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Extensive alteration of fungal gene transcript accumulation and elevation of G-protein-regulated cAMP levels by a virulence-attenuating hypovirus

(chestnut blight/hypovirulence/mRNA differential display/GTP-binding protein/biological control)

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ABSTRACT **Persistent infection of the chestnut blight fungus *Cryphonectria parasitica* with the prototypic hypovirus CHV1-713 results in attenuation of fungal virulence (hypovirulence) and reduced accumulation of the GTP-binding (G) protein α subunit CPG-1. Transgenic cosuppression of CPG-1 accumulation in the absence of virus infection also confers hypovirulence. We now report the use of mRNA differential display to examine the extent to which virus infection alters fungal gene transcript accumulation and to assess the degree to which modification of CPG-1 signal transduction contributes to this alteration. More than 400 PCR products were identified that either increased (296 products) or decreased (127 products) in abundance as a result of virus infection. Significantly, 65% of these products exhibited similar changes as a result of CPG-1 cosuppression in the absence of virus infection. We also report that both virus infection and CPG-1 cosuppression elevate cAMP levels 3- to 5-fold. Additionally, it was possible to mimic the effect of virus infection and CPG-1 cosuppression on transcript accumulation for representative fungal genes by drug-induced elevation of cAMP levels. These results strengthen and extend previous indications that hypovirus infection causes a significant and persistent alteration of fungal gene expression/transcript accumulation. They further show that this alteration is primarily mediated through modification of the CPG-1 signaling pathway and suggest that, similar to mammalian G_i α subunits, CPG-1 functions as a negative modulator of adenylyl cyclase. Finally, these results suggest a role for G-protein-regulated cAMP accumulation in hypovirus-mediated alteration of fungal gene expression.**

Heterotrimeric GTP-binding (G) proteins serve as critical links in signal transduction pathways that enable eukaryotic organisms to respond to a variety of extracellular events (1). It is likely that G-protein-mediated signal transduction is intimately involved in the responses evoked by chemical and environmental cues generated in the context of host–pathogenic fungus interactions on both sides of the equation, i.e., host defenses and fungal pathogenic responses. In this regard, Choi *et al.* (2) reported that infection of the chestnut blight fungus *Cryphonectria parasitica* with a virulence-attenuating mycovirus of the genus *hypovirus* resulted in significantly reduced accumulation of a G-protein α subunit of the G_i family, designated CPG-1. Moreover, transgenic cosuppression of CPG-1 in the absence of virus infection also resulted in attenuation of fungal virulence. Thus, these results indicated a crucial role for G-protein-linked signal transduction in fungal pathogenesis and provided a mechanistic basis for virus-mediated attenuation of fungal virulence (hypovirulence). Consistent with these views, Wang and Nuss (3) reported that the induction of a *C. parasitica*-encoded cellobiohydrolase I, an enzyme with potential for degrading plant host cell wall components, is also regulated through the CPG-1 pathway and prevented by virus infection.

Hypovirus infection results in a range of persistent phenotypic changes in addition to hypovirulence that can include suppressed asexual sporulation (4, 5), reduced pigmentation (5–7), and altered colony morphology (8). Studies from several laboratories have also demonstrated hypovirus-mediated down-regulation of a number of specific fungal genes, e.g., the gene *lac-1*, encoding the phenol oxidase laccase (9, 10); *vir2*, encoding a putative pheromone (11); *cbh-1*, encoding the cellobiohydrolase I CBH-1 (3); and *cpg-1*, encoding CPG-1 (2). There is now mounting evidence to suggest that the altered expression of several of these genes (e.g., *lac-1* and *cbh-1*) is the result of virus-mediated disruption of signal transduction cascades (2, 3, 10, 12). The modification of such regulatory pathways is presumably responsible for the constellation of phenotypic changes observed in hypovirulent strains, including virulence attenuation.

