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Molecular Analysis of the Laccase Gene from the Chestnut Blight Fungus and Selective Suppression of Its Expression in an Isogenic Hypovirulent Strain

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The gene encoding laccase in the chestnut blight fungus, *Cryphonectria parasitica*, has been cloned and characterized. The predicted *C. parasitica* laccase amino acid sequence (591 aa) was 57% identical to the *Neurospora crassa* laccase sequence and contained four potential copper-binding regions that are conserved in a number of copper-binding proteins. Treatment of a virulent *C. parasitica* strain with 3 μM cycloheximide resulted in a marked increase in laccase mRNA accumulation, whereas identical treatment of an isogenic strain that contained a hypovirulence-associated virus failed to significantly increase laccase mRNA levels. In contrast, the accumulation of mRNAs encoding β-tubulin, actin, or glyceraldehyde-3-phosphate dehydrogenase was not appreciably altered by either the presence of a hypovirulence-associated virus or treatment with cycloheximide. These results provide evidence that the expression of a specific fungal gene encoding a known protein product is selectively modulated by a hypovirulence-associated virus.

Additional keywords: benzenediol:oxygen oxidoreductase, *Endothia parasitica*, transmissible hypovirulence.

Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) is a copper-containing phenol oxidase found in many plant and fungal species (Mayer 1987). Although this enzyme has been purified from several sources and characterized biochemically (Fahraeus and Reinhammer 1967; Reinhammer 1970; Froehner and Eriksson 1974; Lerch *et al.* 1978; Minuth *et al.* 1978; Law and Timberlake 1980), and the genes encoding the *Neurospora crassa* Shear et Dodge, *Aspergillus nidulans* (Eidam) G. Wint., and *Coriolus hirsutus* (Wulfen ex Fries) Quelet laccases have been cloned and sequenced (Germann *et al.* 1988; Aramayo and Timberlake 1990; Kojima *et al.* 1990), the biological function of this enzyme remains unclear. In fungi, laccase activity has been implicated as playing a role in sporulation (Leatham and Stahmann 1981), pigment production (Leonard 1971; Clutterbuck 1972), lignin degradation (Ander and Eriksson 1976; Kirk and Shimada 1985), and pathogenesis (Marbach *et al.* 1985; Geiger *et al.* 1986). Recent interest in laccase synthesis in the chestnut blight fungus, *Cryphonectria parasitica* (Murrill) Barr, stems from reports that hypovirulent strains of the fungus are deficient in laccase activity (Rigling *et al.* 1989; Hillmann *et al.* 1990).

Hypovirulent *C. parasitica* strains exhibit a number of distinguishing characteristics in addition to reduced laccase activity and reduced virulence. These can include altered colony morphology (Anagnostakis 1982; Anagnostakis 1984; Elliston 1985), reduced pigmentation (Anagnostakis 1982; Anagnostakis 1984; Elliston 1985), suppressed sporulation (Anagnostakis 1984; Elliston 1978) and reduced levels of cellulase activity (Hillman *et al.* 1990), and oxalate accumulation (Havir and Anagnostakis 1983). Available evidence indicates that the genetic information responsible for the hypovirulence phenotype resides on viral-like double-stranded (ds) RNA genetic elements (Van Alfen *et al.* 1975; Day *et al.* 1977; Anagnostakis and Day 1979), recently termed hypovirulence-associated viruses (HAV) (Shapira *et al.* 1991). The transmissibility of these genetic elements and the accompanying hypovirulence phenotype during hyphal anastomosis provides the basis for biological control of chestnut blight under appropriate conditions (described in Anagnostakis 1982).

Recent studies have provided considerable insight into the structural and functional properties of HAV dsRNAs. For example, the large dsRNA present in hypovirulent strain EP713, L-dsRNA (12,712 bp) was shown to contain two coding domains designated ORF A and ORF B, consisting of 622 and 3,165 codons, respectively (Shapira *et al.* 1991). Both open reading frames (ORF) encode polyproteins that undergo autoproteolytic processing mediated by two papainlike protease activities (Choi *et al.* 1991a; Choi *et al.* 1991b; Shapira and Nuss 1991). Putative helicase and RNA-dependent RNA polymerase domains have also been identified (Koonin *et al.* 1991). Significantly, it was recently shown that certain traits associated with the hypovirulent phenotype could be conferred to an isogenic virulent strain by transformation with a cDNA copy of ORF A (G. H. Choi and D. L. Nuss, unpublished). These results are consistent with the proposal that the hypovirulence phenotype is a consequence of the action of specific HAV-encoded gene products on fungal gene expression rather than the result of a general response of the fungus to the physical presence of HAV dsRNA.

A detailed understanding of the mechanisms involved in HAV-mediated modulation of fungal gene expression requires the identification of specific fungal genes that serve as targets of such modulation. The demonstration that laccase activity is suppressed in hypovirulent *C. parasitica*

strains prompted us to consider the gene encoding this enzyme as a candidate target gene. In this report, we describe the isolation and characterization of a *C. parasitica* laccase gene and demonstrate that the induction of laccase mRNA accumulation is suppressed in an isogenic strain harboring an HAV dsRNA.

MATERIALS AND METHODS

Fungal strains and growth conditions. *C. parasitica* strains EP155 (virulent, dsRNA-free, ATCC 38755) and EP713 (isogenic to EP155, hypovirulent, contains dsRNA, ATCC 52571) were maintained as described previously (Hillman *et al.* 1990). Inoculum for induction experiments was prepared by growing cultures in potato-dextrose broth (PDB, Difco Laboratories, Detroit, MI) at 25° C, in the dark, without shaking. Once the cultures reached stationary phase, mycelial clumps were disrupted by grinding with a Polytron (Kinematica, Lucerne, Switzerland), and the cultures were diluted with 1 vol of PDB and stored at 4° C. Cultures used to examine the induction of laccase mRNA accumulation were initiated by inoculating 50 ml of PDB in 250-ml Delong flasks (Delong Equipment Co., Atlanta, GA) with 0.05 vol of inoculum. Growth conditions were as described for the inoculum except that the growing hyphae were dispersed by gently pipetting twice a day. After 40–48 hr, a zero time sample was removed, and cycloheximide was added to a final concentration of 3 μM from a 3 mM stock solution prepared in ethanol. An equivalent volume of ethanol was added to uninduced cultures. The flasks were swirled gently and incubated as described above.

