Regulation of Constitutively Expressed and Induced Cutinase Genes by Different Zinc Finger Transcription Factors in Fusarium solani f. sp. pisi (Nectria haematococca)*

Daoxin Li, Tatiana Sirakova, Linda Rogers, William F. Ettinger‡, and P.E. Kolattukudy§

From the Departments of Biochemistry and Molecular and Cellular Biochemistry and Neurobiotechnology Center, The Ohio State University, Columbus, Ohio 43210

Cutin monomers, generated by the low levels of constitutively expressed cutinase, induce high levels of cutinase that can help pathogenic fungi to penetrate into the host through the cuticle whose major structural polymer is cutin. We cloned three highly homologous cutinase genes, cut1, cut2, and cut3, from Fusarium solani f. sp. pisi (Nectria haematococca). Amino acid sequence deduced from the nucleotide sequence of cut1 and cut2/3 matched with that of the peptides from cutinase 1 and cutinase 2, respectively, isolated from F. solani pisi grown on cutin as the sole carbon source. Induction of β-glucuronidase gene fused to the promoters of the cutinases integrated into F. solani pisi genome indicates that cut2 is constitutively expressed and induced under starvation, whereas cut1 is highly induced by cutin monomers. A palindrome binding protein (PBP) previously cloned binds only to palindrome 1 of cut1 promoter but not palindrome 1 of cut2/3 which contains two base substitutions. PBP is thought to interfere with the binding of CTF1α, the transcription factor involved in induction, to cut1 promoter and thus keep cut1 gene repressed until induced by cutin monomers. Because PBP cannot bind palindrome 1 of cut2, this gene is not repressed. CTF1α does not transactivate cut2 promoter. A new Cys$_6$Zn$_2$ motif-containing transcription factor, CTF1β, that binds palindrome 2 was cloned and sequenced. In yeast, CTF1β transactivates cut2 promoter but not cut1 promoter unless its palindrome 1 is mutated, unlike CTF1α which transactivates cut1. Thus, CTF1β is involved in the constitutive expression of cut2 that causes production of low levels of cutin monomers that strongly induce cut1 using CTF1α as the transcription factor.

Fungal infection of plants can be assisted by extracellular cutinases that help the pathogen penetrate through the outermost cuticular barrier of the host (1, 2). Conidia of highly virulent pathogens, which can directly penetrate through the cuticle, have low levels of cutinase that release small amounts of cutin monomers when the conidia contact the host surface (3). These monomers transcriptionally activate the expression of an inducible cutinase gene that is responsible for the production of high levels of cutinase that assist the infection peg to gain entry into the host through the cuticle (4). A cis element essential for the inducible expression of cutinase gene was found to be located at −159 bp in the promoter of this gene (5) in Fusarium solani f. sp. pisi (Nectria haematococca). In this region, two overlapping palindromes were found. Palindrome 2 was found to be necessary for the inducible cutinase gene expression (5). A protein that binds the palindromic region, called palindrome binding protein (PBP), (6) and a cutinase transcription factor 1α (CTF1α) which selectively binds palindrome 2 and transactivates the cutinase promoter (7), have been cloned. CTF1α, a 101-kDa protein, contains a Cys$_6$Zn$_2$ binuclear cluster motif, sharing homology to the Cys$_6$Zn$_2$ binuclear cluster DNA-binding domains of transcription factors from yeast and filamentous fungi. Whether the constitutively expressed cutinase and the inducible cutinase are encoded by the same or different genes is not known, and the nature of the transcription factors that are involved in the constitutive expression of cutinase is unknown. Two different but very similar cutinases had been isolated from F. solani pisi (8, 9), and the gene previously cloned matched the amino acid sequence of one of these proteins (10), suggesting that another gene encodes the other. Such a gene had not been previously cloned, although Southern analysis had indicated the presence of multiple cutinase genes in the F. solani pisi genome (11). In this paper, we describe cloning of two highly homologous genes in addition to the one that encodes the inducible cutinase. The newly cloned cutinase gene sequences contain a segment that matches the amino acid sequence of a peptide from cutinase 2, isolated from the fungus. The palindrome 1 of the additional cutinase gene contains two nucleotide differences from that of the previously cloned inducible cutinase gene (cut1). We show that PBP is unable to bind the palindrome 1 of the newly cloned genes. We report cloning of a second cutinase transcription factor (CTF1β), also containing a Cys$_6$Zn$_2$ binuclear cluster motif, that binds palindrome 2. We demonstrate that in yeast CTF1β transactivates the promoter of cut2 but is not effective in transactivating cut1 promoter, except when its palindrome 1 is mutated and thus incapable of binding PBP and/or other repressor. Thus, CTF1β activates the constitutive expression of cut2, and the cutin monomers generated by this enzyme together with CTF1α