Although it is now firmly established that fungal gene expression is altered as a result of hypovirus infection, questions of magnitude and mechanism remain. We have applied the technique of mRNA differential display (13) to examine the extent to which the prototypic hypovirus CHV1-713 alters fungal gene transcript accumulation and to assess the degree to which disruption of the CPG-1 signaling pathway contributes to such changes. Stimulated by reports that mammalian $G_i\alpha$ subunits negatively regulate adenylyl cyclase activity (14–16), we also examined the effect of CHV1-713 infection and CPG-1 cosuppression on cAMP accumulation. Using the information gained from those studies, it was also possible to simulate the effect of CHV1-713 infection (referred to simply as "virus infection" throughout this article) on transcript accumulation for several fungal genes by drug-induced elevation of cAMP levels. The data support the emerging view that hypovirus infection significantly alters the pattern of fungal gene expression by chronically disrupting cellular signal transduction pathways. Implications of these results are discussed in terms of current efforts to understand and control fungal pathogenesis.

MATERIALS AND METHODS

Fungal Strains. *C. parasitica* strains EP155 (American Type Culture Collection no. 38755), EP713 (American Type Culture Collection, no. 52571), and G1310, a transgenic strain derived from EP155 rendered hypovirulent as a result of cosuppression of the gene *cpg-1*, which encodes the G-protein α subunit

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Abbreviations: G protein, GTP-binding protein; IBMX, 3-isobutyl-1-methylxanthine.

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CPG-1 (2), were maintained on Difco potato dextrose agar (PDA) as described (17).

RNA Isolation. RNA was isolated from mycelia after 6 days of growth on cellophane membranes overlaying PDA at 22°C with a 14-hr light/10-hr dark cycle (900 lux) as described (17, 18). Total RNA was further subjected to two rounds of LiCl fractionation to separate single-stranded from double-stranded RNA, and the single-stranded RNA fraction was treated with RQ1 DNase (Promega) to remove any residual DNA.

Differential Display and Probe Isolation. mRNA differential display was performed with the aid of a RNAmap mRNA differential display system (GenHunter, Brookline, MA) following the manufacturer's instruction. Briefly, each 20-μl cDNA reaction mixture contained 0.5 μg of single-stranded RNA, 1 μM anchored primer ($T_{12}MN$), all four dNTPs (each at 20 μM), and 100 units of Moloney murine leukemia virus reverse transcriptase. After incubation for 60 min at 37°C, reaction mixtures were inactivated by incubation at 95°C for 5 min. Two microliters of the heat-inactivated mixtures was then used in a 20-μl amplification reaction mixture containing all four dNTPs (each at 2.0 μM), 0.2 μM arbitrary primer (AP-primer), 1 μM $T_{12}MN$ primer, 1 μl of [α-^{33}P]dATP (1200 Ci/mmol; 1 Ci = 37 GBq), and 1 unit of *Taq* polymerase (AmpliTaq, Perkin-Elmer). PCR parameters were 94°C for 30 sec, 40°C for 2 min, 70°C for 30 sec for 40 cycles followed by a 5-min elongation at 72°C.

PCR aliquots of 3.5 μl were mixed with 2 μl of loading dye, heated at 80°C for 2 min, and subjected to electrophoretic separation on a 6% denaturing DNA sequencing gel. Selected differentially expressed PCR products were excised from the gel and eluted. Recovered DNA fragments were reamplified using the appropriate anchored and arbitrary primer sets and cloned into either the TA plasmid vector (Stratagene) or the *Sma*I site of pUC19. Double-stranded inserts were sequenced by the dideoxynucleotide chain-termination method (19) using the Applied Biosystems *Taq* DyeDeoxy termination cycle sequencing kit and a model 373A DNA sequencing apparatus (Applied Biosystems).

Northern Blot Analysis. Samples containing 10 μg of total single-stranded RNA prepared from the appropriate fungal strains as described above were subjected to Northern blot analysis as outlined by Larson *et al.* (10). Probes were prepared from cloned inserts released from TA cloning vectors by digestion with *Eco*RI or from pUC19 cloning vectors by digestion with *Bam*HI and *Eco*RI and labeled as described (20).