Identification and characterization of the gene encoding laccase. The probe used to screen the *C. parasitica* genomic library was generated by polymerase chain reaction (PCR) amplification of a 720-bp fragment corresponding to the 240 C-terminal amino acid coding domain of *N. crassa* laccase (Germann *et al.* 1988). Template DNA was prepared from *N. crassa* obtained from the Fungal Genetics Stock Center, Kansas City, KS (FGSC 2489). PCR (Saiki *et al.* 1988) was performed with reagents from the GeneAmp kit (Perkin-Elmer-Cetus Instrs., Norwalk, CT) for 30 cycles with the following parameters: denaturation for 1.5 min at 94° C, annealing for 2 min at 62° C, extension for 3 min at 72° C. The PCR product was treated with T4 DNA polymerase (Sambrook *et al.* 1989), cloned into the *Sma*I site of pUC19, and subjected to sequence analysis before use. All sequence analyses were performed by the dideoxy termination method (Sanger *et al.* 1977) using Sequenase reagents (U.S. Biochemical Co., Cleveland, OH). Library screening, subcloning, and other routine procedures were performed according to standard protocols (Sambrook *et al.* 1989).

For primer extension analysis, a 17-nucleotide oligodeoxynucleotide, complementary to the laccase gene sequence at map positions 125–141, was 5′-end-labeled with [γ^{32}P] ATP by T4 polynucleotide kinase (Sambrook *et al.* 1989). One picomole of the labeled primer was hybridized with 0.9 μg of poly(A)$^+$ RNA in 10 μl of a solution containing 0.2 M NaCl and 5 mM 1,4-piperazine-bis[2-ethanesulfonic acid] (PIPES), pH 6.4. The mixture was heated at 65° C for 3 min and transferred to 50° C for 2 hr. The reaction mixture was added to 90 μl of a solution containing 50 mM Tris-HCl, pH 8.3, 10 mM dithiothreitol (DTT), 6 mM $MgCl_2$, 0.5 mM each deoxynucleotide triphosphate, and 25 μg/ml of actinomycin D. cDNA synthesis was initiated by the addition of 10 U of avian myeloblastosis reverse transcriptase (Promega Corp., Madison, WI). After incubation at 42° C for 1 hr, the reaction mixture was precipitated with ethanol. The pellet was suspended in standard sequencing sample buffer, heat denatured, and analyzed on an 8% polyacrylamide-7 M urea sequencing gel.

The precise intron/exon junctions were located by sequence analysis of PCR-amplified laccase cDNA generated from an EP155 cDNA library or poly(A)$^+$ mRNA. cDNA was synthesized as described by Xu *et al.* (1987) without prior treatment with methylmercuric hydroxide. Appropriate sets of primers (sequences available upon request) were employed to amplify laccase cDNA sequences under the following conditions: denaturation at 94° C for 1.0 min, annealing at 50° C for 30 s, and extension at 72° C for 30 s for a total of 30 cycles. The PCR products were subcloned into pUC19 for sequence analysis.

Nucleic acid preparation and analysis. Total nucleic acids were prepared from *C. parasitica* cultures by the method of Borges *et al.* (1990) with minor modifications. Liquid-grown mycelium (1–2 g) was harvested, blotted dry, and powderized in liquid nitrogen with a mortar and pestle. The mycelial powder was transferred to a 50-ml polypropylene centrifuge tube containing 15 ml of cold buffer (4 mM spermidine, 10 mM EDTA, 0.1 M NaCl, 0.5% sodium dodecyl sulfate (SDS), 10 mM freshly added β-mercaptoethanol, and 40 mM Tris-HCl, pH 8.0), and shaken vigorously. The mixture was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform. The aqueous phase was amended with 0.1 vol of 3 M sodium acetate (pH 5.5), and nucleic acids were precipitated by incubating on ice for 10 min after the addition of 2 vol of cold ethanol. The precipitate was collected by centrifugation at 10,000 rpm in a Beckman JS-13 rotor (Beckman Instruments, Fullerton, CA) for 10 min, rinsed with 75% ethanol, and dissolved in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). These preparations were used for Southern hybridization experiments after digestion with appropriate restriction endonucleases.

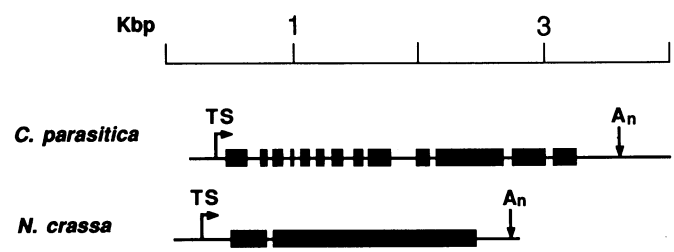

Fig. 1. Organization of the *Cryphonectria parasitica* laccase gene (*lac-1*). The relative positions of the transcription start sites (TS), polyadenylation sites (A_n), exons (thick lines), and introns (thin lines between exons) are compared for both the *C. parasitica* and the *Neurospora crassa* laccase genes. The second intron of the *C. parasitica* laccase corresponds to the single intron present in the *N. crassa* laccase gene (Germann *et al.* 1988).