* This work was supported in part by National Science Foundation Grant IBN-9816868. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF417004, AF417005, and U51672.

‡ Present address: Dept. of Biology, Gonzaga University, Spokane, WA.

§ To whom correspondence should be addressed: Neurobiotechnology Center, Ohio State University, 206 Rightmire Hall, 1060 Carmack Rd., Columbus, OH 43210. Tel.: 614-292-5682; Fax: 614-292-5379; E-mail: kolattukudy.2@osu.edu.

1 The abbreviations used are: PBP, palindrome binding protein; CAT, chloramphenicol acetyltransferase; CTF, cutinase transcription factor; DBD, DNA-binding domain; GST, glutathione S-transferase; orf, open reading frame.
transcriptionally activate the cut1 gene to cause highly induced expression of cutinase.

**EXPERIMENTAL PROCEDURES**

**Materials and Bacterial Strains—**F. solani pisi (isolate T-8) was grown, and genomic DNA was isolated as described before (5). All plasmids were propagated in *Escherichia coli* DH5α (Invitrogen). Chemically competent *Escherichia coli* (Stratagene, La Jolla, CA) and *Bacillus subtilis* (ATCC 6738) were from Amersham Biosciences. Duralon UV membranes were from Stratagene (La Jolla, CA). Restriction and modification enzymes and T4 DNA polymerase were from Invitrogen.

**Amino Acid Sequence of Cutinase 1 and Cutinase 2—**Cutinase 1 and cutinase 2 were purified from the culture filtrates of cutin-grown *F. solani pisi* containing each of the cloned genes, and were reduced, alkylated, and subjected to reverse phase high pressure liquid chromatography on a Nova-Pac C4, Radial-Pac column (Waters Associates) with a linear gradient of 5–65% acetonitrile in water containing 0.1% trifluoroacetic acid. A major 14C-labeled peptide fraction was further fractionated with a 0–50% linear gradient of isopropanol alcohol in water containing 0.1% phosphoric acid, followed by high pressure liquid chromatography using 0–50% acetonitrile in water containing 0.1% trifluoroacetic acid. The 14C-labeled peptides were subjected to amino acid sequencing by Edman degradation in a Beckman sequencer.

**Genomic Library Construction, Screening, and Sequencing of Cutinase Genes—**Genomic DNA was partially digested with Sau3A1 and EcoRI (0.6% in agarose gel). DNA fragments ranging from 9 to 20 kb were gel-purified, ligated to the Lambda FIX II vector (Stratagene), and packaged in vitro into λ particles with Gigapack II Goldplack extract (Stratagene) to create a library that contained 2 × 10^8 recombinant phages. The library was screened by plaque hybridization under low stringency hybridization conditions at 37°C with standard reagents (12) and 35% formamide. Cutinase cDNA labeled with [α-32P]dCTP with the random primer labeling system Rediprime II (Amersham Biosciences) was used as a probe. Membranes were washed twice at room temperature for 15 min in 2× SSPE (0.8 M NaCl, 10 mM Na2HPO4, 1 mM EDTA, pH 7) containing 0.1% SDS and once at 37°C for 15 min in 1× SSPE, 0.1% SDS, prior to exposure to x-ray films. Positive plaques were identified by autoradiography and recovered from agar plugs. DNA purified from positive phage clones was digested with SstI and subjected to Southern blot analysis using the same probe. DNA fragments hybridizing with the probe were isolated from the agarose gel with Geneclean III (Qiagen, Carlsbad, CA), subcloned into pBluescript KS vector, and sequenced by using a Sequenase 2.0 DNA sequencing kit from United States Biochemical Corp. (Cleveland, OH) as recommended by the supplier.