Measurement of cAMP Levels. For studies with phosphodiesterase inhibitors, fungal colonies were grown on cellophane membranes overlaying PDA amended with 2 mM caffeine (Sigma), 2 mM theophylline (Sigma), or 2.5 mM 3-isobutyl-1-methylxanthine (IBMX) (Fluka). A 0.5-g quantity of freshly harvested 7-day-old mycelia was ground to a fine powder with a mortar and pestle in liquid nitrogen, resuspended in 3 ml of Hanks' balanced salt solution (without calcium and magnesium) containing 5 mM EDTA and 1 mM theophylline and thoroughly mixed on ice for 10 min. The extract was then centrifuged at 10,000 × *g* for 10 min, and the resulting supernatant was retained for further analysis. The cAMP content of 0.1 ml of mycelial extract was determined with a [^{125}I]cAMP scintillation proximity assay system (Amersham) following instructions of the manufacturer.

RESULTS

Hypovirulent *C. parasitica* strain EP713 was originally generated by introduction of the cytoplasmically replicating prototypic hypovirus CHV1-713 into virulent virus-free strain EP155 via anastomosis (8). Thus, these two strains are considered to have identical nuclear genetic backgrounds, although nuclear fingerprinting analysis has not been performed. Strain G1310, rendered hypovirulent due to transgenic cosuppression of CPG-1 accumulation, was also derived from strain EP155 and differs genetically from EP155 only in that it contains several (estimated at one to three copies; data not shown) integrated sense orientation copies of *cpg-1* embedded in transformation vector pCPXHY1 (2). Thus, it was predicted that differences in the accumulation of differential display PCR products for the three strains would be primarily, if not exclusively, due to the influence of persistent virus infection or CPG-1 cosuppression.

The differential display protocol used in this study employed four anchored primers, degenerate in the penultimate position, combined with a total of 20 different arbitrary primers to give a total of 80 combinations. As indicated in Fig. 1*a*, inspection of even a relatively small portion, approximately 6% of the total display potential, revealed a surprisingly large number of differences in PCR product accumulation between strains EP155 and EP713. A thorough examination of display gels generated with all 80 possible primer pairs revealed approximately 139 PCR products that changed in intensity by more then 4-fold as a result of virus infection (comparison of EP155 and EP713). Examples of significant intensity changes are clearly illustrated in Fig. 1*b*. When parameters were adjusted to include differences that were in excess of 2-fold, this number conservatively increased to more than 400. Inspection of the display patterns for strain G1310 RNA revealed the interesting observation that nearly 65% of the changes in PCR product intensity that were related to virus infection (EP713 display) were also conferred by cosuppression of CPG-1 accumulation (included under 713/1310-common in Table 1). In addition, a subset of PCR products exhibited changes in abundance that were display-specific (Table 1). That is, approximately 27% and 21% of the total observed differences were specific for the EP713 and G1310 displays, respectively. Even when considering these additional specific variations, more than 50% of the total differentially accumulating PCR products were found to exhibit similar changes in intensity in both the EP713 and G1310 displays.

Previous reports of hypovirus-mediated alteration in the expression of specific fungal genes have focused primarily on examples of reduced transcript accumulation (2, 3, 9–11). Thus, it was surprising to find that the majority of the changes observed in the EP713 display were due to increased intensity (296 products) rather than to decreased intensity (127 products) (Table 1). Since increased accumulation of host gene transcripts could also contribute to alterations in fungal phenotype, we were stimulated to clone several PCR products that exhibited increased intensity in the EP713 display. The positions of two such PCR products, 13-1 and 7-7, are indicated by asterisks in Fig. 1 *b* and *c*, respectively. Northern blot analysis using the cloned 13-1 and 7-7 PCR products as probe revealed the increased accumulation of corresponding mRNAs of approximately 0.8 kb and 3.2 kb, respectively, in the virus-infected and CPG-1 cosuppressed strains (Fig. 2), as predicted from the differential display analysis. As illustrated in a subsequent section of this paper, such probes provide novel reagents (reporter genes) with which to examine regulatory mechanisms underlying virus-mediated enhancement of fungal gene expression.