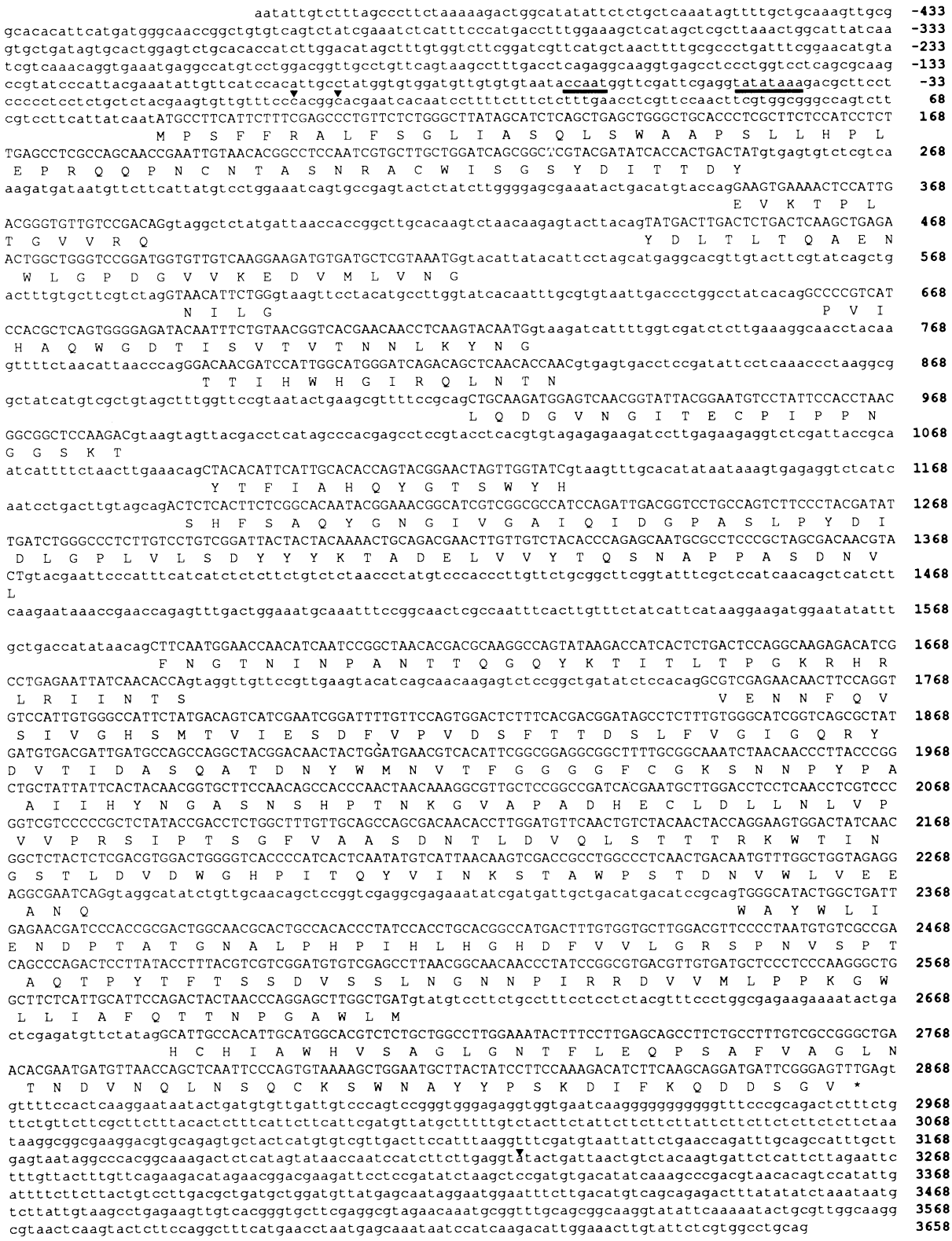

Fig. 2. Nucleotide sequence of the *Cryphonectria parasitica* laccase gene. The deduced amino acid sequence is shown below the nucleotide sequence. CCAAT and TATA motifs within the 5′-flanking region are underlined. Transcription initiation sites and the polyadenylation sites are indicated by (▼) within the 5′ and 3′ portions of the gene, respectively. Introns are indicated in lowercase letters. The first transcription site is designated as nucleotide 1. The sequence presented extends from an *Ssp*I site 504 nt upstream of the transcription start site to the *Pst*I site at map position 3,658.

C. parasitica RNA was prepared for northern analysis by a method originally developed for *N. crassa* (O. Yarden and C. Yanofsky, in press) following several modifications. Mycelia were harvested from liquid culture by gentle vacuum filtration through Miracloth (Calbiochem Corp., La Jolla, CA), and excess liquid was removed by blotting. The drained mycelial pads were inserted into 1.5-ml screw-top microcentrifuge tubes containing 0.5 g of 0.5 mM zirconium beads, 350 μl of water-saturated phenol, and 500 μl of extraction buffer (150 mM sodium acetate, pH 5.0, 100 mM LiCl, 4% SDS, 10 mM EDTA, and 20 mM β-mercaptoethanol [adapted from Lucas *et al.* 1977]). The tubes were immediately shaken for 1 min in a Mini Bead Beater (Biospec Products Inc., Bartlesville, OK) set at high speed, allowed to cool, and shaken a second time for 2 min at low speed. Chloroform/isoamyl alcohol (350 μl) was added, and the samples were shaken an additional 2 min at low speed. After centrifugation to separate the phases, the phenol phase was re-extracted with 250 μl of extraction buffer. The aqueous phases were then combined and extracted with phenol-chloroform until no interface was present; the final aqueous phase was then extracted once with chloroform. RNA was precipitated by adding 0.33 vol of 8 M LiCl and incubated overnight at 4° C. The precipitate was recovered by centrifugation, washed with 80% ethanol, thoroughly drained, resuspended in 2 mM EDTA, and stored at −70° C. RNA concentrations were determined photometrically. Samples containing 15 μg of RNA were denatured, subjected to electrophoresis through a formaldehyde-1.5% agarose gel, and transferred to a nylon membrane (GeneScreen Plus, DuPont, Wilmington, DE). Blots were hybridized with probes specific for *C. parasitica* laccase, actin, β-tubulin, and glyceraldehyde-3-phosphate dehydrogenase (*gpd-1*) transcripts in four independent, sequential hybridization reactions under stringent conditions as specified by the membrane manufacturer.

