The EMBO Journal vol.11 no.12 pp.4539-4548, 1992

Regulatory pathways governing modulation of fungal gene expression by a virulence-attenuating mycovirus

Thomas G.Larson, Gil H.Choi and Donald L.Nuss[1]

Department of Molecular Oncology and Virology, Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110, USA

[1]Corresponding author

Communicated by A.van Kammen

A viral double-stranded RNA associated with virulence attenuation (hypovirulence) in the chestnut blight fungus (*Cryphonectria parasitica*) has been shown by DNA-mediated transformation to be responsible for transmissible hypovirulence. In addition to reduced virulence, the fungal strain harboring this virus exhibits a diverse array of characteristics, termed hypovirulence-associated traits, which distinguish it from an isogenic virus-free strain. We have investigated one of these traits, suppressed *lac-1* (laccase) transcript accumulation. Two different and opposing regulatory pathways appear to govern *lac-1* transcript levels in the virus-free strain: a stimulatory pathway was found to be dependent on the inositol trisphosphate (IP_3) and calcium second messenger systems. A second pathway limiting transcript accumulation was shown to require ongoing protein synthesis. Additionally, changes in the level of *lac-1* transcript accumulation were found to be related to modulation of promoter activity and this activity was shown to be suppressed in the virus-containing strain. We conclude that this hypovirulence-associated virus interferes with transduction of an IP_3-calcium-dependent signal that is required for stimulation of *lac-1* transcription. The perturbation of such signal transduction pathways by hypovirulence-associated viruses may account for the manifold symptoms associated with transmissible hypovirulence.

Key words: Cryphonectria parasitica/hypovirulence-associated virus/laccase/second messengers/signal transduction

Introduction

The fungus *Cryphonectria parasitica* is the etiological agent responsible for the blight that has ravaged the American chestnut (for reviews see Anagnostakis, 1982b; Griffin, 1986; MacDonald and Fulbright, 1991). *C.parasitica* strains harboring viral-like double-stranded (ds) RNA genetic elements often exhibit reduced levels of virulence (hypovirulence) (Day *et al.*, 1977). In addition to reduced virulence, these strains also exhibit a number of distinguishing characteristics that are collectively referred to as hypovirulence-associated traits (Hillman *et al.*, 1990). Although the exact constellation of traits varies among different hypovirulent strains, it can include suppressed sporulation, altered colony morphology, reduced pigmentation, diminished oxalate accumulation and reduced cellulase and laccase activity (Anagnostakis, 1982b; Anagnostakis, 1984; Elliston, 1985; Rigling *et al.*, 1989; Hillman *et al.*, 1990). Recent DNA-mediated transformation studies employing full-length and partial cDNA clones of the large viral dsRNA (L-dsRNA) isolated from the hypovirulent *C.parasitica* strain EP713, provided the first direct evidence that viral RNA is responsible for the hypovirulence phenotype (Choi and Nuss, 1992a,b). These studies also identified a specific viral coding domain that is responsible for several hypovirulence-associated traits.

The mechanism by which a hypovirulence-associated virus alters host phenotype is currently unknown, but appears to involve the modulation of transcript accumulation from specific host genes (Powel and Van Alfen, 1987; Rigling and Van Alfen, 1991; Choi *et al.*, 1992). *Lac-1*, which encodes the phenoloxidase laccase, is one such gene and has been well characterized in *C.parasitica* (Rigling *et al.*, 1989; Rigling and Van Alfen, 1991; Choi *et al.*, 1992). Exposure to low levels of the protein synthesis inhibitor cycloheximide (CHX) has been observed to stimulate laccase expression in both *C.parasitica* (Choi *et al.*, 1992), and *Neurospora crassa* (Froehner and Eriksson, 1974; Linden *et al.*, 1991). In the case of *C.parasitica*, CHX has been shown to increase the level of *lac-1* transcript in a virus-free virulent strain, EP155, but not in the isogenic EP713 strain which harbors a hypovirulence-associated virus (Choi *et al.*, 1992). Exposure to the antifungal and immunosuppressive agent cyclosporin A (CspA) has also been shown to stimulate *lac-1* transcript accumulation in the virus-free EP155 strain of *C.parasitica*, but not in the isogenic virus-containing EP713 strain (Larson and Nuss, 1992). Additionally, the increase in *lac-1* transcript levels elicited by CspA was shown to stem primarily from an increase in transcription activity (Larson and Nuss, 1992).

Since *lac-1* transcript levels are suppressed by the hypovirulence-associated virus carried by strain EP713, the attenuation of the gene's expression may serve as a model system for understanding the origin of many hypovirulence-associated traits. To this end, we have begun to characterize the regulatory pathways that govern *lac-1* expression. In this paper we present information indicating that *lac-1* transcription is governed by at least two different and opposing pathways: one regulatory pathway stimulates *lac-1* transcription and appears to be dependent on the calcium and inositol triphosphate (IP_3) second messenger systems, while the other pathway limits *lac-1* transcription. CHX and CspA both appear to stimulate *lac-1* transcription by deactivating the inhibitory pathway, while the hypovirulence-associated virus present in the EP713 strain of *C.parasitica* appears to suppress *lac-1* transcription by interfering with the stimulatory pathway. Since calcium is widely used as a second messenger and governs many different intracellular processes (for reviews see Hokin, 1985; Berridge, 1987), the ability of this virus to disrupt signal transduction may explain the capacity of a single viral coding region to cause the diverse array of hypovirulence-associated traits displayed by strain EP713.

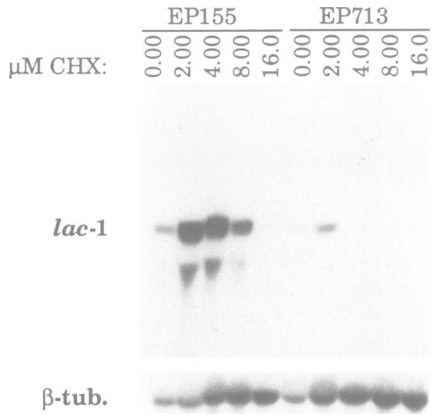

Fig. 1. Dose–response of *lac-1* transcript accumulation to CHX in strains EP155 and EP713. Strains EP155 and EP713 were grown in stationary cultures containing 50 ml of PDB for 40 h and exposed to the indicated concentrations of CHX for an additional 24 h. The zero dose cultures received ethanol vehicle equivalent in volume to that used in the 16 μM dose cultures. Total RNA samples from strains EP155 and EP713 were denatured and separated by electrophoresis using a 1.2% agarose–formaldehyde gel. The RNA was transferred to a nylon membrane and hybridized with a *lac-1* probe. Following autoradiography, the blot was stripped and rehybridized with a β-tubulin probe. The hypovirulent strain EP713 contains the hypovirulence-associated virus dsRNA, but is otherwise isogenic to the virulent strain EP155 (Anagnostakis and Day, 1979; Hillman *et al.*, 1990).

Results

Dose response to CHX

We previously reported that *lac-1* transcript accumulation in *C.parasitica* is reproducibly stimulated by exposure to CHX (Choi *et al.*, 1992). In the previous study we used 3 μM CHX based on the observation that this concentration induces the maximal production of both the laccase (Froehner and Eriksson, 1974) and tyrosinase (Horowitz *et al.*, 1970) enzyme activities in *N.crassa*. Since this dose was determined in a different organism by measuring enzymatic activity, we wished to establish the optimal dose for stimulating *lac-1* transcript accumulation in *C.parasitica*. The data presented in Figure 1 demonstrate that maximal *lac-1* transcript accumulation resulted from treatment with 2–4 μM CHX in the virus-free (virulent) *C.parasitica* strain EP155. Additional experiments confirmed that maximal accumulation occurred between 2.5 and 3 μM CHX and that accumulation ceased at ~10 μM (data not presented). Similar results were obtained for the isogenic virus-containing EP713 strain, except that the accumulation of the *lac-1* transcript was significantly lower than in the EP155 strain (Figure 1) even though maximal accumulation was stimulated by approximately the same concentration of CHX in the two strains. Since maximal transcript accumulation was observed at ~3 μM CHX, this concentration was used as the standard concentration for all other experiments unless otherwise noted.

The inhibition of protein synthesis stimulates lac-1 transcript accumulation

To demonstrate that CHX affects *lac-1* transcription by inhibiting protein synthesis, we examined the effect of CHX on both protein synthesis and the accumulation of the *lac-1* transcript in CHX-resistant mutants of *C.parasitica* EP155.

