelial values (Fig. 3A). This deletion also reduced the conidial β-galactosidase value. The light-response curve resembled that of DE1559. These findings locate a presumed light-independent mycelial repression site in the segment from −778 to −353. Deletion to nt −265 (DE265) rendered the construct insensitive to light and resulted in a further elevation of the dark β-galactosidase level (Fig. 3A). The conidial β-galactosidase level dropped by a factor of two. This deletion appears to have removed a DNA segment or segments required for the response to light, and it also removed a conidiation activation site. Further deletion to nt −236 (DE236), or to nt −191 (DE191), had no effect on the dark or light values (Fig. 3A). However, the −191 deletion reduced the conidial β-galactosidase value to the lowest level observed, suggesting that there is a second conidiation activation site located between nt −236 and −191.

A sequence designated CRS-B (conidiation regulatory sequence) is present in the upstream region of *con-6*, a gene that displays regulatory responses similar to those of *con-10* (White and Yanofsky, 1993). A factor was detected in mycelial extracts of *N. crassa* that bound to CRS-B (White and Yanofsky, 1993). Two copies of CRS-B are present in the upstream region of *con-10* (White and Yanofsky, 1993). The first copy, at −281, was deleted in DE265 (Fig. 3A). Comparison of the conidial β-galactosidase values for DE353 vs DE265 indicates that deletion of this segment led to a 60% decrease in conidial expression. We also deleted a 45-bp segment from the upstream region of DE778 that contains the second CRS-B site, at −217 (Fig. 3B). This construct retains the CRS-B site at −281. The construct obtained was integrated at the *his-3* locus and *con-10-lacZ* expression of the resulting strain, DE778Δ45, was compared with that of its homologous control strain, DE778 (Fig. 3B). DE778Δ45 and DE778 showed identical light and dark mycelial β-galactosidase levels but DE778Δ45 responded only weakly to conidiation (340 units vs 2000 units). Both copies of CRS-B were deleted in DE191 (Fig. 3A). Conidial expression of *con-10-lacZ* was at its lowest in DE191 (Fig. 3A). These findings suggest that deletion

of either or both CRS-B sites leads to an appreciable reduction in conidial expression.

Either Copy of a Second Repeat Sequence Is Required for Maximal Expression of con-10

Two copies of a 17-bp sequence, with opposite orientations, are present in the *con-10* clone. One copy is located upstream of the transcription start site, at position −377, and the other copy is located in the first intron, at position +166 (Fig. 4, top). We searched for a factor or factors that bound at this sequence by performing DNA band shift analyses. We used various extracts and a labeled synthetic double-stranded DNA fragment containing the 17-bp repeat sequence (designated 171–172, Table 1). The extracts employed were from mycelia induced to conidiate in the light for 12 or 24 hr and from 14-hr mycelia grown vegetatively in the light (Fig. 4, left). A major shifted band was detected with each extract (Fig. 4 left, arrow D). The extract from vegetative mycelia gave a second band that migrated slightly faster than the major band (Fig. 4 left, arrow C). Presumably mycelia contain a second protein that binds to some portion of the 17-bp repeat sequence. A third band was detected with the three extracts (Fig. 4 left, arrow B), just above the free DNA (Fig. 4 left, arrow A). This band was not observed reproducibly in gels run with the same extracts. The explanation for this variability was not investigated.

DNase I protection experiments were performed with extracts from conidiating mycelia grown in the light and with the natural 193-bp *Hin*cII-*Bam*HI intron DNA fragment that contains one copy of the 17-bp sequence. A conidiating mycelia extract was used since it gave only a single shifted species (Fig. 4, left, arrow D). Approximately 10 bp of the 17-bp repeat sequence were protected from DNase I cleavage by bound protein (Fig. 4, right). The protected nucleotides correspond to the first 10 nucleotides (5′-TGCCAAGACA-3′) of the 17-bp repeat.