**Isolation of Phage Clones for CTF1β—**The 27 discrete clones identified in the original tertiary screen for *pb* (6) were tested with *pb* gene-specific primers, and the phage clones that yielded no PCR products were further investigated. Phage DNA purified from these clones was double digested with EcoRI and BamHI, EcoRI and SauI, EcoRI and SstI, or EcoRI and XhoI. The phage clones whose DNA differed in restriction patterns were probed with 32P-labeled palindrome 1 of 12 nucleotides and palindrome 2 of 17 nucleotides as a probe by using spotting the gnt11 library (6, 7) and another *gnt11* library constructed similarly with oligo(DT)2 using standard procedures (12).

**Subcloning and Sequence Analysis—**An oligo(DT)2 primed cDNA library was constructed from poly(A)+ RNA purified from an ultrasonic lysate of the cultures received 0.2 ml of minimal medium with or without 80 μg/ml of cutin hydrolysate (sonicated) and were incubated for 72 h at 24°C. An aliquot of the medium from each sample was assayed for cutinase activity (9). For each sample, the total incubation mixture was homogenized in 0.5 ml of 3× β-glucuronidase buffer (1× β-glucuronidase buffer: 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1% sodium lauryl sarcosine, and 0.1% Triton X-100 in 50 mM sodium phosphate buffer, pH 7.0, (20)), using a Mini-BeadBeater. The homogenate was centrifuged at 15,000 × g for 10 min, and the supernatant and the insoluble fraction were examined by SDS-PAGE. Total protein from 100 ml of isopropyl-β-D-thiogalactopyranoside-induced bacterial culture of two randomly selected clones was subjected to SDS-PAGE on 12.5% gel and transferred to nitrocellulose, and the filter was treated and tested for binding to the concatenated palindromic element as described before (6, 14).

**Probes for the gus Gene in the Promoters of cut1, cut2, or cut3 in F. solani pisi—**The start codon and 404-bp promoter region of cut1, cut2, and cut3 were amplified with *Phv* polymerase, introducing BamHI sites at the 5′ and 3′ ends. The amplified DNA products were ligated into the BamHI site of plasmid pGUS2.15, yielding cut1gus, cut2gus, and cut3gus. After sequencing the introduced promoter regions and the junction to the gus gene, a hygromycin resistance gene (hgy) fused to a constitutive promoter from *Cochliobolus heterosporus* (16) was cloned into the EcoRI sites of the constructs as the selection marker for *F. solani pisi* transformation.

**F. solani pisi conidia (107) were inoculated into 500 ml of mineral medium containing 2% glucose.** After the culture was shaken for 24 h at 24°C, mycelia were harvested by filtration through a Whatman No. 1 filter and washed twice with 0.6 M MgCl2. Protoplast preparation and transformation were done as described (17). The transformed protoplasts were plated on mineral medium containing 2% glucose, 1.2 μg sorbitol, and 2% agar. An overlay of 1% agar containing hygromycin B (300 μg/ml) (Calbiochem) was added after 24 h of incubation. Stable transformants were selected as before (17) and verified by PCR using primers designed to amplify the hygromycin gene portion or the *gus* gene. A Southern blot of the junction portion of the construct was hybridized with 32P-labeled palindrome 1 of 12 nucleotides and palindrome 2 of 37 nucleotides containing both overlapping palindromic elements (−159 to −178 bp) from cut1 was prepared as described (6). The palindromic element of cut2 (containing both palindromes) was prepared as described with the synthetic 37-mer, aattCGAAATGGACGGCGAGCTCG...
CGAGGTCGGCTTTCAG and its complement. Binding of recombinant PBp to these DNA fragments was tested essentially as described (6). In Vivo Transactivation of lacZ Gene in Yeast by CTF1β—Full-length cfβ was amplified by PCR as separate 5’ and 3’ end fragments. For the 5’ end amplified fragment, BamHI was used to digest the 5’ end and NeII was used to cut the 3’ end. These two BamHI- and NeII-digested fragments were simultaneously cloned into the BamHI sites of the GAL4 DNA-binding domain hybrid cloning vector pGBT9 (CLONTECH, Palo Alto, CA) to generate pGBT9/CF1β(1–882), which contained the DNA-binding domain of GAL4 fused in-frame to cfβ. For comparison with CTF1α, fragments of full-length cfα were first amplified by PCR as separate 5’ and 3’ end fragments. These two fragments were then joined by SOEing (splicing by overlap extension) PCR (22) and cloned in-frame into the PstI site of pGBT9 to produce pGBT9/CTF1α(1–909). Additionally, the EcoRI fragment of lacf/51 was directly cloned into the EcoRI site of pGBT9 to generate pGBT9/CTF1α(1–519) that contained the DNA-binding domain of GAL4 fused in-frame to CTF1α (amino acids 1–519).