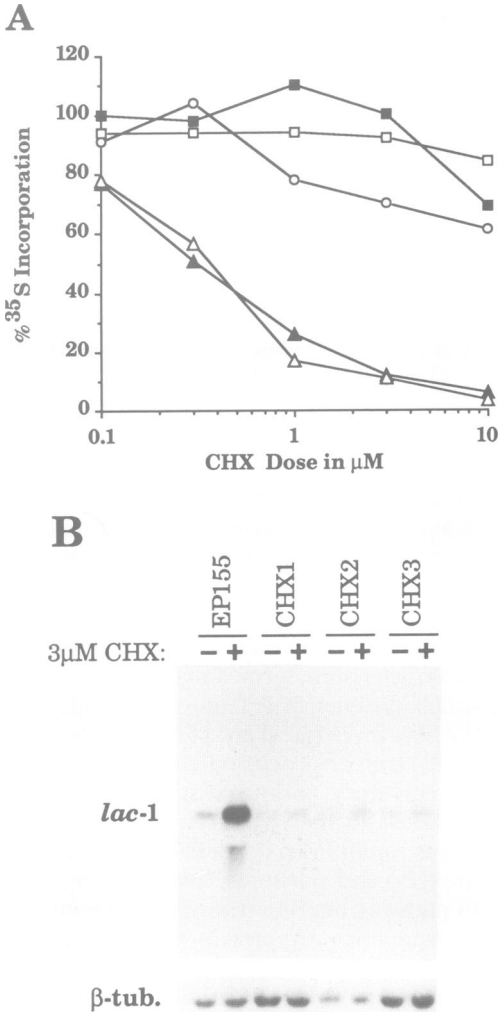

Fig. 2. Response of CHX-resistant mutants to CHX. **A.** Incorporation of [^{35}S]methionine by cultures of strain EP155 (filled triangles), strain EP713 (open triangles), and CHX-resistant strains CHX1 (filled squares), CHX2 (open squares) and CHX3 (open circles) exposed to various doses of CHX relative to unexposed cultures. Fungi were grown as described in Figure 1 and transferred to an equivalent volume of MMLT broth (Anagnostakis, 1982a) with tannic acid omitted after 40 h. Cultures were preincubated 30 min at room temperature with the indicated concentrations of CHX before adding [^{35}S]methionine. Incubations were continued for 2 h after which time the labelled protein was precipitated by adding TCA to a final concentration of 10%. After extracting unincorporated isotope, the precipitated protein was solubilized with NaOH and the specific activity was determined. Incorporation is expressed as a percentage of the specific activity of the protein in controls receiving no CHX. Each data point represents the mean of at least two trials. **B.** Accumulation of *lac-1* transcript in strains EP155, CHX1, CHX2 and CHX3 with and without exposure to CHX. CHX-resistant mutants, CHX1, CHX2 and CHX3, and the wild type parental strain, EP155, were exposed to 3 μM CHX for 24 h as described in Figure 1. Controls were incubated with an equivalent volume of ethanol vehicle. Total RNA was analysed as described in Figure 1.

The affect of CHX on protein synthesis was examined in the virulent (virus-free) EP155 and hypovirulent (virus-infected) EP713 strains and in three UV-induced, CHX-resistant mutants of EP155 with otherwise normal phenotypes. The three mutant strains were significantly more resistant to the effect of CHX on the incorporation of [^{35}S]methionine into acid-insoluble protein, as shown in

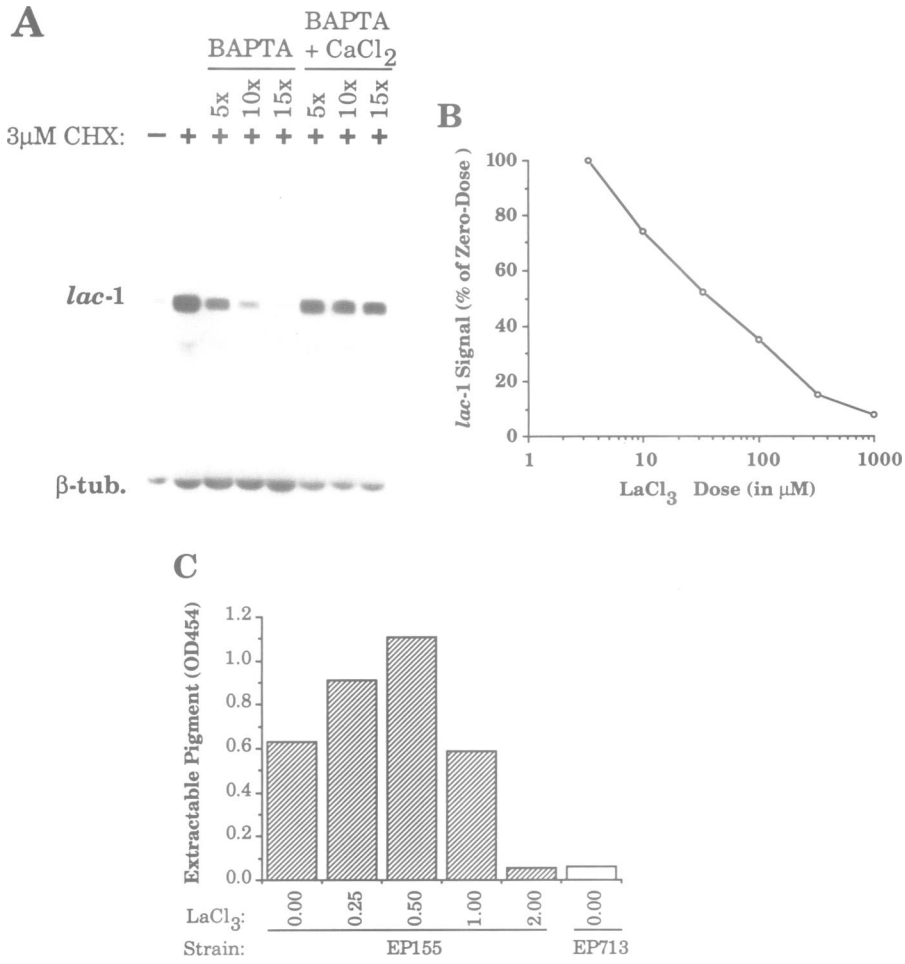

Fig. 3. The effect of compounds that perturb calcium metabolism on *lac-1* transcript levels and pigmentation in strain EP155. **A**. Effect of BAPTA on CHX-mediated *lac-1* transcript accumulation. EP155 cultures were prepared as described in Figure 1 and exposed for 24 h to 3 μM CHX combined with increasing amounts of BAPTA or BAPTA + $CaCl_2$. The concentration of BAPTA was increased as a multiple of the available calcium concentration in the medium (0.13 mM) where 5 × = 0.65 mM BAPTA, 10 × = 1.3 mM BAPTA and 15 × = 2.0 mM BAPTA. $CaCl_2$ was added to give a final concentration equivalent to the BAPTA concentration. Total RNA was analysed as described in Figure 1. **B**. Effect of $LaCl_3$ on CHX-mediated *lac-1* transcript accumulation. EP155 cultures were prepared and exposed to 3 μM CHX and the indicated concentrations of $LaCl_3$ for 24 h. Total RNA was subjected to hybridization analysis as described in Figure 1 and the *lac-1* and β-tubulin signal intensities were determined by photodensitometry. Each *lac-1* signal was normalized relative to the corresponding β-tubulin signal from the same gel lane and is expressed as a percentage of the normalized *lac-1* signal from a control culture receiving CHX but no $LaCl_3$. **C**. Effect of $LaCl_3$ on pigmentation in EP155. Cultures were grown on cellophane disks covering PDA containing the indicated concentrations of $LaCl_3$ in mM. Pigments were extracted and diluted 1:40 (v/w fungus) and the concentration was determined photometrically as described by Hillman *et al.* (1990). Optical density of pigment extracted from strain EP713 grown on PDA without supplements is presented for comparison.

Figure 2A. At the 3 μM dose used for the stimulation of *lac-1* transcript accumulation, the CHX-resistant mutants appeared to incorporate >75% of the label that was incorporated by untreated controls while the EP155 and EP713 strains incorporated only 10−12% of the control values. At 10 μM CHX, incorporation by the EP155 and EP713 strains was reduced to ~5% of that incorporated by untreated controls, while incorporation by the mutants was still >60% of the control values. When the CHX-resistant strains were tested for the CHX-mediated accumulation of the *lac-1* transcript, transcript levels were found to be similar in the cultures regardless of whether or not they were exposed to CHX (Figure 2B). The resistance of the mutants to both the inhibition of protein synthesis by CHX and the stimulation of *lac-1* transcript accumulation by CHX, establishes that CHX increases *lac-1* transcript levels through the inhibition of protein synthesis rather than through an alternative mechanism. Additionally, the similar response of EP155 and EP713 to the inhibitory effect of CHX on protein synthesis supports the conclusion that the failure of CHX to elicit *lac-1* transcript accumulation in EP713 to a level equivalent to that observed in EP155 does not stem from differences in the sensitivities of the two strains to the compound.