To examine the regulatory significance of the repeat sequence, we prepared constructs with and without this sequence, each containing the *con-10–lacZ* translational fusion (Fig. 5). Deletion of the intron with one copy of the repeat sequence (DE1559ΔI) reduced light induced expression by a factor of two but did not affect dark expression or conidial expression appreciably. Deletion of both copies (compare DE353 with DE353ΔI) resulted in a severe reduction in light-induced expression, dark expression, and conidial expression. To determine if either the 17-bp sequence or the footprinted 10-bp sequence was necessary for high-level expression, these two sequences were added at the 5′ end of deletion DE353ΔI, to give constructs DE353ΔI17F and DE353ΔI10F. Although the 17-bp sequence restored much of the activity to DE353 (Fig. 5), the 10-bp sequence only increased conidial expression. It appears from these findings that multiple factors interact with segments of the 17-bp repeat sequence and influence *con-10* expression.

As a further test of the importance of the 17-bp repeat

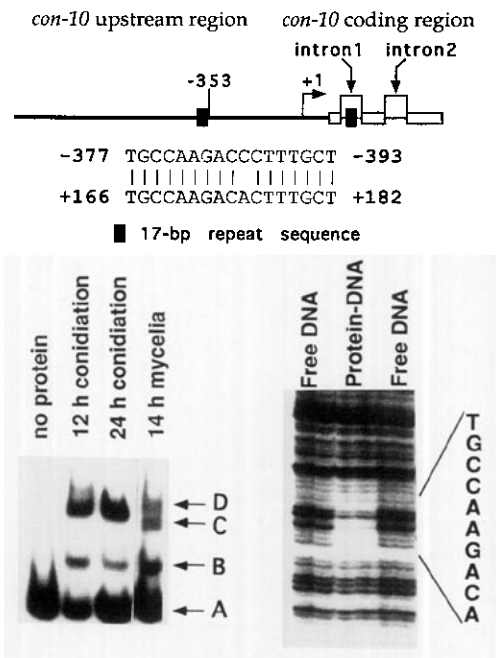

FIG. 4. Band shift assay and footprint for protein(s) that bind to the 17-bp repeat sequence. Top: Schematic diagram of the *con-10* transcriptional unit. The gene consist of three exons (filled boxes) and two introns. The transcription start site is marked (+1). The two copies of the 17-bp repeat sequence have opposite orientations; they are indicated by black rectangles. The sequence and location relative to the transcription initiation site are also indicated in the alignment. Bottom, left: Band shift of DNA fragment 171–172 containing the 17-bp repeat sequence. No protein: control, no protein extract; 12 h conidiation: extract from cultures induced to conidiate in the light for 12 hr (a 12-hr conidiating culture); 24 h conidiation: extract from cultures induced to conidiate in the light for 24 hr (a 24-hr conidiating culture); 14 h mycelia: extract from mycelia grown for 14 hr in the light (14 h mycelia). Arrows indicate the positions of the free DNA fragment (arrow A) and protein–DNA complexes (arrows C–D). The 200-bp intron fragment containing the 17-bp repeat was shown to effectively compete with the 17-bp labeled synthetic DNA (data not shown). The band marked by arrow B appeared in all lanes with added protein. This band was not observed reproducibly using the same extract. The significance of band B is unclear. See Materials and Methods for a description of the electrophoresis conditions and other procedures. Bottom right: DNase I footprinting of a complex formed between a factor in crude extracts prepared from conidiating mycelia grown in the light (conidial crude extracts) and the 193-bp *Hin*cII–*Bam*HI *con-10* intron DNA fragment. Free DNA: DNase I cleavage pattern of DNA 5′-labeled at the *Bam*HI end; Protein-DNA: DNase I digestion pattern of a protein–DNA complex (complex D) isolated from a band shift gel. Cleavage products were visualized by electrophoresis (see Materials and Methods). The protected sequence corresponds to nucleotides 1–10 of the 17-bp repeat sequence displayed.