RESULTS

Identification and characterization of the *C. parasitica* laccase gene. Screening of a *C. parasitica* genomic DNA library with a 720-bp PCR amplicon corresponding to the 240 amino acid carboxyl-terminal coding domain of the *N. crassa* laccase gene resulted in the identification of 12 prospective positive clones. Of these, four clones (λLAC1, λLAC12, λLAC14, λLAC15) were further characterized and shown, by restriction and Southern hybridization analysis, to contain independent overlapping inserts. An overview of the *C. parasitica* laccase gene organization, as revealed by sequence analysis of a series of contiguous subclones extending in both directions from the region that hybridized to the *N. crassa*-specific probe, is presented in Figure 1.

Two transcription start sites for the *C. parasitica* laccase gene were identified by primer extension experiments (Fig. 2). A canonical TATA box was identified 42 nt upstream of the first transcription start site, and the sequence between the two elements was found to be pyrimidine-rich (31 of 42 residues). This is similar to the organization of the *N. crassa* laccase gene in which a TATA box motif was found 42 nt upstream of the transcription start site, and the intervening sequence contained 29 pyrimidine residues. In contrast to the *N. crassa* laccase gene, the *C. parasitica* laccase gene contained a CCAAT motif 64 nt upstream of the first transcription start. The first ATG codon located downstream of the identified transcription start sites (map positions 86–88) also constituted the initiation codon of

Table 1. Introns identified in *Cryphonectria parasitica* genes

Gene	Intron	Size (nt)	5′ Splice site	Internal consensus	3′ Splice site
lac-1[a]	1	97	GTGAGT	ACTGAC- - - 6- - -CAG	
	2	54	GTAGGC	TCTAAC- - - 11- - -CAG	
	3	67	GTACAT	GCTGAC- - - 13- - -TAG	
	4	60	GTAAGT	CCTGGC- - - 6- - -CAG	
	5	61	GTAAGA	TCTAAC- - - 7- - -CAG	
	6	88	GTGAGT	ACTGAA- - - 10- - -CAG	
	7	108	GTAAGT	TCTAAC- - - 6- - -CAG	
	8	55	GTAAGT	CCTGAC- - - 6- - -CAG	
	9	214	GTACGA	GCTGAC- - - 7- - -CAG	
	10	58	GTAGGT	GCTGAT- - - 7- - -CAG	
	11	71	GTAGGC	GCTGAC- - - 10- - -CAG	
	12	72	GTATGT	ACTGAC- - - 13- - -TAG	
gpd-1[b]	1	150	GTAAGT	GCTGAC- - - 24- - -TAG	
	2	72	GTAAGT	GCTGAC- - - 18- - -CAG	
epn-1[c]	1	98	GTGAGT	ACTGAC- - - 13- - -AAG	
	2	89	GTTGGT	ACTAAC- - - 19- - -TAG	
	3	89	GTGAGT	ACTAAT- - - 20- - -CAG	
Consensus			$GT^{A}_{G}{}^{A}_{G}GT$	$NCT^{G}_{A}AC$	$^{C}_{T}AG$
Neurospora crassa[d]			$GTA^{A}_{C}GT$	$^{A}_{G}CT^{A}_{G}AC$	$^{C}_{T}AG$
Saccharomyces cerevisiae[e]			GTATGT	TACTAAC	YAG

[a] This report.
[b] Choi and Nuss 1990.
[c] G. H. Choi, R. Shapira, and D. L. Nuss, unpublished.
[d] Germann *et al.* 1988.
[e] Langford *et al.* 1984.

the predicted laccase coding region based on amino acid sequence similarities with the predicted *N. crassa* laccase amino acid sequence (Germann *et al.* 1988). The nucleotide sequence context of this ATG codon, ATCAATATGC, is similar to the consensus sequence, $_{AG}TCA^{A}{}_{C}AATGG$, derived for favorable translation initiation of *N. crassa* genes (Roberts *et al.* 1988).

The predicted *C. parasitica* laccase coding domain consisted of 592 codons, including the termination codon at map positions 2,864–2,866, and contained a total of 12 introns. By contrast, the *N. crassa* laccase gene was reported to contain a single intron (Germann *et al.* 1988) that upon inspection was found to correspond to the second intron found in the *C. parasitica* laccase gene (Fig. 1). The presence and extact boundaries of the predicted introns were confirmed by sequence analysis of PCR-amplified laccase cDNA generated from a *C. parasitica* strain EP155 cDNA

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Nc   1 MKFLGIAALVAGLLAPPLVLAAPAPGTEGVNLLTPVDKRQDSQAERYGGG 50
Cp   1 MPSF.FRALFSGLIASQLSWAAPS.......LLHPLEPRQQP........ 34