The stimulation of lac-1 transcript accumulation by CHX is calcium-dependent

While testing the effects of various compounds on the accumulation of *lac-1* transcript in CHX-treated *C.parasitica*, we noticed that substances known to perturb calcium-mediated signal transduction also affected *lac-1* transcript accumulation. The addition of the calcium chelator 1,2-bis-(*o*-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid (BAPTA) (Tsien, 1980) to cultures of the virus-free EP155 strain together with 3 μM CHX inhibited the stimulation of *lac-1* transcript accumulation in a dose-dependent manner

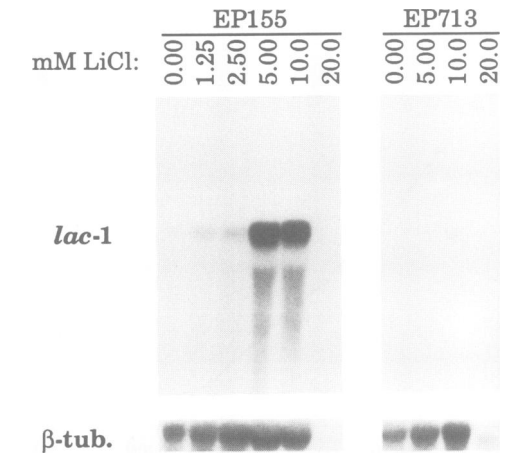

Fig. 4. Dose–response of *lac-1* transcript accumulation to LiCl in strains EP155 and EP713. Cultures were prepared and grown for 40 h as described in Figure 1. LiCl was added to the concentrations indicated and the cultures were incubated for an additional 24 h. RNA was analysed as described in Figure 1.

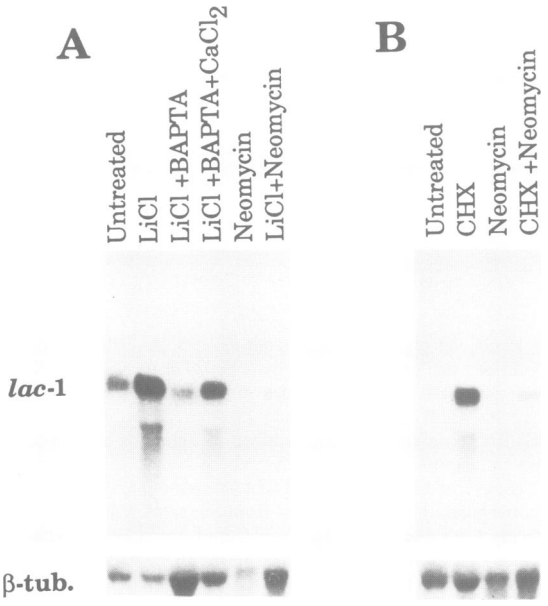

Fig. 5. The effect of compounds that perturb calcium and IP_3 metabolism on the LiCl- and CHX-mediated increase of *lac-1* transcript levels in strain EP155. **A.** Effect of 2 mM BAPTA and 0.5 mM neomycin on *lac-1* transcript accumulation mediated by 10 mM LiCl. **B.** Effect of 0.5 mM neomycin on CHX-mediated transcript accumulation. EP155 cultures were prepared and grown for 40 h as described in Figure 1. The indicated substances were added and the cultures were grown for an additional 24 h. RNA was analysed as described in Figure 1.

(Figure 3A). Transcript levels dropped to the level observed in uninduced controls when the BAPTA concentration exceeded 2 mM ($\sim 15 \times$ the calcium concentration in the medium used for the experiments). When sufficient calcium chloride was provided to neutralize the BAPTA that was added to the cultures, the chelator no longer inhibited *lac-1* transcript accumulation (Figure 3A), indicating that the effect of the BAPTA was specifically due to calcium chelation. Similar results were observed when ethylene glycol-bis-(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid (EGTA) was added along with the CHX, although significantly higher concentrations (10 mM) were required due to the limited chelating capacity of EGTA (Tsien, 1980) at the low pH of the fungal medium (pH = 5.2) (data not presented).

In addition to the calcium chelators BAPTA and EGTA, the effect of the calcium channel blocker, lanthanum (La; Nathan *et al.*, 1988) was investigated. When $LaCl_3$ was added to EP155 cultures in combination with 3 μM CHX, the accumulation of *lac-1* transcript was reduced in a dose-dependent manner (Figure 3B). The inhibition by $LaCl_3$ was linear in the range from $\sim 3-300$ μM when plotted as a percent of an uninhibited, CHX-stimulated control against the logarithm of $LaCl_3$ concentration. Transcript accumulation was reduced to the level observed in the uninduced controls at ~ 1 mM $LaCl_3$. $LaCl_3$ was also found to affect the development of pigmentation in the EP155 strain. When the fungus was grown on solid medium containing $LaCl_3$, an increase in extractable pigments was observed when $LaCl_3$ concentrations were < 1 mM (Figure 3C). Extractable pigments were significantly reduced when $LaCl_3$ concentrations exceeded 1 mM and were similar to those found in the hypovirulent strain at a concentration of 2 mM (Figure 3C). $LaCl_3$ concentrations higher than 2 mM reduced the radial growth rate of the EP155 mycelium, while concentrations of 5 mM and above were found to inhibit growth completely (data not presented). These results suggest that blocking calcium channels in EP155 can mimic some of the hypovirulence-associated traits displayed by the virus-harboring EP713 strain.

IP_3 levels affect lac-1 transcript accumulation

Since calcium mobilization is stimulated by IP_3 (Hokin, 1985; Berridge, 1987), we investigated the effects of several compounds that inhibit the IP_3 cycle on *lac-1* transcript accumulation. Lithium has been shown to enhance the accumulation of IP_3 by blocking dephosphorylation of the compound (Berridge *et al.*, 1982). Chronic exposure to LiCl has been observed to stimulate an increase in IP_3 levels in *N. crassa* with maximum accumulation observed at 10 mM LiCl (Hanson, 1991). Exposure of the virus-free EP155 strain to LiCl resulted in a significant stimulation of laccase transcript accumulation at concentrations of 5–10 mM (Figure 4). In contrast, exposure of the virus-harboring EP713 strain to similar concentrations of LiCl failed to elicit a similar increase in *lac-1* transcript levels (Figure 4), demonstrating that the stimulation of *lac-1* transcript accumulation by LiCl is suppressed in the presence of the virus. These LiCl concentrations did not affect β-tubulin transcript levels in either strain, suggesting that the stimulatory effect of lithium on transcript levels and its apparent inhibition by the presence of virus are limited to a specific subset of genes. Concentrations of LiCl in excess of 20 mM failed to increase laccase transcript levels in either strain, however β-tubulin transcript levels also were reduced (Figure 4), suggesting that these concentrations of lithium have a non-specific effect on transcription.

Neomycin appears to block the hydrolysis of phosphatidylinositol bisphosphate (PIP_2) into IP_3 and diacylglycerol (Downes and Mitchell, 1981; Carney *et al.*, 1985; Cockcroft *et al.*, 1987; Slivka and Insel, 1987). Since an increase in IP_3 levels can trigger calcium mobilization, neomycin can inhibit signal transduction through calcium-dependent pathways (Carney *et al.*, 1985; Vergara *et al.*, 1985; Penner, 1988). As shown by the data presented in Figure 5A, 0.5 mM neomycin blocked the stimulation of

lac-1 transcript accumulation by LiCl, suggesting that without IP_3 generation, lithium was unable to stimulate *lac-1* transcription. BAPTA effectively blocked the stimulation of *lac-1* transcript accumulation by LiCl, indicating that the lithium-induced accumulation of IP_3 was affecting *lac-1* transcript levels through a calcium-dependent mechanism. When sufficient $CaCl_2$ to saturate the BAPTA was added to the cultures, the chelator no longer blocked the accumulation of *lac-1* transcript, confirming that the effect of the BAPTA in this case was specifically due to calcium chelation.