Suppression of CPG-1 Accumulation Results in Elevated cAMP Levels. Mammalian homologues of *C. parasitica* CPG-1, members of the G_i family of G-protein α subunits, function to reduce cAMP levels by inhibiting adenylyl cyclase (14). The observation that CPG-1 accumulation is severely reduced in a hypovirus-infected strain (2) suggested the possibility that virus infection results in elevated cAMP levels. Measurement of intracellular cAMP accumulation confirmed this prediction, showing a consistent 3- to 4-fold higher level of cAMP in strain EP713 than in the isogenic virus-free strain EP155 (Fig. 3). The consequence of transgenic cosuppression of CPG-1 on

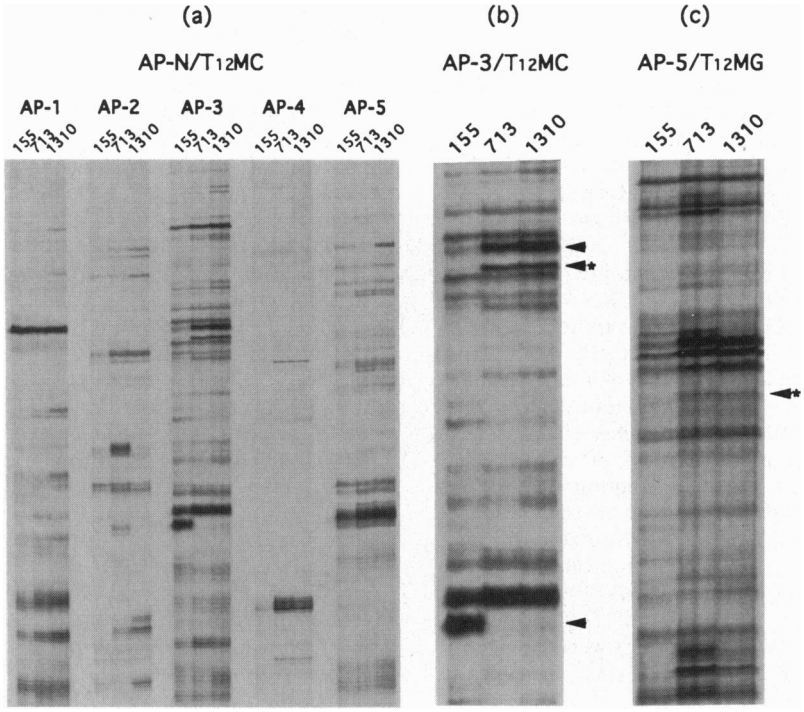

FIG. 1. Differential display of mRNAs from *C. parasitica* strains EP155 (155), EP713 (713), and G1310 (1310) grown under parallel culture conditions. (*a*) Portion of a sequencing gel autoradiograph displaying PCR products generated with the same anchored primer ($T_{12}MC$) and five different arbitrary primers as indicated above the lanes. Differentially accumulating PCR products are abundant and easily observable without marking. The portion of the gel shown represents approximately 1/16 of the total display potential provided by the mRNA differential display kit (GenHunter, Brookline, MA). (*b*) Enlargement of a portion of the autoradiograph presented in *a* that contains examples of PCR products that exhibit a similar increase or decrease in accumulation as a result of hypovirus infection or CPG-1 cosuppression. The asterisk indicates the band from which probe 13-1 was cloned. (*c*) Portion of a display from which probe 7-7 (asterisk) was isolated. Both the 13-1 and 7-7 probes were used in Northern blot analyses described in Figs. 2 and 4.

cAMP levels, in the absence of virus infection, was also examined. cAMP levels were found to be significantly elevated (4- to 5-fold) in strain G1310 and in a second CPG-1-cosuppressed transformant, strain G1318 (2) (Fig. 3). These observations suggest that *C. parasitica* CPG-1, like mammalian $G_i\alpha$ subunits, down-regulates cAMP levels, presumably by inhibiting adenylyl cyclase, and that virus-mediated suppression of CPG-1 accumulation results in elevated intracellular cAMP levels.