    51 GGGGCNSPTNRQCW.SPGFNINTDYELGTPNTGKTRRYKLTLTETDNWIG 99
    35 ...NCNTASNRACWISGGSYDITTDYEVKTPLTGVVRQYDLTLTQAENWLG 81

   100 PDGVIKDKVMMVNDKIIGPTIQADWGDYIEITVINKLKSNGTSIHWHGMH 149
    82 PDGVVKEDVMLVNGNILGPVIHAQWGDTISVTVTNNLKYNGTTIHWHGIR 131

   150 QRNSNIQDGVNGVTECPIPPRGGSKVYRWRATQYGTSWYHSHFSAQYGNG 199
   132 QLNTNLQDGVNGITECPIPPNGGSKTYTFIAHQYGTSWYHSHFSAQYGNG 181

   200 IVGPIVINGPASANYDVDLGPFPLTDYYYDTADRLVLLTQHAGPPPSNNV 249
   182 IVGAIQIDGPASLPYDIDLGPLVLSDYYYKTADELVVYTQSNAPPASDNV 231

   250 LFNGFAKHPT.TGAGQYATVSLTKGKKHRLRLINTSVENHFQLLLVNHSM 298
   232 LFNGTNINPANTTQGQYKTITLTPGKRHRLRIINTSVENNFQVSIVGHSM 281

   299 TIISADLVPVQPYKVDSLFLGVGQRYDVIIDANQAVGNYWFNVTFGGSKL 348
   282 TVIESDFVPVDSFTTDSLFVGIGQRYDVTIDASQATDNYWMNVTFGGGGF 331

   349 CGDSDNHYPAAIFRYQGAPKALPTNQGVAPVDHQCLDLNDLKPVLQRSLN 398
   332 CGKSNNPYPAAIIHYNGASNSHPTNKGVAPADHECLDLLNLVPVVPRSIP 381

   399 TNSIALNTGNTIPITLDGFVWR..VNGTAININWNKPVLEYVLTGNTNYS 446
   382 TSGFVAASDNTLDVQLSTTTRKWTINGSTLDVDWGHPITQYVINKSTAWP 431

   447 QSDNIVQVEGVNQWKYWLIENDPDG.AFSLPHPIHLHGHDFLILGRSPDV 495
   432 STDNVWLVEEANQWAYWLIENDPTATGNALPHPIHLHGHDFVVLGRSPNV 481

   496 TAISQTRYVFDPAVDMARLNGNNPTRRDTAMLPAKGWLLIAFRTDNPGSW 545
   482 SPTAQTPYTFTSS.DVSSLNGNNPIRRDVVMLPPKGWLLIAFQTTNPGAW 530

   546 LMHCHIAWHVSGGLSNQFLERAQDLRNSISPADKKAFNDNCDAWRAYFPD 595
   531 LMHCHIAWHVSAGLGNTFLEQPSAFVAGLNTNDVNQLNSQCKSWNAYYPS 580

   596 NAPFPKDDSGLRSGVKAREVKMKW* 620
   581 KDIFKQDDSGV*..... 592
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Fig. 3. Alignment of the deduced amino acid sequences for *Cryphonectria parasitica* and *Neurospora crassa* laccases. The *N. crassa* sequence is presented on the top line and the *C. parasitica* on the bottom line. Identical matches are indicated by (|), whereas conserved and neutral amino acid substitutions are denoted by a colon and a period, respectively. Note the large alignment gaps within the N-terminal domain and the presence of 13 C-terminal residues in the *N. crassa* sequence that are absent in the *C. parasitica* sequence. There is evidence that formation of the mature form of *N. crassa* laccase involves removal of 49 amino acid residues from the N-terminus and 13 residues from the C-terminus of the encoded sequence (Germann *et al.* 1988). The divergence in sequence similarity within these regions suggests that the two related enzymes may be processed via alternative pathways.

library or poly(A)$^+$ mRNA. Intron sizes ranged from 55 to 214 nt, comparable to that reported for other fungal genes (Germann *et al.* 1988; Roberts *et al.* 1988). A survey of 17 introns compiled from three *C. parasitica* genes, the genes for laccase (*lac-1*, this study), glyceraldehyde-3-phosphate dehydrogenase (*gpd-1*) (Choi and Nuss 1990), and endothiapepsin (*epn-1*, G. H. Choi, B. P. Rae, R. Shapira, D. M. Pawlyk, and D. L. Nuss, unpublished), revealed the conserved sequences $GT^{A}{}_{G}{}^{A}{}_{G}GT$ and $^{C}{}_{T}AG$ for the 5′ and 3′ splice sites, respectively (Table 1). Additionally, an internal consensus sequence $CT^{G}{}_{A}AC$, which may be involved in lariat formation (Langford *et al.* 1984), was identified 6–24 nt upstream of the 3′ splice sites. These consensus sequences are in agreement with those found for other fungal introns (Roberts *et al.* 1988) (Table 1).

The *C. parasitica* laccase polyadenylation site was mapped to position 3,226 also by sequence analysis of PCR-amplified laccase cDNA. In this case, the 3′ primer contained nineteen 3′-terminal T residues, and the 5′ primer was complementary to map positions 1,986–2,002. There was no evidence of a typical polyadenylation signal, AATAAA (Proudfoot and Brownlee 1976), in the region upstream of the polyadenylation site, a situation common to many fungal genes. However, two pyrimidine-rich regions were observed at map positions 2,949–3,008 and 3,018–3,066. Similar thymidine- and cytosine-rich regions were also found in the 3′ noncoding region of the *N. crassa* laccase gene (Germann *et al.* 1988).

Comparison of the predicted amino acid sequences for the *N. crassa* and *C. parasitica* laccases revealed a 73% similarity with a 57% level of identity (Fig. 3). Regions of high similarity include four domains considered to contain copper-binding ligands that are conserved among a number of multicopper oxidases (Messerschmidt and Huber 1990). These regions are highly conserved in all four fungal laccases for which sequence information is available and share considerable sequence similarity with multicopper oxidases from plant and animal sources (Fig. 4). The high level of conservation for these regions among the fungal laccases contrasts with the overall level of amino acid similarity (e.g., comparison of the *C. parasitica* laccase and the *A. nidulans* laccase I amino acid sequences [Aramayo and Timberlake 1990] revealed values of 51% similarity and only 28% identity).

Comparison of the predicted *N. crassa* laccase amino acid sequence with that of the purified mature form of the protein revealed evidence for both N-terminal and C-terminal processing (Germann *et al.* 1988). Although the *N. crassa* laccase sequence contains a putative signal peptide sequence, additional processing events appear to result in the removal of a total of 49 N-terminal residues from the precursor protein (Germann *et al.* 1988). Moreover, there is evidence for the processing of 13 residues from the C-terminus of the precursor. Interestingly, the predicted *C. parasitica* laccase sequence is 28 residues shorter than that predicted for the *N. crassa* laccase, and gaps in the alignment of the two sequences fall within these terminal domains (Fig. 3). Whereas the N-terminal portion of the *C. parasitica* sequence contains the hallmarks of a signal peptide (von Heijne 1986) (i.e., a positively charged residue at position 6 followed by a hydrophobic core region as revealed by

hydropathy analysis) (data not shown) and several potential dipeptide cleavage sites, it also contains two large alignment gaps, comprising 15 residues, within the region corresponding to the 49 residues that are processed from the N-terminal portion of the *N. crassa* laccase sequence. Additionally, the *C. parasitica* laccase sequence lacks a sequence corresponding to the 13-residue oligopeptide that is processed from the C-terminus of *N. crassa* laccase. These dissimilarities suggest that different pathways may be involved in the post-translational processing of these related enzymes. A comparison of the processing pathways for the laccases from these two ascomycetes may provide insights into general mechanisms involved in post-translational modification and secretion of fungal proteins.