The conclusion that calcium mobilization is required for CHX to elicit an increase in *lac-1* transcript levels, suggests that IP_3 generation may also be involved in the response to CHX. This hypothesis was tested using neomycin to block PIP_2 hydrolysis in CHX-treated cultures. As with the LiCl-mediated increase in *lac-1* transcript levels, 0.5 mM neomycin suppressed the CHX-mediated increase in transcript levels, suggesting that IP_3 generation stimulates the calcium mobilization required for CHX to elicit an increase in *lac-1* transcript levels. Cultures were exposed to the combination CHX and LiCl to determine if the combination of the two compounds would cause a further increase in *lac-1* transcript accumulation. All concentrations of LiCl tested (0.5−10 mM) suppressed both the β-tubulin and the *lac-1* transcript levels when combined with 3 μM CHX (data not presented), suggesting that the combination of the two compounds has a non-specific inhibitory effect on transcription.

Culture density affects stimulation of lac-1 transcript accumulation by CHX and lithium

Ringling and Van Alfen (1991) have shown that *lac-1* transcription increases when liquid cultures enter late exponential phase. To explore the relationship between the induction of *lac-1* transcript accumulation and culture density in more detail, culture density was varied by adding different volumes of inoculum to a series of cultures. This method allowed the culture density to be varied, while incubation time was held constant. As indicated in Figure 6, the cultures inoculated with 2.5 and 3 ml volumes of inoculum reached stationary phase by 40 h. Under these conditions, *lac-1* expression increased ~3-fold as the culture approached stationary phase and then began to decline (Figure 6). This pattern is similar to that previously reported for uninduced cultures where samples were removed as the density of a single culture increased over time (Rigling and Van Alfen, 1991). The accumulation of transcript in the LiCl-treated cultures also showed an ~3-fold increase, although overall transcript levels were significantly higher than those in the uninduced cultures and the transcript levels continued to increase after the cultures reached stationary phase (Figure 6). The accumulation of *lac-1* transcript in the CHX-treated cultures showed a more substantial 7-fold increase, which also continued after the culture reached stationary phase (Figure 6). The continued increase in *lac-1* transcript levels in the LiCl- and CHX-treated cultures after stationary phase suggests that the compounds disturb the normal regulation of *lac-1* transcript accumulation.

The lac-1 promoter encodes the information required for the induction and suppression of transcript accumulation

To determine whether the *lac-1* promoter contains the information necessary for transcript levels to respond to CHX

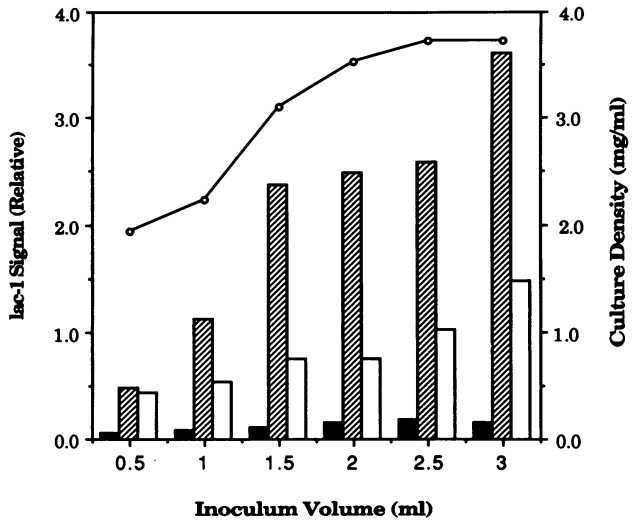

Fig. 6. Effect of culture density on the accumulation of *lac-1* transcript in uninduced EP155 cultures (solid bars) and in EP155 cultures induced with CHX (cross-hatched bars) and LiCl (open bars). EP155 cultures were prepared as described in Figure 1 except that the inoculum volumes were varied as indicated. At 48 h after inoculation, mycelia were collected from the uninduced set of cultures to determine the culture density and prepare RNA. The remaining cultures were induced with either 3 μM CHX or 10 mM LiCl. These were incubated for an additional 12 h before determining culture density and preparing RNA. The density of the induced cultures did not change significantly during this incubation. Culture density was determined by collecting mycelia from a 10 ml volume of culture on Miracloth (Calbiochem) using gentle vacuum filtration. Each sample was blotted until a constant weight was obtained. RNA was prepared and analysed by dot blotting. β-tubulin and *lac-1* signals were quantified by photodensitometry and the *lac-1* signals were normalized relative to the corresponding β-tubulin signals.

stimulation, the promoter of the gene was spliced to a non-homologous reporter gene. A cassette consisting of the *Escherichia coli hph* (hygromycin phosphotransferase) coding region fused to the *Aspergillus nidulans trpC* terminator was used as the reporter. As indicated by the diagram presented in Figure 7, two different constructs were made by fusing the reporter cassette to either a 229 bp amplicon of the *C.parasitica lac-1* promoter (cloned in pLP8 and carried by transformant LPH8) or to a longer 2.2 kb amplicon (cloned in pLP1 and carried by transformant LPH1). Both versions of the promoter contain the putative TATA and CCAAT promoter elements, *lac-1* transcription start site and 85 bp of the 5′ untranslated region of the *lac-1* transcript (Choi *et al.*, 1992). As the data presented in Figure 7 indicates, the two fusions behaved differently when transformed into EP155. The transcript level produced by the fusion with the 2.2 kb promoter segment was regulated in LPH1 transformants in a manner consistent with the regulation of the endogenous *lac-1* gene: transcript levels increased in cultures exposed to 3 μM CHX, but this increase was suppressed by 2 mM BAPTA and 0.5 mM neomycin (Figure 7). The fusion with the short promoter segment was transcribed in LPH8 transformants at a low level that did not change when cultures were exposed to 3 μM CHX (Figure 7). These observations indicate that while the short promoter is functional, it does not respond to CHX stimulation. The response of the long promoter to CHX stimulation indicates that the information required for the response is located in the *lac-1* promoter at least 144 bp upstream from the transcription start site. The localization of the

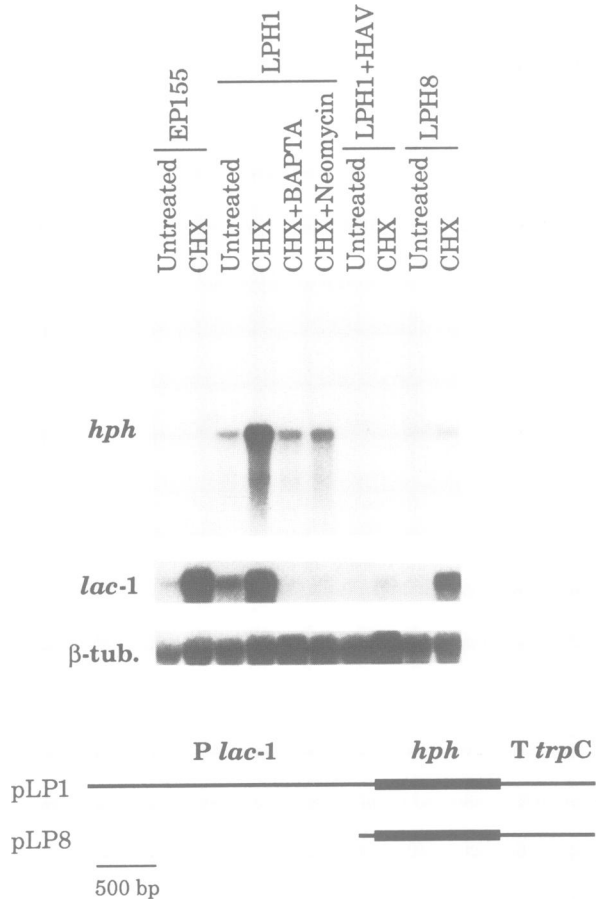

Fig. 7. Transcription activity of the *lac-1* promoter. Cultures of EP155, an LPH1 transformant, an LPH1 transformant containing the hypovirulence-associated virus from strain EP713 (LPH1 + HAV) and an LPH8 transformant, were prepared as described for Figure 1. At 48 h 3 μM CHX alone or in combination with 2 mM BAPTA or 0.5 mM neomycin was added to the indicated cultures and the incubation was continued for an additional 24 h. RNA was analysed as described in Figure 1 except that the blot was hybridized to a probe specific for the *hph* transcript and subjected to autoradiography before stripping and rehybridizing to the *lac-1* probe.

information required for the CHX-mediated stimulation of transcription to the *lac-1* promoter region also suggests that the changes in *lac-1* transcript accumulation resulted from the modulation of transcription activity rather than a change in transcript stability.