Representative Fungal Transcripts That Accumulate in Response to Virus Infection also Accumulate in Response to Drug-Induced Elevation of cAMP Levels. An elevation in cAMP levels is often considered within the context of positive regulation or induced expression of specific sets of responsive genes (21). To establish linkage between elevated cAMP levels and altered gene expression patterns in virus-infected *C. parasitica*, especially for virus-mediated enhanced gene expression, we employed cloned probes 13-1 and 7-7 to examine the effect of drug-induced elevations of cAMP levels on the accumulation of the corresponding mRNAs by Northern blot analysis. Treatment of strain EP155 with several inhibitors of cAMP phosphodiesterase, including caffeine, theophylline (22), and IBMX (23), elevated intracellular cAMP concentrations, ranging from 3.2-fold with IBMX to nearly 8-fold with theophylline (Table 2). Northern blot analysis revealed a significant increase in the accumulation of both 13-1 and 7-7 mRNAs in all three treated cultures (Fig. 4). Additionally, there was a positive correlation between the magnitudes of

Table 1. Inventory of differential display PCR products

	No. products			
	≥4-fold		2- to 4-fold	
Category	Decrease	Increase	Decrease	Increase
713-total	31	108	96	188
713/1310-common*	23	64	58	129
713-unique	8	44	38	59
1310-unique	10	42	32	31

*Differential PCR products that exhibited similar changes in the 713 and 1310 profiles relative to the profile generated from strain EP155 mRNA.

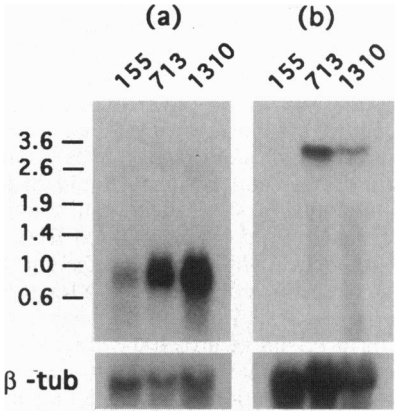

FIG. 2. Northern blot analysis of mRNAs corresponding to two arbitrary probes selected by differential display as exhibiting increased accumulation in virus-infected and CPG-1-cosuppressed *C. parasitica* strains. Single-stranded RNAs isolated from strains EP155 (155), EP713 (713), and G1310 (1310) were hybridized with cloned differentially accumulated probes 13-1 (*a*) or 7-7 (*b*). After autoradiography, the blots were stripped and rehybridized with a probe for *C. parasitica* β-tubulin (β-tub). Positions of RNA size markers (kb) are indicated at the left (*a*).

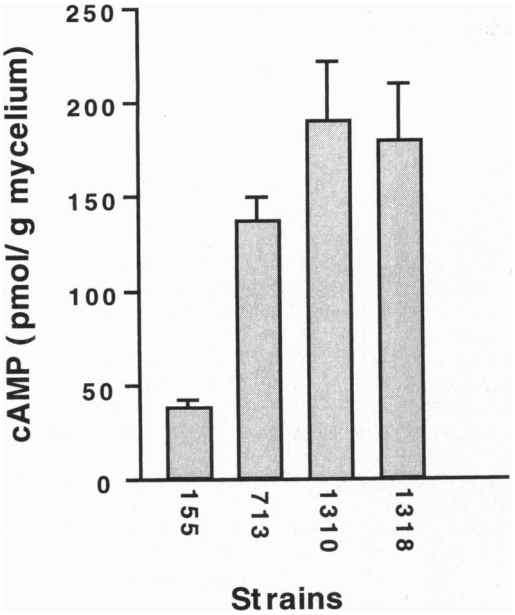

FIG. 3. Effect of virus infection and CPG-1 cosuppression on intracellular cAMP levels. cAMP levels were determined for strains EP155 (155), EP713 (713), G1310 (1310), and G1318 (1318) grown under parallel conditions using the cAMP scintillation proximity assay kit (Amersham). Strain G1318 is an additional transgenic *C. parasitica* strain that exhibits profound CPG-1 cosuppression (2). Values represent means of three replicates.

cAMP elevation and mRNA accumulation. This was especially evident for 13-1 mRNA (Fig. 4*a*).