Laccase gene expression. Because laccase activity has been reported to be suppressed in hypovirulent strains of *C. parasitica* (Rigling *et al.* 1989), including in strain EP713, which harbors the recently characterized HAV L-dsRNA (Hillman *et al.* 1990), it was of interest to examine the regulation of laccase gene expression at the level of mRNA accumulation in paired virulent and hypovirulent *C. parasitica* strains. As a preliminary experiment, Southern blot analysis was performed on genomic DNA of the isogenic virulent and hypovirulent strains EP155 and EP713. Identical patterns consisting of anticipated 3.3- and 1.3-kbp bands, corresponding to the 5′ and 3′ portions of the laccase gene, were observed for both strains (Fig. 5). Combined, the two fragments span the laccase gene from 1,976 nt upstream of the first transcription start site through intron 12 (map positions −1,976–2,669). This result indicates the absence of closely related genes in the *C. parasitica* genome and confirms that both strains EP155 and EP713 contain the same structural gene. Additional Southern analysis indicated that there was no apparent structural rearrangement of the laccase gene due to the presence of the HAV dsRNA and that the laccase gene is likely present as a single copy gene in each strain (data not shown).

Initial studies, designed to measure laccase mRNA accumulation in *C. parasitica* by northern hybridization analysis, indicated that laccase gene expression was under complex regulatory control. The level of accumulation appeared to be influenced by a variety of conditions including the culture medium, the age of the culture, and exposure to light. Whereas laccase mRNA accumulation was generally found to be substantially lower in strain EP713 than in EP155, the degree of variability observed within one set and between different sets of conditions complicated the interpretations.

The report that laccase enzymatic activity was consistently induced in vegetatively growing cultures of *N. crassa* after treatment with low concentrations of protein synthesis inhibitor cycloheximide (Froehner and Eriksson 1974) prompted us to perform similar experiments with *C. parasitica* in an effort to identify conditions under which laccase gene expression was subject to less variability. As shown in Figure 6, addition of 3 μM cycloheximide to cultures of the virulent *C. parasitica* strain EP155 resulted in a time-dependent increase in the accumulation of a ≈2.3-kb RNA species corresponding to laccase mRNA. An increase in band intensity was evident by 6 hr after cycloheximide addition and was found to reach a level of approximately 15- to 20-fold by 24 hr. Although some variability was observed in the uninduced level of laccase mRNA and consequently the final magnitude of induction, the addition of 3 μM cycloheximide consistently resulted in a significant increase in laccase mRNA accumulation.

To test whether the induction of laccase mRNA accumulation was selective, or whether the results reflected a general increase in mRNA accumulation, blots were rehybridized with probes specific for other *C. parasitica* mRNAs. These included probes for mRNAs that encode two structural proteins, β-tubulin and actin, and an mRNA encoding the highly expressed protein, glyceraldehyde-3-phosphate dehydrogenase. The levels of β-tubulin and actin mRNAs (1.9 and 1.8 kb, respectively) were found to change little during the 24-hr incubation period with the small variation observed most likely being related to differences in sample loading. The level of *gpd-1* mRNA (1.5 kb) also remained relatively stable over the incubation period, exhibiting a slight increase in accumulation by the 24-hr timepoint. This comparison makes it clear that the induction in *C. parasitica* laccase mRNA accumulation after the addition of low concentrations of cycloheximide is under the control of a selective regulatory pathway.

Having established that laccase mRNA accumulation is consistently induced in a virulent *C. parasitica* strain after the addition of cycloheximide, it was of interest to examine whether the induction would be affected by the presence of a HAV. Consequently, laccase mRNA accumulation was compared for the isogenic virulent and hypovirulent *C. parasitica* strains EP155 and EP713. As typified in Figure 6, treatment of hypovirulent strain EP713 with cycloheximide resulted in only a very modest increase in laccase mRNA accumulation. Note that there was no significant difference in the accumulation of the β-tubulin, actin, or *gpd-1* mRNAs either between the two strains or within the same strain after treatment with low concentrations of cycloheximide. The combined results indicate that

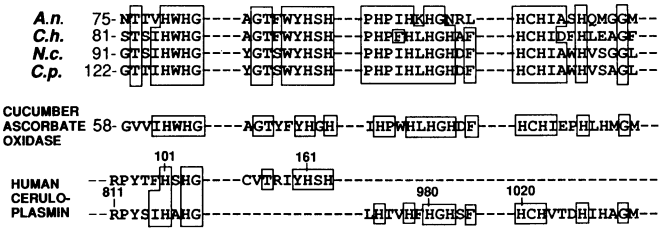

Fig. 4. Amino acid sequence comparison within conserved regions of several multicopper oxidases. Strongly conserved regions thought to contain copper ligands for a number of multicopper oxidases are shown. Identical amino acid residues are boxed. Dashes represent gaps introduced for optimal alignments. The top four sequences correspond to the four fungal laccase genes cloned to date, which include the following sources: A.n. = *Aspergillus nidulans* (Aramayo and Timberlake 1990); C.h. = *Coriolus hirsutus* (Kojima *et al.* 1990); N.c. = *Neurospora crassa* (Germann *et al.* 1988), and C.p. = *Cryphonectria parasitica* (Fig. 2). Sequences of conserved regions for multicopper oxidases from a plant (Ohkawa *et al.* 1989) and animal (Takahashi *et al.* 1984) source are shown in the bottom portion of the figure. The numbers at the left indicate the number of residues from the N-terminus, included as points of reference. Due to the complexity of the human ceruloplasmin sequence comparison, map positions are provided throughout the sequences presented.

cycloheximide-mediated induction of fungal laccase gene expression involves regulation at the level of mRNA transcription or accumulation and that this regulatory pathway is altered in an isogenic *C. parasitica* strain that harbors a HAV.