To investigate the effect of the virus on transcription by the two promoters, viral dsRNA was introduced into the transformants by anastomosis with EP713. When the virus-containing transformants were grown on hygromycin-containing medium, the growth rates of LPH1 transformants, but not the LPH8 transformants, were significantly reduced relative to the growth rates on medium where the antibiotic was omitted (data not presented). This result suggests that the virus suppresses expression of the *hph* gene from the construct with the long promoter, but not from the construct with the short promoter. Virus-mediated suppression of the transcript levels in an LPH1 transformant was confirmed by hybridization analysis of RNA from cultures exposed to 3 μM CHX and from untreated cultures (LPH1 + HAV, Figure 7). These results suggest that as with the information required for the *lac-1* promoter to respond to CHX, the information required for the virus-mediated suppression of *lac-1* transcript accumulation is located in the *lac-1* promoter at least 144 bp upstream of the transcription start site.

Discussion

CHX may stimulate lac-1 transcription by inhibiting translation of a negative regulatory factor

CHX inhibits protein synthesis at the translational level in eukaryotes by binding to the 60S ribosomal subunit (for a brief review, see Vázquez, 1979). Low concentrations of CHX and other protein synthesis inhibitors have been shown to stimulate the expression of the *N.crassa* phenoloxidase enzymes tyrosinase (Horowitz *et al.*, 1970) and laccase (Froehner and Eriksson, 1974). To account for this phenomenon, both Horowitz *et al.* (1970) and Froehner and Eriksson (1974) proposed that the inhibition of protein synthesis blocks the translation of a labile regulatory factor responsible for repressing expression of the tyrosinase and laccase enzymes. We have considered a similar explanation for the increased *lac-1* transcript levels observed in CHX-treated cultures of *C.parasitica* (Choi *et al.*, 1992) and have suggested that the mechanism may be analogous to the one proposed by Bauerle and Baltimore (1988) to account for the CHX-mediated activation of NF-κB. In our model, CHX would elicit an increase in *lac-1* transcript levels by blocking the translation of an inhibitory transcription factor that is similar to IκB.

Although the primary effect of CHX is on translation (Vázquez, 1979), the compound also has been shown to stimulate the signal transduction pathways that activate the transcription of certain genes (Mahadevan and Edwards, 1991; Edwards and Mahadevan, 1992). Froehner and Eriksson (1974) and Horowitz *et al.* (1970) used additional inhibitors of protein synthesis to rule out the possibility that the increase in laccase (Froehner and Eriksson, 1974) and tyrosinase (Horowitz *et al.*, 1970) enzyme activities observed in *N.crassa* may stem from a secondary effect of CHX treatment. As a complementary approach to the use of additional inhibitors of protein synthesis, we employed *C.parasitica* mutants specifically resistant to the inhibition of translation by CHX. The insensitivity of both protein translation (Figure 2A) and *lac-1* transcript levels (Figure 2B) to the effects of CHX in these mutants, indicates that the compound induced *lac-1* transcript accumulation by inhibiting protein synthesis and not through some other mechanism. This result supports the hypothesis that CHX blocks the synthesis of a protein that functions as a negative regulator of *lac-1* transcript accumulation. Our previous results indicated that the accumulation of *lac-1* transcript becomes apparent ~6 h following the addition of CHX to EP155 cultures (Choi *et al.*, 1992), suggesting that it takes approximately this period of time for the concentration of this putative regulator to drop below the level required to effectively suppress *lac-1* transcript levels.

CHX has been shown to increase mRNA levels (Meister *et al.*, 1979) possibly by stabilizing polysomes (Edwards and Mahadevan, 1992). To determine whether *lac-1* transcript levels increase in CHX-treated *C.parasitica* in response to a specific increase in promoter activity or to a general increase in transcript stability, the effect of CHX on transcript levels was investigated in transformed fungi using a non-homologous reporter fused to two versions of the *lac-1*

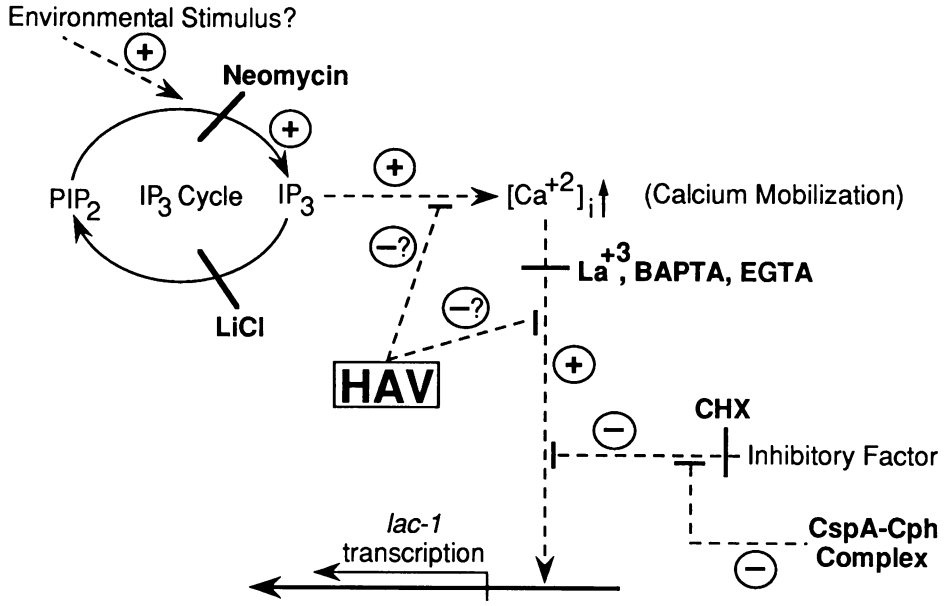

Fig. 8. Schematic diagram illustrating possible roles of calcium and IP_3 second messenger systems and of a putative negative regulatory factor in governing *lac-1* promoter activity. Possible sites of action are indicated for compounds that inhibit *lac-1* transcription (neomycin, La^{3+}, BAPTA and EGTA) as well as for compounds that stimulate *lac-1* transcription (CHX, CspA and LiCl). The hypovirulence-associated virus from strain EP713 (HAV) may suppress *lac-1* transcription by interfering with signal transmission through these systems (see text). +, indicates pathways that either stimulate other pathways or stimulate *lac-1* transcription; −, indicates pathways that inhibit other pathways.

promoter. The ability of CHX to elicit a significant increase in transcript levels from the reporter gene driven by the 2.2 kb version of the *lac-1* promoter indicates that promoter activity plays a crucial role in the increase in transcript levels. On the other hand, the inability of CHX to affect a change in transcript levels from the construction with the short promoter suggests that the enhancement of mRNA stability plays a relatively minor role in the increased accumulation of transcript. The differential response of the long and short promoter fusions to CHX suggests that the element responsible for the response of the *lac-1* promoter to CHX resides at least 144 bp upstream from the transcription start site.

Stimulation of lac-1 transcription is dependent on the IP_3 and calcium second messenger systems

Signal transduction through the IP_3 and calcium-dependent second messenger systems is involved in a large number of processes that allow eukaryotic cells to respond to environmental stimuli such as the binding of hormones and pheromones (for reviews see Hokin, 1985; Berridge, 1987; Van Haastert *et al.*, 1991). The abrogation of the CHX-mediated stimulation of *lac-1* transcription by the calcium chelator BAPTA (Figure 3A) and by the calcium channel blocker lanthanum (Figure 3B), indicates that stimulation of *lac-1* transcription may be dependent on calcium mobilization. BAPTA also suppresses the CHX-stimulated increase in the *lac-1* promoter fusion transcript observed in fungi transformed with the long promoter construct (Figure 7), establishing that it is the stimulation of *lac-1* transcription which is calcium-dependent. Froehner and Eriksson (1974) noted that the production of laccase by *N.crassa* was significantly reduced at low calcium concentrations, suggesting that calcium mobilization may also be necessary for laccase expression in *N.crassa*.