TABLE 1
OLIGONUCLEOTIDES USED FOR PLASMID CONSTRUCTION AND BAND SHIFT ANALYSES

Oligonucleotide	Sequence 5′–3′	Purpose
10F	CTAGTGCCAAGACA	Oligonucleotides used to construct pDE353ΔI10F
10R	CTAGTGTCTTGGCA	
17F	CTAGTGCCAAGACACTTTGCTA	Oligonucleotides used to construct pDE353ΔI17F
17R	CTAGTAGCAAAGTGTCTTGGCA	
171	GATCCCACACTGCCAAGACACTTTGCTA	Oligonucleotides used in band shift experiments
172	GATCTAGCAAAGTGTCTTGGCAGTGTGG	
70	ACCTCTTCCTTGGGGCGGTTGGCGAAGTTG	Intron deletion mutagenesis primer
45	CCCTTTGCATCTCGCTGCAGAGATCGGA	−236 to −192 deletion mutagenesis primer

sequence we deleted the intron bearing the 17-bp repeat from DE191, to give construct DE191ΔI (Fig. 5). Removal of the intron reduced the β-galactosidase level in the light, in the dark, and in conidia. These findings suggest that the 17-bp sequence serves as an enhancer of *con-10* expression under the conditions tested.

DISCUSSION

Expression of *con-10* is influenced by several events, including exposure to light and induction of and progression through the stages of conidiation. In the present study we used a translational fusion of *con-10* to *E. coli lacZ* to assess the effects of genetic deletions on *con-10* expression under different environmental and developmental conditions. A construct with the translational fusion plus the 1.5-bp segment upstream of *con-10*, when integrated at the *his-3* locus, responded in a manner entirely consistent with the results of previous transcriptional analyses of endogenous *con-10* expression (Roberts *et al.*, 1988; Sachs and Yanofsky, 1991; Lauter and Russo, 1991; Lauter and Yanofsky, 1993).

It should be noted that potential position effects on expression of *con-10–lacZ* fusions were minimized by integrating each construct at exactly the same location in the *his-3* locus (M. Plamann and C. Yanofsky, unpublished; White and Yanofsky, 1993). In support of the validity of this procedure, we found that independent isolates bearing each integrated *con-10–lacZ* construct gave virtually identical expression values. Our findings with *con-10–lacZ* differ from observations with comparable *con-6–lacZ* integrated constructs (White and Yanofsky, 1993). In the latter case presumed identical transformants exhibited very different levels of *con-6–lacZ* expression. In *N. crassa* it has been observed that when multiple copies of a foreign gene are introduced by transformation, the introduced copies of the gene can be modified in some manner rendering them nonfunctional (Pandit and Russo, 1992). Similarly, when a gene introduced by transformation contains sequences homologous to an endogenous gene, expression of both the introduced copy and the endogenous copy is sometimes impaired (Romano and Macino, 1992; T. Schmidhauser and C. Yanofsky, unpublished). This phenotypic inactivation has been termed "quelling" (Romano and Macino, 1992). Apparently the DNA segment containing *con-6*, but not the segment with *con-10*, contains one or more modifiable sites which can result in quelling. A quelling-like phenomenon called "cosuppression," has also been observed in plants (for review see, Flavell, 1994).

In induction experiments with visible light, we found that there were two stages to the *con-10–lacZ* response. Exposure of mycelium to light for a period as short as 5 sec induced expression; exposure for 1 to 15 min elicited a maximal, first stage response. Exposure to light for 30 min, or administering light for two 1-min periods separated by 28 min in the dark, produced a second stage