DISCUSSION

In the recent reports concerning phenol oxidase activity in strains of *C. parasitica* (Rigling *et al.* 1989; Hillman *et al.* 1990), several independent assays were used to establish that the activity measured was of the laccase type. Based on the high degree of amino acid sequence similarity between the predicted protein encoded by the gene identified and characterized in this report and the *N. crassa* laccase sequence (Fig. 3), it is clear that the corresponding *C. parasitica* laccase gene has been cloned. Furthermore, Southern blot analysis of genomic DNA failed to detect any *C. parasitica* genes of closely related sequence. Therefore, it is likely that the product of the gene described in this report is responsible for the majority, if not all, of the *C. parasitica* laccase activity.

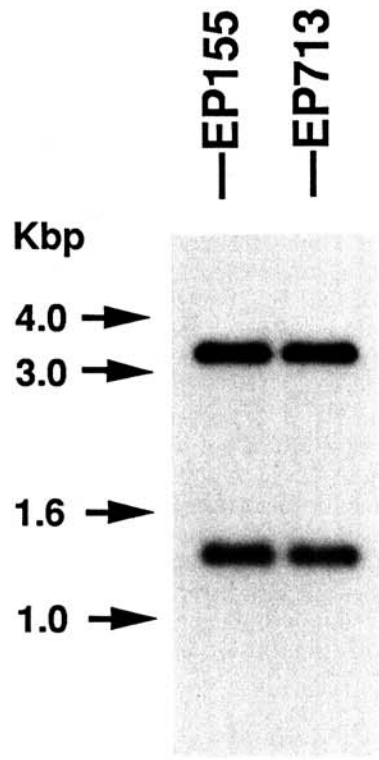

Fig. 5. Southern hybridization analysis of the *Cryphonectria parasitica* laccase gene. Total nucleic acid preparations (5 μg) from virulent *C. parasitica* strain EP155 and from the isogenic hypovirulent strain EP713 were digested with restriction endonucleases *Pst*I and *Xho*I separated on a 0.7% agarose gel and transferred to a Hybond-N membrane by capillary blotting. Blots were probed sequentially with two ^{32}P-labeled DNA fragments (Feinberg and Vogelstein 1983) corresponding to the 850-bp *Eco*RI-*Sal*I fragment spanning map positions 1,374–2,224 (probe 1) and the 2.2-kbp *Eco*RI fragment that extends from position −832 to position 1,374 (probe 2). The 1.3- and 3.3-kbp hybridization bands were specific for probes 1 and 2, respectively. The migration positions of DNA marker bands are indicated at the left.

The function(s) of laccase in *C. parasitica* remains to be determined. Laccase activity has been shown to be associated with pigment production in several fungi. For example, laccase is probably involved in the production of a brown pigment during fruiting body formation in the basidiomycete *Schizophyllum commune* (Leonard 1971). A role for laccase in the production of the green pigment associated with spores of the ascomycete *A. nidulans* has been confirmed by genetic means (Clutterbuck 1972). In this regard, hypovirulent *C. parasitica* strain EP713 exhibits both reduced pigmentation and reduced laccase activity. This suggests a possible relationship between laccase production and pigmentation that merits further investigation.

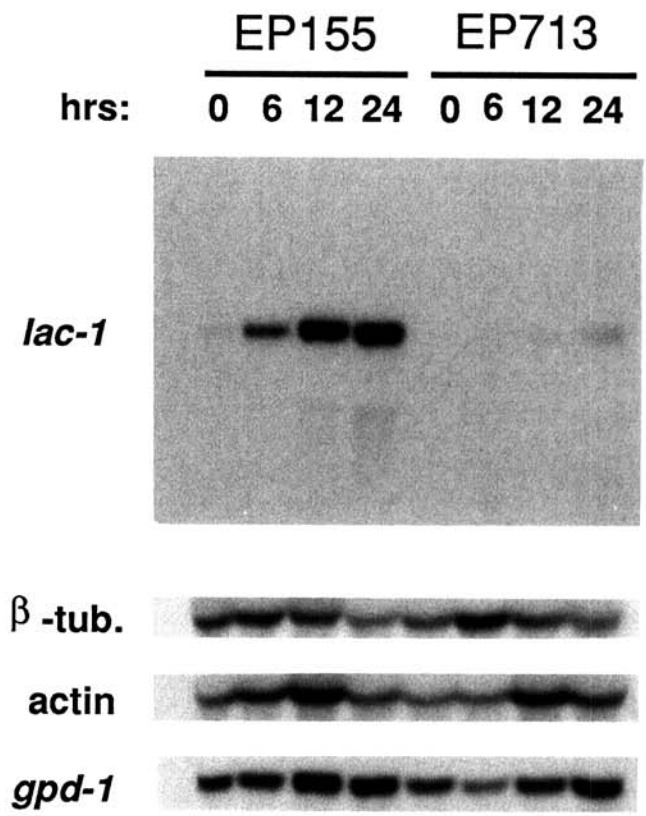

Fig. 6. Northern hybridization analysis of EP155 and EP713 RNA after addition of 3 μM cycloheximide. RNA prepared from strains EP155 and EP713 at various times after the addition of 3 μM cycloheximide were separated through a formaldehyde-1.5% agarose gel, transferred to a nylon membrane, and hybridized sequentially with several different probes. The laccase-specific probe (*lac-1*) consisted of a 850-bp *Eco*RI-*Sal*I genomic subfragment that encompassed exon 10 and most of exon 11. Probes specific for *Cryphonectria parasitica* β-tubulin and actin transcripts were obtained by screening a *C. parasitica* cDNA library with PCR amplicons of the *Neurospora crassa* β-tubulin gene (Orbach *et al.* 1986) and the yeast actin gene, respectively (Gallwitz and Sures 1980; Ng and Abelson 1980). The cloning and characterization of the *C. parasitica* β-tubulin and actin cDNAs will be described elsewhere (T. G. Larson, G. H. Choi, and D. L. Nuss, unpublished). The probe specific for the glyceraldehyde-3-phosphate dehydrogenase mRNA (*gpd-1*) consisted of the 1.9-kbp *Bgl*II-*Eco*RI fragment corresponding to *gpd-1* map positions 236–2,167 as presented in Choi and Nuss 1990. Estimated sizes of the different mRNAs were as follows: *lac-1* = 2.3 kb; β-tubulin = 1.9 kb; actin = 1.8 kb; *gpd-1* = 1.5 kb. The times after addition of cycloheximide at which the RNA samples were harvested are indicated at the top of the blot.