To support the hypothesis that *lac-1* transcription is dependent on calcium mobilization, we examined the effect of IP_3 accumulation on *lac-1* transcript levels. As with other eukaryotes (Hokin, 1985; Berridge, 1987), IP_3 triggers calcium mobilization in fungi such as *N.crassa* (Cornelius *et al.*, 1989). Hanson (1991) has demonstrated that chronic LiCl exposure causes an increase in intracellular IP_3 levels in *N.crassa*. Treatment of *C.parasitica* with LiCl causes an increase in *lac-1* transcript levels, demonstrating that LiCl stimulates *lac-1* transcription (Figure 4) and suggesting that IP_3 may play a role in regulating *lac-1* transcript levels. Interestingly, *lac-1* transcript levels reached a maximum at 10 mM LiCl (Figure 4), which is the same LiCl concentration that produces the maximum accumulation of IP_3 in *N.crassa* (Hanson, 1991), raising the possibility of a correlation between maximal IP_3 levels and maximal *lac-1* transcript levels. The ability of compounds that block signal transduction either distal (neomycin) or proximal (BAPTA) to the IP_3 second messenger to abrogate the stimulation of *lac-1* transcription by both LiCl (Figure 5A) and CHX (Figures 3A, 5B and 7) further supports a role for IP_3 as a regulator of *lac-1* transcription.

At least two regulatory pathways govern lac-1 transcription

Our results indicate that the *lac-1* promoter responds to at least two regulatory signals: one is a stimulatory signal mediated by the IP_3−calcium second messenger systems and the other is an inhibitory signal mediated by a labile, negative regulatory factor. Such a promoter would be inactivated when the calcium−IP_3 second messenger systems are debilitated by BAPTA, $LaCl_3$ and neomycin and would be 'derepressed' when synthesis of the negative regulatory factor is blocked by the inhibition of translation (Figure 8). As mentioned above, the details of this model could be mechanistically similar to the one proposed for NF-κB regulation by Bauerle and Baltimore (1988). In this

version of the model, activator and inhibitor factors would form an inactive cytoplasmic complex, similar to that proposed for the complex between NF-κB and IκB (Bauerly and Baltimore, 1988). Calcium mobilization would cause the complex to dissociate, allowing the activating factor to travel to the nucleus to activate *lac-1* transcription. Although this analogy to the NF-κB system is consistent with our results, other models are possible and cannot be ruled out at this time.

Although LiCl and CHX can significantly increase the expression of *lac-1*, it is doubtful that these compounds play a role in the natural regulation of the gene. Rigling and Van Alfen (1991) have shown that *lac-1* transcript levels increase in uninduced *C.parasitica* liquid cultures as they approach stationary phase. We observed a similar result in cultures where the cell density varied while incubation time remained constant (Figure 6), indicating that growth conditions, such as nutrition or gas exchange, may regulate *lac-1* transcription in nature. This observation suggests a role for a receptor capable of detecting the environmental stimulus that elicits an increase in *lac-1* expression and converting information about the intensity of this stimulus into IP_3 generation by modulating phospholipase C activity. Although this scenario is speculative, it is consistent with current knowledge regarding receptor-mediated signal transduction (Hokin, 1985; Berridge, 1987). Evidence supporting this scenario is provided by the stimulation of *lac-1* transcription by LiCl: LiCl has been shown to amplify agonist-dependent increases in IP_3 levels (Berridge *et al.*, 1982), suggesting that the compound may amplify an IP_3-mediated signal responsible for stimulating *lac-1* transcription. This hypothesis is consistent with the ability of compounds that block signal transduction either distal (neomycin) or proximal (BAPTA and $LaCl_3$) to the IP_3 second messenger to squelch the LiCl-mediated increase in *lac-1* transcript levels (Figure 5A).

Additional support for the transduction of a culture-dependent stimulus comes from the observation that CHX elicits maximal transcript accumulation when added to cultures at stationary phase (Figure 6). A similar observation was made by Feldman and Thayer (1974), who noted that maximal tyrosinase synthesis was induced when protein synthesis inhibitors were added to stationary phase *N.crassa* cultures. The results are consistant with the hypothesis that the addition of a translation inhibitor blocks synthesis of a putative regulatory factor that normally limits activation of the *lac-1* promoter. As the available inhibitory factor is turned over, its concentration drops below that required to limit *lac-1* promoter activity, resulting in a 'superinduction' of *lac-1* transcription. The maximal *lac-1* transcript levels observed at stationary phase support the hypothesis that the environmental stimulus modulating *lac-1* expression reaches a maximum at this point. The decline of *lac-1* transcript levels observed in unstimulated cultures as they approach stationary phase suggests that the proposed inhibitory factor may play a role in dampening the response of the *lac-1* promoter to the environmental stimulus.

Our previous results indicate that CspA stimulates *lac-1* transcription by affecting the same regulatory pathway as CHX, but through a different mechanism (Larson and Nuss, 1992). These conclusions suggest that as with CHX, CspA may interfere with the activity of the negative regulatory factor proposed to limit *lac-1* promoter activation (Figure 8). CspA has been shown to abrogate the transmission of some calcium-dependent signals (Mattila *et al.*, 1990; Baldari *et al.*, 1991), but not others (Mattila *et al.*, 1990). This observation suggests that the calcium-dependent pathway proposed to be responsible for stimulating *lac-1* transcription could belong to a class that is resistant to the action of CspA. In mammalian cells, the calcium-dependent signals blocked by CspA have been shown to be required for the activation of specific transcription factors (Emmel *et al.*, 1989; Mattila *et al.*, 1990; Flanagan *et al.*, 1991). This observation suggests the possibility that CspA stimulates *lac-1* transcription by blocking a calcium-dependent signal that governs the activity of the negative regulatory factor proposed to limit *lac-1* transcription. For example, expression of the putative regulatory factor could require a calcium-dependent transcription factor, allowing CspA to block synthesis of the factor at the level of transcription, much as CHX appears to block it at the level of translation. Alternatively, CspA could neutralize the negative regulatory factor by squelching a calcium-dependent signal that is required to maintain it in an active state. Dumont *et al.* (1990) have reported that CspA blocks calcium-dependent signals that constrain T cell activation, supporting the possibility that CspA might interfere with a calcium-dependent signal that functions to limit the activation of *lac-1* transcription. The possibility that the activity of the putative negative regulator is governed by a calcium-dependent pathway, provides a possible mechanism for the dampening of *lac-1* transcription as cultures enter stationary phase, as observed in uninduced cultures (Figure 6; Rigling and Van Alfen, 1991).

Evidence that hypovirulence-associated viruses perturb regulatory pathways

The hypovirulence-associated traits exhibited by the virus-harboring EP713 strain appear to result from the action of the L-dsRNA ORFA coding region (Choi and Nuss, 1992a). In the case of laccase activity, the presence of the virus suppresses the CHX-mediated (Choi *et al.*, 1992; Figure 1). CspA-mediated (Larson and Nuss, 1992) and LiCl-mediated (Figure 4) increases in *lac-1* transcript levels. This suppression appears to be at the level of promoter activity as demonstrated by the inability of CHX to elicit a significant change in the accumulation of reporter transcript in LPH1 transformants harboring viral dsRNA (LPH1 + HAV, Figure 7). The similarity between the effect of the virus and the inhibitors of the IP_3 and calcium second messenger systems suggests that the virus codes for a product that interferes with either IP_3- or calcium-dependent signal transmission (Figure 8). The perturbation of such a fundamental regulatory system may explain the ability of a single viral coding region to produce such manifold effects on host metabolism. In support of this hypothesis, the addition of the calcium channel blocker $LaCl_3$ to solid culture medium was observed to suppress the pigmentation of mycelia grown on the medium in a manner that is similar to the suppression of pigmentation that results from viral infection (Figure 3C). The ability of $LaCl_3$ to suppress pigmentation and to prevent stimulation of *lac-1* transcription suggests that both processes may be regulated by the calcium second messenger system.

The possibility that hypovirulence-associated viruses interfere with *C.parasitica* metabolism by perturbing the IP_3–calcium second messenger systems is supported by similar observations in other organisms. For example, the

influenza A virus has been reported to cause human neutrophil deactivation by impairing the mobilization of internal calcium reverses (Hartshorn *et al.*, 1988). Additionally, both the human immunodeficiency virus (Perez *et al.*, 1991) and feline leukemia virus (Wright *et al.*, 1989) appear to interfere with the IP_3−calcium-dependent signal transduction pathway. Thus, if hypovirulence-associated viruses do interfere with IP_3−calcium-dependent signalling, they may serve as models to help understand how other viruses affect signal transduction pathways.

Perspective

While the model for the regulation of *lac-1* transcription presented in this report is necessarily tentative, it provides a conceptual framework for generating testable hypotheses that can be used for guiding future research. The ability to manipulate the *lac-1* promoter and test the effects of various alterations on promoter activity in transformed fungi, should facilitate defining the elements that respond to the positive and negative regulatory signals responsible for governing *lac-1* transcription. These elements can in turn be used to define the regulatory factors themselves and to begin to elucidate the signal transduction pathways that control these factors. The recent development of an infectious cDNA clone of the hypovirulence-associated virus carried by *C.parasitica* strain EP713 (Choi and Nuss, 1992b) should also allow progress on defining the interactions between viral gene products and components of the signal transduction pathways that are responsible for regulating *lac-1* transcription. Defining the mechanism responsible for suppression of *lac-1* transcription should not only provide a basis for understanding the source of other traits caused by the presence of hypovirulence-associated viruses but may also provide a basis for understanding the mechanism underlying hypovirulence itself.