Plasmids pGBT9/CTF1α(1–519), pGBT9/CTF1α(1–909), and pGBT9/CTF1α(1–882) were introduced into the yeast reporter strain SJY526 (CLONTECH) to express the hybrid proteins of GAL4 BD-CTF1α(1–519) and GAL4 BD-CTF1α. Plasmids pVA3 and pLAM5 (CLONTECH) expressing the hybrid protein of GAL4 DNA-binding domain fused to amino acids 72–390 of murine p53 protein or hybrid protein of GAL4 DNA-binding domain fused to amino acids 66–230 of human lamin C, respectively, and vector pGBT9 were introduced into SJY526 as negative controls. For positive control, plasmid pCL1 expressing the wild-type GAL4 DNA-binding domain fused to amino acids 66–230 of human lamin C was directly introduced into SJY526 according to the supplier’s instructions. Filter binding assays for β-galactosidase activity of lacZ gene product were performed according to the manufacturer’s instructions.

In Vivo Transactivation of cut1 and cut2 Promoter by CTF1β—For expression of CTF1β in yeast without the in-frame fusion of GAL4 protein, a GAL4 DNA activation domain hybrid cloning vector, pGAD424, was modified as follows. A HindIII-EcoRV linker containing an EcoRI site was ligated to HindIII- and EcoRV-digested pGAD424 to produce pEXP(55–11032 H/11032 – HindIII + EcoRI + EcoV), which was then cut with EcoRI and EcoRV. The EcoRI and EcoRV fragment of pGAD424 was released from pGAD424 by digestion with EcoRI and EcoRV, gel-purified, and ligated to EcoRI and EcoRV-digested pEXP(HindIII + EcoRI + EcoV). The resulting plasmid was designated as pEXPX(L), which allows for leucine selection and expression of genes driven by the ADH1 promoter.

To clone the full-length cfβ in pEXPX(L), pGBT9/CTF1β(1–882) was digested with BamHI. The full-length cfβ fragment was isolated and ligated to BamHI-digested pEXPX(L) to produce pEXPX(L)/CTF1β.

The promoter of cut2 was amplified by PCR with a sense primer (5’-GAC GAG GCC GCG CGG GAG ACG TTC CAT CC-3’) containing a NotI site, an antisense primer (5′-GGC GAG AGG AGT GGT GAA ACG GGC-3′), and cut2 DNA as template. The PCR fragments were cut with NotI and phosphorylated with T4 DNA kinase. This fragment was ligated to EcoRI and EcoRV-digested pGAD424 by digestion with EcoRI and EcoRV, gel-purified, and ligated to EcoRI and EcoRV-digested pEXPX[L] (EcoRI + EcoV). The resulting plasmid was designated pEXPX[cut2p], which was then cut with NotI and Sall, and the resulting fragment was gel-purified and ligated to a similarly digested pRS413, a yeast centromere plasmid (YEp) that allows for histidine selection in yeast (Stratagene; Ref. 7). The resultant plasmid was designated pYcut2p.