Within the context of host-pathogen interaction, laccase could potentially contribute to pathogen-mediated degradation of lignified zones (Lewis and Yamamoto 1990) generated by the host as a physical defense mechanism. It is well established that hypovirulent *C. parasitica* strains are deficient in their ability to form the deep necrotic cankers associated with infection by virulent strains (Hebard *et al.* 1984; reviewed by Griffin 1986). It is possible that this deficiency is related to the reduced level of laccase activity produced by hypovirulent strains. The availability of the cloned laccase gene and the information gained from its characterization provide new avenues for determining the role of this enzymatic activity, both in the life cycle of *C. parasitica* and in host-pathogen interaction.

There has been a continuing debate as to whether the hypovirulence phenotype in *C. parasitica* is the result of a general debilitation due to the presence of viral-like genetic elements or whether the hypovirulence-associated elements modulate specific regulatory pathways involved in nuclear gene expression. Powell and Van Alfen (1987a; 1987b) have previously reported the differential accumulation of specific proteins and poly(A)$^+$ RNAs between virulent and hypovirulent strains of *C. parasitica*. However, there has been no subsequent report concerning the nature of the polypeptides or the coding potential of the RNA species identified in those studies. In this report, we provide evidence for HAV-mediated modulation of the expression of a specific, well characterized fungal gene encoding a known protein product. Of equal importance, we were able to demonstrate that the expression of three additional fungal genes, those encoding β-tubulin, actin, and glyceraldehyde-3-phosphate dehydrogenase, was not significantly altered in hypovirulent strains under the same conditions. These results support the proposal that the hypovirulence phenotype is a consequence of the altered expression of a specific subset of fungal genes rather than a general debilitation of the fungus. Moreover, a recent report that hypovirulence-associated characteristics can be conferred by transformation of a virulent, virus-free strain with a cDNA copy of a portion of the HAV dsRNA (G. H. Choi and D. L. Nuss, in press) suggests that the altered expression of fungal genes is due to the action of specific viral gene products rather than a general response to the physical presence of the viral RNA.

The mechanism involved in cycloheximide-mediated induction of fungal laccase mRNA accumulation and its suppression in hypovirulent *C. parasitica* strain is currently unknown. An explanation forwarded by Froehner and Eriksson (1974) that the induction of the *N. crassa* laccase was due to derepression of laccase synthesis was based on a model proposed by Horowitz *et al.* (1970) concerning the induction of another *N. crassa* phenol oxidase, tyrosinase. As noted by Froehner and Eriksson (1974), both of these phenol oxidase activities were induced by low concentrations of protein synthesis inhibitors, the cycloheximide dose response curves for the induction of both activities were similar, and the relative effectiveness of three different protein synthesis inhibitors on induction of the two enzymes was comparable. The hypothesis proposed by Horowitz *et al.* (1970) suggests that in rapidly growing cultures, the *N. crassa* phenol oxidase genes are repressed by a rapidly turning-over protein repressor. The addition of low concentrations of protein synthesis inhibitors is thought to reduce the effective concentration of the repressor resulting in derepression of these genes. Mechanistically, this could be analogous to the induction of gene expression after cycloheximide-mediated activation of transcription factor NFκB in mammalian cells (Wall *et al.* 1986; Schreck *et al.* 1991). Additional evidence that *N. crassa* tyrosinase and laccase are regulated by a common mechanism comes from genetic studies in which the expression of both activities was shown to be affected similarly by the same mutation, which, interestingly, appears not to be a structural gene mutation (Froehner and Eriksson 1974). The results presented here on the *C. parasitica* laccase gene extend the observations on fungal laccase gene expression by showing that derepression involves an increase in laccase mRNA accumulation. This observation, in turn, suggests that regulation is at the level of transcription or mRNA stability. It should be possible, now that the promoter regions for both the *N. crassa* and the *C. parasitica* laccase genes have been cloned and characterized, to begin to identify the control elements involved in the regulation of fungal laccase gene expression.

The observation that laccase mRNA accumulation did not increase appreciably in a cycloheximide-treated HAV-containing isogenic hypovirulent strain provides additional evidence that this virus may modulate the expression of specific fungal genes at the level of mRNA transcription. An alternative mechanism involving interference with signal transduction pathways, direct interaction of HAV-encoded polypeptides with repressor molecules, or stabilization of mRNA turnover must also be considered. In this regard, several recent developments have provided new opportunities for examining the molecular basis of hypovirulence. These include the cloning, complete sequence analysis and partial elucidation of the expression strategy of a HAV dsRNA (Choi *et al.* 1991a; Choi *et al.* 1991b; Koonin *et al.* 1991. Shapira *et al.* 1991; Shapira and Nuss 1991), the cloning of a number of *C. parasitica* genes (Choi and Nuss 1990; this report; G. H. Choi, B. P. Rae, R. Shapira, D. M. Pawlyk, and D. L. Nuss, unpublished), and the development of an efficient transformation system for *C. parasitica* (Churchill *et al.* 1990; Hillman *et al.* 1990). The availability of a characterized gene that appears to be the target of HAV-mediated modulation, the laccase gene, and several well characterized nontarget genes, *gpd-1*, actin, β-tubulin, adds an additional important dimension for future studies.

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