Materials and methods

Fungal strains and growth conditions

C.parasitica strains EP155 (virulent and virus-free, ATCC 38755) and EP713 (isogenic to EP155, hypovirulent and contains viral dsRNA, ATCC 52571) were maintained as previously described by Hillman *et al.* (1990). Stationary liquid cultures in 50 ml of potato dextrose broth (PDB, Difco) were inoculated and maintained as previously described by Choi *et al.* (1992). Unless specified otherwise, compounds to be tested for their effect on *lac-1* transcript accumulation were added to 40 h after inoculation and following an additional 24 h incubation, mycelia were harvested for RNA extraction. CHX (Sigma) was added to cultures from a 30 mM ethanol stock. BAPTA (Molecular Probes) was added from a 200 mM stock prepared in distilled water and filter sterilized. $CaCl_2$, $LaCl_3$ and LiCl (Sigma) were added from 1, 0.25 and 8 M stocks, respectively, which were also prepared in distilled water and filter sterilized. Neomycin (Calbiochem) was added directly to the cultures as a solid.

Isolation of CHX-resistant mutants of EP155

CHX-resistant mutants were obtained by UV mutagenesis using a modification of a procedure originally described by Neuhäuser *et al.* (1970). EP155 conidia were collected from a mycelium grown on potato dextrose agar (PDA, Difco) as previously described by Hillman *et al.* (1990) and spread on 15 ml PDA methionine biotin (PDAmb, Anagnostakis, 1982a) plates. The plated conida were irradiated with 350 J/M^2 at 280 nm using a Statalinker apparatus (Stratagene). This dose gave an approximate kill rate of 50%. After the irradiated plates were incubated in the dark at 25°C for 16 h, they were overlaid with 10 ml of PDAmb containing 87.5 μM of CHX (35 μM final concentration). The plates were incubated as above and growing mycelia were subcultured to individual PDA plates containing 35 μM CHX. CHX-resistant isolates were obtained at a frequency of 4×10^{-6} per irradiated spore. All isolates were tested for growth on minimal medium agar [MMLT (Anagnostakis, 1982a) with tannic acid omitted] for normal sporulation and pigmentation on PDA, for normal germination of spores on PDAmb with and without CHX, and for normal laccase production on Bavendamm's medium (Bavendamm, 1928). Single spore isolates from strains passing these tests were saved for further analysis.

Construction of LPH1 and LPH8 transformants

LPH strains 1 and 8 (*lac-1* promoter *hph* fusion) were constructed by transforming EP155 with plasmids pLP1 and pLP8. Plasmids pLP1 and pLP8 were assembled by replacing the *A.nidulans trpC* promoter region of pUCDH25 with the *KpnI*−*ClaI* fragments containing *C.parasitica lac-1* promoter amplicons. The pUCDH25 plasmid was constructed by subcloning the *SspI*−*SphI* fragment of pDH25 containing the promoter and terminator sequences from the *A.nidulans trpC* gene fused to the *E.coli hph* coding region (Cullen *et al.*, 1987) into the *SmaI*−*SphI* sites of pUC19 (Yanisch-Perron *et al.*, 1985). For plasmid pLP1, 2.2 kb of the *C.parasitica lac-1* promoter was amplified by PCR from a genomic subclone using primers LP1 (5′AAAAAGGTACCTTCTAGAAAAGAGCCTCT-3′) and LP2 (5′-AAAACATCGATAATGAAGGACGAAGAC-3′). For plasmid pLP8, a 229 bp segment of the *lac-1* promoter was amplified using primers LP2 and LP9 (5′-GCTAGCGGTACCCTCAGCGCAAGCCGTATC-3′). These primers introduced *KpnI* (underlined in primers PL1 and PL8) and *ClaI* (underlined in primer PL2) restriction sites into the termini of the amplicons. PCR (Saiki *et al.*, 1988) was performed using a GeneAmp kit (Perkin−Elmer Cetus Instruments) for 30 cycles using the following parameters: denaturation for 1.5 min at 94°C, annealing for 30 s at 50°C and extension for 1 min at 72°C. The PCR products were treated with T4 DNA polymerase and cloned into pUC19 (Yanisch-Perron *et al.*, 1985) using standard methods (Sambrook *et al.*, 1989). *C.parasitica* strains LPH1 and LPH8 were prepared by transforming EP155 spheroplasts with pLP1 and pLP8, respectively (Churchill *et al.*, 1990) and transformants were selected on 40 μg/ml hygromycin (Calbiochem). Single spore isolates of the transformants were prepared and used for all experiments.

LPH1 and LPH8 strains carrying the hypovirulence-associated virus from strain EP713 were prepared by anastomosis (Anagnostakis and Day, 1979) on PDA. Transmission of the virus to the transformants was verified by testing for suppressed pigmentation on PDA and suppressed laccase production on Bavendamm's medium.

RNA preparation and analysis

RNA was prepared using a Mini-Beadbeater apparatus (Biospec Products, Inc.) as previously described (Choi *et al.*, 1992), but with the following modifications: the harvested mycelia were disrupted as before, except that the extraction tubes contained 1 g 0.5 mm zirconium beads and the extraction buffer was 4% SDS, 150 mM sodium acetate, pH 5.0, 100 mM LiCl, 10 mM EDTA, 10 mM EGTA and 20 mM β-mercaptoethanol. Total RNA (10 μg/sample) was separated by formaldehyde−agarose gel electrophoresis as previously described by Choi *et al.* (1992) and transferred to nylon membranes (Gene Screen Plus, DuPont) using a positive pressure blotting apparatus (Stratagene) as directed by the manufacturer. Dot blots were prepared by denaturing samples in formaldehyde−formamide sample buffer using standard methods (Sambrook *et al.*, 1989) and applying them to nylon membranes (Gene Screen Plus, DuPont) as directed by the manufacturer using a dot blot manifold (Minifold, Schleicher and Schuell). Blots were hybridized as previously described (Choi *et al.*, 1992). Probes for the *lac-1* and β-tubulin transcripts were prepared as previously described by Choi *et al.* (1992). The probe for the *lac-1* promoter−*hph* fusion transcript was prepared from the 790 bp *Eco*RI−*Bam*HI fragment of pUCDH25 that carries the majority of the *hph* coding region. Photodensitometry was performed using an UltroScan XL laser densitometer (LKB Pharmacia). Where necessary, autoradiographs exposed for different time periods were scanned to insure that the signal fell within the linear response range of the film. The Northern blots present in each figure were prepared using RNA extracted from cultures inoculated, grown, induced and harvested in parallel.

Protein synthesis assay

The inhibition of protein synthesis by CHX was assessed by measuring [^{35}S]methionine incorporation. Isotope incorporation was determined using a modification of a procedure described by Perlman and Feldman (1982). Liquid cultures were inoculated and incubated as described above. After 40 h, 5 ml vol of the cultures were distributed into sterile 15 ml screwcap centrifuge tubes for use in the incorporation assays. The fungus was pelleted by centrifugation at 2000 *g* for 10 min and resuspended in 5 ml of minimal medium (Anagnostakis, 1982a). CHX or vehicle was added to each tube and following a 30 min incubation, 3 μCi of [^{35}S]L-methionine (Amersham SJ.1015) was added. After an additional 2 h incubation, isotope incorporation

was stopped by adding 100% (w/v) trichloroacetic acid (TCA) to each tube to a final concentration of 10%. Following a 10 min incubation, the fungus was pelleted by centrifugation at 2000 *g* for 15 min. The pellets were then washed with 10% TCA and transferred to screw top microfuge tubes containing 0.5 g 0.5 mm zirconium beads. Unincorporated isotope was extracted from the pellets by adding 1 ml 10% TCA and shaking for 50 s with a Mini-Beadbeater apparatus set on low. The fungus was pelleted by microcentrifugation at 8000 r.p.m. for 10 min and the supernatant was removed by aspiration. This washing process was repeated twice. The precipitated protein was then solubilized by adding 0.5 ml of 0.5 N NaOH, shaking with the Mini-Beadbeater for 50 s on low and incubating at room temperature overnight. After the insoluble debris was removed by microcentrifugation at 8000 r.p.m. for 10 min, the supernatants were transferred to clean tubes and were neutralized by adding 0.5 ml of 0.5 N HCl. The radioactivity of each sample was determined by liquid scintillation counting and the protein content was determined by the Bradford assay (Bradford, 1976) using reagents from Bio-Rad.