Plasmids pCAT(5) containing the wild-type cut1 promoter/cut gene and pYcut2p were introduced into yeast strain YPH499 (Stratagene; Ref. 7) to yield yeast strains YPH499-pYCAT and YPH499-pYcut2p.

For transcription by CTF1β, pYEXPX(L)/CTF1β was introduced into YPH499-pYCAT and YPH499-pYcut2p as described (Stratagene; Ref. 7). Yeast transformants were selected on leucine- and histidine-lacking minimal medium (7). Growth of yeast transformants and CAT assays were done as described (7).

RESULTS

Multiple Cutinase Genes and Identification of Proteins Encoded by Them—Constitutive expression of low levels of cutinase by F. solani pisi had been noted (3), and such an expression is probably responsible for the low level of cutinase carried by the conidia of the pathogenic isolates of this organism. It is possible that the gene that encodes the constitutively expressed enzyme is different from that which encodes the inducible one. The presence of the multiple cutinase genes was indicated by Southern analysis of the genome of F. solani pisi (11) and by the production of multiple cutinases (8). However, only the highly inducible cutinase gene had been cloned (10). Therefore, we first examined a genomic DNA library for cutinase genes. Screening of a Lambda FIX II library with the previously cloned full-length cutinase cDNA as the probe under low stringency revealed six clones that hybridized. DNA from these phage clones, digested with SalI and analyzed by Southern hybridization, showed unique digestion patterns and had different size fragments hybridizing with the cDNA probe. Subcloning and DNA sequencing showed that two of the clones contained overlapping DNA sequences that revealed the previously sequenced cutinase gene that we designate cut1. Two additional cutinase genes were found in the remaining four clones as overlapping sequences. These two newly cloned genes are designated cut2 and cut3.

cut2 and cut3 genes and their 5′-untranslated regions (404 bp) were sequenced. The nucleotide sequences revealed identical size orfs of 693 bp for cut2 and cut3 showing 86 and 85.5% identity to cut1, respectively. Both orfs are interrupted by one 49-bp intron. The position and the nucleotide sequence of the introns in the two genes are identical. cut1 has one 52-bp intron located at the same relative position, but it shows only 65% sequence homology to the cut2/3 intron. cut2 would encode a protein with a calculated molecular mass of 23.93 Da, a pI of 7.68, and shares 93% amino acid identity with cut1. The protein encoded by cut3 has a calculated molecular mass of 24.02 Da, a pI of 6.82, and exhibits 92.1% amino acid identity with cutinase 1. cut2 and cut3 products, that have one amino acid more than the cut1 product, share more identity to each other (98.7%) than to cut1 (Fig. 1).

Amino acid sequencing of the Cys- and His-containing tryptic peptides obtained from cutinase 1 and cutinase 2 isolated from cutin-grown F. solani pisi showed very similar but distinctly different amino acid sequence. Peptides isolated from cutinase 1 and cutinase 2 after reduction and carboxymethylation with 14C-labeled iodoacetamide were sequenced. One of the labeled peptides isolated from cutinase 2 was a 28-mer composed of amino acids 186–213 of the cutinase, and it showed two amino acid differences (Val208 → Ile208 and Ala206 → Thr206), when compared with the amino acid sequence of the corresponding peptide isolated from cutinase 1. Cutinase 1 peptide sequence is found in the orfs of the cut1 gene, and cutinase 2 peptide sequence is found in the orfs of both cut2 and cut3 (Fig. 1).