DISCUSSION

The technique of mRNA differential display has allowed a more comprehensive examination of the effects of a hypovirus infection on fungal gene expression/transcript accumulation than was previously possible (24). It is clear from this analysis that hypovirus CHV1-713 infection causes a pronounced and stable change in the pattern of fungal gene transcript accumulation. A second, somewhat unexpected, finding is that more fungal gene transcripts appeared to increase in abundance as a result of virus infection than appeared to decrease. A third, more predictable, finding is that a significant proportion of the observed changes in fungal gene transcript accumulation can be attributed to disruption of the CPG-1 signal transduction pathway. Measurements of cAMP levels also confirmed predictions that suppressed accumulation of CPG-1, due to either virus infection or transgenic cosuppression, results in elevated intracellular cAMP levels. This finding is consistent with the conclusion that CPG-1, like mammalian $G_i\alpha$ subunits, functions to negatively regulate adenylyl cyclase activity. Finally, by combining the use of probes identified by differential display with phosphodiesterase inhibitors, it was possible to establish linkage between elevated cAMP levels and virus-mediated alteration of fungal gene expression/transcript accumulation.

Table 2. Effects of phosphodiesterase (PDE) inhibitors on *C. paracitica* intracellular cAMP accumulation

PDE inhibitor	cAMP, pmol/g
None	59.60 ± 5.41
Caffeine	256.67 ± 20.82
Theophylline	467.33 ± 38.91
IBMX	192.33 ± 12.50

Values represent the mean ± SD of three replicates.

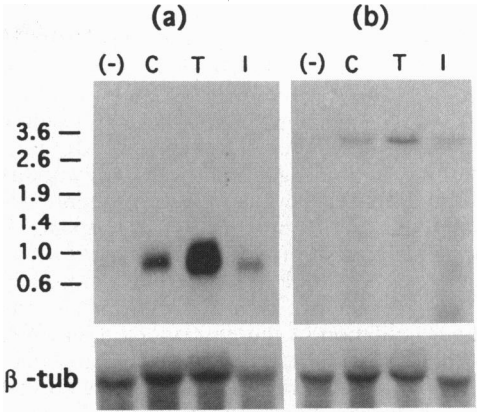

FIG. 4. Northern blot analysis showing enhanced accumulation of mRNAs corresponding to probes 13-1 and 7-7 following phosphodiesterase inhibitor-induced elevation of intracellular cAMP levels. RNA isolated from strain EP155 grown on cellophane overlaying PDA (lanes −), PDA containing 2 mM caffeine (lanes C), PDA containing 2 mM theophylline (lanes T), or PDA containing 2.5 mM IBMX (lanes I) were probed with cloned PCR products 13-1 (*a*) or 7-7 (*b*). Normalization with the β-tubulin probe and position of marker RNAs are as described in Fig. 2.

It is important to emphasize that this report does not attempt to quantify the absolute number of genes that are differentially expressed as a result of virus infection. Indeed, other investigators using differential display have reported that a significant number of differential PCR products hybridize to constitutively expressed mRNAs or fail to hybridize at a detectable level (25–27). For example, Johnson *et al.* (28) estimated that 30% of characterized differential PCR product clones corresponded to differentially expressed mRNAs. Irrespective of these limitations, the results of the differential display analysis clearly challenge a current view that hypovirus infection causes the reduced accumulation of a small number of host mRNAs and proteins (24, 29, 30). Even after extensive adjustments, a value of approximately 400 differential PCR products observed for the EP713 display is not consistent with virus-mediated alteration in the accumulation of a small number of fungal gene transcripts. In addition, the observation that nearly two-thirds of those 400 differential PCR products increased in abundance does not support the view that virus infection results primarily in reduced accumulation of fungal mRNA transcripts. A pronounced alteration in the pattern of fungal mRNA accumulation is, however, consistent with the broad range of phenotypic changes exhibited by hypovirus-infected *C. parasitica* strains.