Quantification of pigment accumulation

Pigment accumulation was quantified using a modification of the technique described by Hillman *et al.* (1990). Fungus was grown on cellophane on PDA. Radial sections of mycelium weighing 0.2 g were placed in a screw cap microfuge tube containing 1 g zirconium beads. Pigments were extracted by adding 1 ml absolute ethanol and shaking the tubes for 1 min with a Mini-Beadbeater apparatus set on high. After microcentrifugation for 10 min at 8000 r.p.m., the supernatants were reserved and each sample was extracted with a second volume of ethanol. The extracts for each sample were combined and diluted with ethanol to give a final volume equal to 40 × the mass of the extracted mycelium. Samples were then made basic by adding NaOH to 0.01 N and the absorbances were determined at 454 nm (Hillman *et al.*, 1990).

Acknowledgements

We wish to thank Diane Pawlyk for excellent technical assistance.

References

Anagnostakis,S.L. (1982a) *Mycologia*, **74**, 826–830.
Anagnostakis,S.L. (1982b) *Science*, **215**, 466–471.
Anagnostakis,S.L. (1984) In Jennings,D.H. and Rayner,A.D.M. (eds) *The Ecology and Physiology of the Fungal Mycelium*. Cambridge University Press, Cambridge, pp. 353–366.
Anagnostakis,S.L. and Day,P.R. (1979) *Phytopathology*, **69**, 1226–1229.
Baldari,C.T., Macchia,G., Heguy,A., Melli,M. and Telford,J.L. (1991) *J. Biol. Chem.*, **266**, 19103–19108.
Bauerle,P.A. and Baltimore,D. (1988) *Science*, **242**, 540–546.
Bavendamm,W. (1928) *Z. Pflanzenkrank. Pflanzenschutz.*, **38**, 257–276.
Berridge,M.J (1987) *Annu. Rev. Biochem.*, **56**, 159–93.
Berridge,M.J., Downes,C.P. and Hanely,M.R. (1982) *Biochem. J.*, **206**, 587–595.
Bradford,M.M. (1976) *Anal. Biochem.*, **72**, 248–254.
Carney,D.H., Scott,D.L., Gordon,E.A. and LaBelle,E.F. (1985) *Cell*, **42**, 479–488.
Choi,G.H. and Nuss,D.L. (1992a) *EMBO J.*, **11**, 473–477.
Choi,G.H. and Nuss,D.L. (1992b) *Science*, **257**, 800–803.
Choi,G.H., Larson,T.G. and Nuss,D.L. (1992) *Mol. Plant–Microbe Interact.*, **5**, 119–128.
Churchill,A.C.L., Ciuffetti,L.M., Hansen,D.R., Van Etten,H.D. and Van Alfen,N.K. (1990) *Curr. Genet.*, **17**, 25–31.
Cockcroft,S., Howell,T.W. and Gomperts,B.D. (1987) *J. Cell Biol.*, **105**, 2745–2750.
Cornelius,G., Gebauer,G. and Techel,G. (1989) *Biochem. Biophys. Res. Commun.*, **162**, 852–856.
Cullen,D., Leong,S.A., Wilson,L.J. and Henner,D.J. (1987) *Gene*, **57**, 21–26.
Day,P.R., Dodds,J.A., Elliston,J.E., Jaynes,R.A. and Anagnostakis,S.A. (1977) *Phytopathology*, **67**, 1393–1396.
Downes,C.P. and Mitchell,R.H. (1981) *Biochem. J.*, **198**, 133–140.
Dumont,F.J., Staruch,M.J., Koprak,S.L., Melino,M.R and Sigal,N.H. (1990) *J. Immunol.*, **144**, 251–258.
Edwards,D.R. and Mahadevan,L.C. (1992) *EMBO J.*, **11**, 2415–2424.
Elliston,J.E. (1985) *Phytopathology*, **75**, 151–158.
Emmel,E.A., Verweij,C.L., Durband,D.B., Higgins,K.M., Lacy,E. and Crabtree,G.R. (1989) *Science*, **246**, 1617–1620.
Feldman,J.F. and Thayer,J.P. (1974) *Biochem. Biophys. Res. Commun.*, **61**, 977–982.
Flanagan,W.M., Corthésy,B., Bram,R.J. and Crabtree,G.R. (1991) *Nature*, **352**, 803–807.
Froehner,S.C. and Eriksson,K.E. (1974) *J. Bacteriol.*, **120**, 450–457.
Griffin,G.J. 1986 (1986) *Hort. Rev.*, **8**, 291–335.
Hartshorn,K.L., Collamer,M., Auerbach,M., Meyrs,J.B., Pavotsky,N. and Tauber,A.I. (1988) *J. Immunol.*, **141**, 1295–1301.
Hanson,B.A. (1991) *Exp. Mycol.*, **15**, 76–90.
Herman,T.E., Kurtz,M.B. and Champe,S.E. (1983) *J. Bacteriol.*, **154**, 955–964.
Hillman,B.I., Shapira,R. and Nuss,D.L. (1990) *Phytopathology*, **80**, 950–956.
Hokin,L.E. (1985) *Annu. Rev. Biochem.*, **54**, 205–235.
Horowitz,N.H., Feldman,H.M. and Pall,M.L. (1970) *J. Biol. Chem.*, **245**, 2784–2788.
Larson,T.G. and Nuss,D.L. (1992) *Proc. Natl. Acad. Sci. USA*, in press.
Linden,R.M., Schilling,B.C., Germann,U.A. and Lerch,K. (1991) *Curr. Genet.*, **19**, 375–381.
MacDonald,W.L. and Fulbright,D.W. (1991) *Plant Disease*, **75**, 656–661.
Mahadevan,L.C. and Edwards,D.R. (1991) *Nature*, **349**, 747–748.
Mattila,P.S., Ullman,K.S., Fiering,S., Emmel,E.A., McCutcheon,M., Crabtree,G.R. and Herzenberg,L.A. (1990) *EMBO J.*, **9**, 4425–4433.
Meister,R.K., Hulman,S.E. and Johnson,L.F. (1979) *J. Cell Physiol.*, **100**, 531–538.
Nathan,R.D., Kanai,K., Clark,R.B. and Giles,W. (1988) *J. Gen. Physiol.*, **91**, 549–572.
Neuhäuser,A., Klingmüller,W. and Kaudewitz,F. (1970) *Mol. Gen. Genet.*, **106**, 180–194.
Penner,R. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 9856–9860.
Perlman,J. and Feldman,J.F. (1982) *Mol. Cell. Biol.*, **2**, 1167–1173.
Perez,V.L., Rowe,T., Justement,J.S., Butera,S.T., June,C.H. and Folks,T.M. (1991) *J. Immunol.*, **147**, 3145–3148.
Powel,W.A. and Van Alfen,N.K. (1987) *Mol. Cell. Biol.*, **7**, 3688–3693.
Rigling,D. and Van Alfen,N.K. (1991) *J. Bacteriol.*, **173**, 8000–8003.
Rigling,D., Heiniger,U. and Hohl,H.R. (1989) *Phytopathology*, **79**, 219–223.
Saiki,R.K., Gelfand,D.H., Stoffel,S., Scharf,S.J., Higuchi,R., Horn,G.T., Mullis,K.B. and Erlich,H.A. (1988) *Science*, **239**, 487–491.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning, A Laboratory Manual*, second edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Slivka,S.R. and Insel,P.A. (1987) *J. Biol. Chem.*, **262**, 4200–4207.
Tsien,R.Y. (1980) *Biochemistry*, **19**, 2396–2404.
Van Haastert,P.J.M., Janssens,P.M.W. and Erneux,C. (1991) *Eur. J. Biochem.*, **195**, 289–303.
Vázquez,D. (1979) *Inhibitors of Protein Synthesis*. Springer Verlag, New York. pp. 155–159.
Vergara,J., Tsien,R.Y. and Delay,M. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 6352–6356.
Wright,K.A., Dezzutti,C.S., Lewis,M.G. and Olsen,R.G. (1989) *Vet. Immunol. Immunopathol.*, **21**, 47–53.
Yanisch-Perron,C., Vieira,J. and Messing,J. (1985) *Gene*, **33**, 103–119.

Received on August 7, 1992