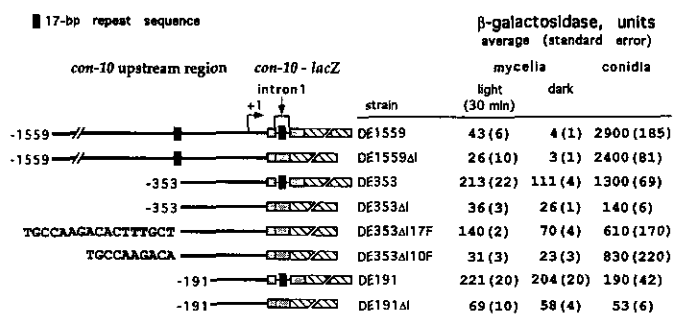

FIG. 5. The effect of the 17-bp repeat sequence on mycelial and conidial expression of *con-10*. Mycelial half pads from strains carrying different deletions upstream of the *con-10–lacZ* fusion, with or without the repeat sequence, or a segment thereof, were either illuminated for 30 min, or kept in the dark as controls. Light and dark exposures (30 min) were terminated by quick freezing half pads in liquid nitrogen. Note that these samples were not incubated in the dark for 30 min prior to freezing. Protein extracts from mycelia, and from conidia, were assayed for β-galactosidase activity. Strains in which the *con-10* intron was deleted by site-directed mutagenesis are indicated by a "ΔI" in their names. Nucleotides added to the end of the *con-10* promoter segment in deletion mutant DE353 are indicated in bold letters. The DE1559, DE353, and DE191 values are those presented in Figs. 3A and 3B.

response. Apparently a period of at least 15 min in the dark or in the light is required before *N. crassa* can give a second stage response. One might speculate that the molecules needed for the first stage response are present and active in dark-grown mycelium, whereas the molecules needed for the second stage response must be synthesized *de novo*. It was shown previously that photoinduction of *con-10* expression becomes progressively less effective as the period of illumination is increased beyond 30 min (Lauter and Yanofsky, 1993). This finding agrees with the observation reported here that light induction of β-galactosidase accumulation is saturated after a 30-min exposure to light. A similar two-step stimulus–response curve has been noted in studies on light-induced carotenoid biosynthesis (photocarotenogenesis) in *N. crassa* (Schrott, 1980). The accumulation of carotenoids upon exposure of mycelium to light for different times followed a two-step curve with thresholds and saturation times similar to those reported here for photoinduction of *con-10* expression (Schrott, 1980). Two component stimulus–response curves have also been described for many photoresponses in the distantly related fungus *Phycomyces blakesleeanus;* these photoresponses include phototropism (Galland and Lipson, 1987), photocarotenogenesis (Bejarano *et al.*, 1991), photomorphogenesis (Corrochano *et al.*, 1988), and light-induced absorbance changes in mycelial extracts (Trad and Lipson, 1987). The influence of light on *con-10* expression is not evident during conidial development. No differences in *con-10–lacZ* expression were observed in mature conidia of strain DE1559 that were formed in the light or in constant darkness (F.-R. Lauter and C. Yanofsky, unpublished). The stage of development at which the light effect on *con-10* expression is lost is not known.

To identify the genetic segments involved in control of *con-10* expression by light and development, we introduced various deletions into the *con-10* upstream region. By combining these deletions with the translational fusion reporter gene, we found that photoinduction and developmental expression of *con-10* are mediated by different DNA segments. Furthermore, it appeared that light and developmental responses were mediated by opposite-acting mechanisms. Thus, the effects of light appeared to influence repression while the effects of development seemed to affect activation (through the CRS-B elements) but also repression (through the mycelial repressor elements). Interestingly, negative regulatory elements have been identified that affect light-regulated plant genes (Li *et al.*, 1994).

In Fig. 6 we have summarized our conclusions concerning the presumed locations of regulatory elements that influence *con-10* expression. We believe there are two genetic segments that contain elements that mediate dark repression. Deletion of one or both of these segments (Fig. 3A) increases mycelial dark expression. Deletion of the DNA segment from nt −778 to −353 increases mycelial *con-10* expression in the dark and in the light. This suggests that this DNA segment contains a site of mycelial repression. In other experiments this presumed mycelial repression site was localized to the region between nt −517 and −353 (L. Corrochano and C. Yanofsky, unpublished), i.e., strains with a deletion of the region upstream of −517 had β-galactosidase levels identical to those of strain DE778. The strain that has lost the mycelial repression site (DE353) was still photoinducible (Fig. 3A), suggesting that the dark repression sites act independently of the mycelial repression site.