A central role for the CPG-1 signal transduction pathway in virus-mediate alteration of fungal gene expression/transcript accumulation was firmly established by the observation that 65% of the changes observed in the EP713 display were also observed in the G1310 display (Fig. 1 and Table 1). The fact that disruption of the CPG-1 pathway by either virus infection or transgenic cosuppression results in a significant change in the apparent mRNA accumulation pattern indicates that this pathway plays an important role in the ability of *C. parasitica* to sense and respond to environmental cues. At this early stage of investigation, we know only that the intact CPG-1 pathway is required for the pathogenic response of *C. parasitica* on a susceptible plant host (2) and for the production of cellulase in response to cellulose substrate (3). It will be of considerable interest to determine the full range of environmental cues that are sensed by, and functions that are regulated through, this important pathway. Cloned probes identified by differential display will greatly assist in this effort.

Differential display also identified a subset of PCR products that exhibited changes in abundance in response to either

hypovirus infection or CPG-1 cosuppression but not to both conditions (Table 1). Some of the EP713-specific differential PCR products could correspond to transcripts derived from the 12.7-kb CHV1-713 genomic RNA. Alternatively, some of the EP713-specific changes could be the result of virus-mediated disruption of regulatory pathways other than the CPG-1 pathway. In this regard, Larson and coworkers (10, 12) have reported that hypovirus infection suppresses laccase production by disrupting an inositol trisphosphate/Ca^{2+}/calmodulin signaling pathway required for *lac-1* transcription. G1310-specific differential products are more difficult to explain. However, one reasonable possibility is that the differential accumulation of the corresponding mRNAs is canceled in the hypovirus-infected strain due to cross-talk between multiple affected regulatory pathways and are, therefore, only observed in the G1310 display. Several of these display-specific differential PCR products are currently being characterized.

A fundamental finding of this study is that intracellular cAMP levels rise in response to suppressed CPG-1 accumulation. Although not yet formally demonstrated, it is highly probable that CPG-1, like its mammalian $G_i\alpha$ homologues, functions as a negative regulator of adenylyl cyclase. The ability to simulate the effect of virus infection on transcript accumulation for several fungal genes by artificially elevating cAMP levels with phosphodiesterase inhibitors (Fig. 4) provides confidence that the changes in cAMP accumulation observed after hypovirus infection are relevant to the observed virus-mediated alterations.

Given the new insights provided by this study, a role of CPG-1 in fungal pathogenesis can be broadly envisioned as follows. In a virus-free *C. parasitica* strain, the level of activated CPG-1 is determined by events at the cell surface involving as yet undefined G-protein-linked receptors and unidentified ligands. By negatively regulating adenylyl cyclase, activated CPG-1 modulates the level of cAMP that in turn modulates gene expression, likely via a protein kinase A-dependent cascade, to elicit appropriate adaptive responses. By suppressing CPG-1 accumulation, virus infection constitutively elevates cAMP levels by relieving the negative regulation of adenylyl cyclase. This effectively compromises the ability of the invading fungus to respond appropriately to events at the cell surface, thereby impeding penetration and canker formation.

A precise understanding of the mechanism by which a hypovirus suppresses CPG-1 accumulation could have important implications for improved biocontrol strategies and antimycotic drug development. The availablility of infectious hypovirus cDNA clones (31) and the recent expansion of hypovirus host range (32) provides opportunities for detailed comparative analysis of the effect of hypovirus infection on pathogenesis-related signal transduction in a variety of pathogenic fungi. As a long-term extension of these studies, one could envision the construction of virulence-attenuating transmissible RNA replicons by installing appropriate hypovirus sequences within RNA replication platforms derived from endogenous mycoviruses of different pathogenic fungi. One can also imagine small molecule-mediated intervention of fungal signal transduction cascades corresponding to the CPG-1 pathway as a general strategy for rational design of antimycotic therapeutic agents.

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