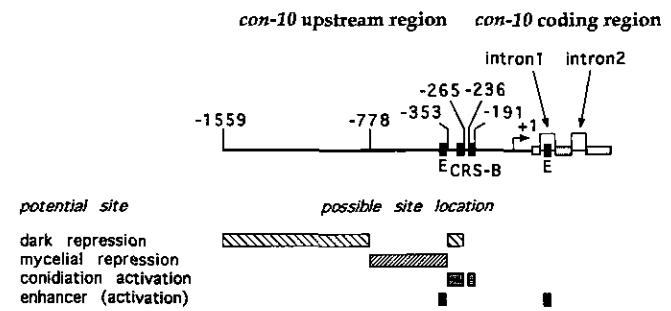

FIG. 6. The locations of genetic segments containing presumed elements that control light and developmental expression of *con-10*. The numbers at the top or over the *con-10* upstream sequence refer to the transcription initiation site (+1) and the ends of each segment. As indicated in the figure, the region between −1559 and −778 contains a mycelial dark repression site; the region between −778 and −353 contains a mycelial repression site (probably between −512 and −353; L.M.C., preliminary observation) and a copy of the enhancer (E) at −377; the region between −353 and −265 contains a mycelial dark repression site, a conidiation activation site, and a copy of the CRS-B element at −281 (these three sites may be identical); the region between −236 and −191 contains a CRS-B element at −217 which may function as a conidiation activation site. The first intron contains a second copy of the enhancer (E) at +166.

Figure 6 also shows the locations of the 17-bp repeat enhancer-like elements. The enhancer appeared to increase expression under all our test conditions (Fig. 5); thus, they appear to be activation sites. A factor was detected in extracts of conidiating cultures that bound to the 17-bp repeat sequence. A second factor that bound at this sequence was present in mycelia. We have no information on the activating or repressing functions of either of these factors. Finally, there are two so-called CRS-B binding sites (Fig. 6). A factor has been detected in mycelium but not in conidiating hyphae that binds at this sequence (White and Yanofsky, 1993). This factor has not been isolated and studied. Our findings indicate that deletion of the segment from −353 to −265 (Fig. 3A), containing one CRS-B element, or −236 to −192

(Fig. 3B), containing the second CRS-B element, markedly reduce conidial expression of the *con-10–lacZ* fusion, suggesting that CRS-B sites are conidiation activation sites.

All of the strains that displayed photoinduction of *con-10–lacZ* expression showed a two stage stimulus–response curve similar to that of strain DE1559. This suggests that the initial response to photoinduction and the time-dependent stage required for maximum photoinduction operate through the same or nearby genetic elements. Interestingly, deletion of nt −1498 to −1017 of the *eas* upstream region abolishes *eas* expression (Kaldenhoff and Russo, 1993) and removes one of the sites we have identified (Table 2) (Lauter *et al.*, 1992; White and Yanofsky, 1993).

Expression of many plant genes is light-regulated, and several conserved sequences important for light regulation have been identified (reviewed in Gilmartin *et al.*, 1990). The sequence ACGT, recognized by some bZIP proteins, is involved in light-regulated expression of several plant genes (see Izawa *et al.*, 1993, and references therein). We have found this sequence at many locations in the *con-10* upstream region (−1544, −1452, −990, −820 and −227). One ACGT element (−227) is in a copy of CRS-B. Another sequence involved in light-regulated expression of plant genes is GATA, which often appears in tandem in plant gene promoters (Gilmartin *et al.*, 1990). There are tandem copies of GATA in the *con-10* upstream region (nt −1519, −1513, −1495, −1464, −1370, and −133). The possible role of these specific sequences in expression of *con-10* remains to be tested.

Deletion of the *con-10* upstream sequence to nt −353 only reduces conidiation-specific expression twofold, suggesting that most of the elements involved in developmental regulation of this gene are located downstream of this position. A gradual reduction of expression upon increasing the size of the upstream deletion was also noted in deletion analysis with promoters of other genes of *N. crassa* (Frederick and Kinsey, 1990a,b) and with promoters of genes of other filamentous fungi (Hamer and Timberlake, 1987; Adams and Timberlake, 1990). We have not determined if there is an inhibitory effect associated with placing foreign vector sequences closer to the transcription initiation site.

Since many environmental signals are known to influence conidiation, it is perhaps not surprising that many sites, with different functions, appear to mediate *con-10* expression. The regulatory proteins that act at these sites may act independently or synergistically. On the basis of our current results we could view conidiation-specific expression of *con-10* as involving the following: relief from repression at a site that can block expression in mycelia; a positive response at two conidial activation sites that mediate expression upon conidiation; and expression enhancement at either of two copies of an enhancer sequence that is responsible for overall expression of the gene in both mycelia and conidia. In mycelia, expression of *con-10* would be blocked by repressors binding at one mycelial repression site and two dark repression sites. It seems likely on the basis of our results, and previous findings (Lauter and Yanofsky,

TABLE 2
SHARED PUTATIVE REGULATORY SEQUENCES

Gene	Sequence	Matches
	.... ********..	
con-10	−1434-TGCTTGTTCGATGCTT	15/16
al-2	242-TGAGAGTTCGATGCTG	14/16
con-6	−515-GTCGGGTTCGATGCAT	13/16
Consensus	TGCGXGTTCGATGCTT	
	*.*****..*.*. ..	
con-10	−1004-GACAAGTGCATGAGACGTT	17/19
con-6 (R)	−116-GACAAGT-CATGAAACAGC	17/19
al-1 (R)	489-GACAAGTG-ATGATGCGCA	16/19
eas (R)	−976-GCCAAGTGCAAGTCAAAGA	15/19
Consensus	GACAAGTGCATGAXACRGA	
	....***.**.***. .*... *.*.	
con-10	−891-CTCATCATCACTTGG-CTTGTCAGGA	25/26
eas	−1117-ATGTTCAACACTTGGA-TAGT-AGGA	19/26
al-1	474-CGCATCATCACTTGT-CTTAAAAAGT	19/26
con-6	−1077- CTTGGTCTTGT-AGGA	15/16
eas	−6-CTCATCATCAG	10/11
Consensus	CTCATCATCACTTGG-CTTGT-AGGA	
	.**.*******.**.**	
con-10	−515-GGGAGCT-TATTCCCCGCGTG	21/21
eas	−752-GGAAGCTGTATTCCC-GC-TG	17/21
con-8	−447-GGGAGCT-TA	10/10
Consensus	GGGAGCT-TATTCCCCGCGTG	
Enhancer (E)		
con-10 (R)	−377-TGCCAAGACCCTTTGCT	
con-10 (IVS)	+166-TGCCAAGACACTTTGCT	
CRS-B	..**.....*...	
con-10	−281-GCTGTCAGAATCTC	11/14
con-10 (R)	−217-GATGTCAACGTGAT	10/14
con-6	−213-GCTGTCAGCATCAT	14/14
al-1	730-ACTGTGCGCATCAT	11/14
al-2 (IVSR)	1402-GCTGTCAGA-TCTG	10/14
eas (R)	−1081-CATGCCAGCATCAT	11/14
Consensus	GCTGTCAGCATCAT	

Note. Each sequence is numbered relative to the transcription initiation site except those for *al-1* and *al-2*, which are numbered according to their published sequence. "R" indicates that the sequence was present in the reverse orientation. "IVS" indicates that the sequence was found in an intron. Matches to the consensus sequence are indicated at the right of each alignment. A star over a nucleotide indicates that it is shared in all the aligned sequences; a dot over a nucleotide indicates that it is shared in most of the sequences. The sequences were obtained from the following references: *con-6* (White and Yanofsky, 1993), *con-8* (Roberts and Yanofsky, 1989), *con-10* (Roberts *et al.*, 1988), *eas* (Eberle and Russo, 1992), *al-1* (Schmidhauser *et al.*, 1990), *al-2* (Schmidhauser *et al.*, 1994).

1993), that light acts by relieving dark repression independently of relieving mycelial repression.

We have compared the sequences of the genetic segments containing elements controlling expression of *con-10* with sequences from the presumed regulatory regions of other developmentally and light-regulated genes of *N. crassa*. There are many short sequences suggestive of regulatory elements that are present in the presumed regulatory regions for these genes; putative shared regulatory elements are presented in Table 2. Some of these presumed regulatory elements may serve similar functions in other organisms.

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