Comparison of Promoters of Cutinase Genes—The 5′-flanking region that is known to contain the promoter of cut1 (5, 17) showed a high degree of identity with the corresponding segment of cut2 and cut3 (Fig. 2). The promoter segments of cut2 and cut3 were about 94% identical, whereas they showed 80–83% identity to cut1 promoter. They contained the G-rich element found to be essential for the high level of the inducible
Regulation of Cutinase Genes by Different Zn Finger Proteins

Fig. 2. The promoter sequences of the three cutinase genes from *F. solani pisi*. The palindrome 1 and palindrome 2 sequences are indicated.

cutinase gene expression and the silencer of the cutinase promoter (5). Comparison of the palindromic sequences at −159 bp, which have been found to be essential for the inducible expression of the cutinase gene, showed that palindrome 2 is conserved in all three cut genes. However, palindrome 1 in cut2 and cut3 contains two nucleotide substitutions (Fig. 2).

cut2/3 Palindrome 1 Does Not Bind PBP—We had cloned and expressed previously a protein designated palindrome binding protein (PBP) that binds the palindromic segment of cut1 promoter. However, whether PBP bound to only one of the palindromes was not known. Examination of the binding to the two palindromes individually by gel retardation assay showed that PBP bound only to palindrome 1 and not palindrome 2 (Fig. 3A). To test whether the two nucleotide substitutions in the palindrome 1 of cut2/3 affect PBP binding, we performed gel retardation tests with the palindrome segment of cut1 or cut2/3. Only palindrome 1 of cut1 bound to PBP but not the palindrome 1 of cut2/3 (Fig. 3B).

Cloning of CTF1β—Because the phage clones encoding PBP were isolated using the fragment containing both palindromes, the phage clones obtained during that screening may also contain clones that would encode CTF1. The tertiary screening for PBP clones yielded 27 discrete clones. PCR test indicated that 10 of the phage clones belonged to those encoding PBP (data not shown). The remaining 17 clones showed 5 distinctly different restriction patterns, and the representative clones from each group were designated as ctf1 8, ctf1 11, ctf1 15, ctf1 22, and ctf1 26. When tested by Southwestern hybridization (14), all these 5 types of clones showed binding to the concatenated palindrome 2 fragment (data not shown). DNA inserts from these phage clones were subcloned into pBS KS to generate pCTF1–8, pCTF1–11, pCTF1–15, pCTF1–22, and pCTF1–26. Initial sequencing of these clones indicated that pCTF1–8 was a partial clone of pCTF1–15, pCTF1–22 was a partial clone of pCTF1–26, and pCTF1–11 was a distinct clone. The inserts in pCTF1–15 and pCTF1–11 were completely sequenced, and the deduced polypeptides revealed the presence of Cys6Zn(II)2 binuclear cluster DNA-binding motifs in their N termini. The lack of in-frame stop codons for both DNA inserts indicated that neither clone represented a complete open reading frame. The polypeptide encoded by the DNA insert in pCTF1–15 was designated CTF1β and further studied (7). The one encoded by pCTF1–11 was designated CTF1γ (Fig. 4A). Further screening of Agt11 libraries identified three additional overlapping clones for CTF1γ (Fig. 4A). The complete sequencing of the four overlapping clones for CTF1β revealed a contiguous cDNA sequence of 4234 bp containing a complete open reading frame of 2646 bp that would encode a putative acidic protein of 882 amino acids with a calculated pI of 6.33 and molecular weight of 98,180 (Fig. 4B). CTF1β contains 10 potential consensus sequences for phosphorylation by casein kinase II (23), two potential sites for phosphorylation by the cAMP-dependent protein kinase A (24), and four potential phosphorylation sites for protein kinase C (Fig. 4C) (25). Two consensus sites, PPS and PSSF, for potential mitogen-activated protein kinase phosphorylation (26) were also identified. Two potential asparagine-glycosylation sites (27) are observed. PSORT identified one likely nuclear localization signal RRKK (28) in CTF1β (Fig. 4A). A Cys6Zn(II)2 binuclear cluster domain is located at the N terminus from amino acid residues 51–76 of CTF1β (Fig. 5). CTF1α and CTF1β showed only 17% overall amino acid identity.

Binding of CTF1β to Palindrome 2 in Cutinase Promoter—To test the binding of CTF1β to the palindromic fragment, GST fusion vector was used to express the amino acids 6–341 of CTF1β. This GST fusion protein contains the DNA-binding domain of CTF1β. Protein samples from two bacterial transformants were subjected to DNA binding assay by Southwestern hybridization (14). Only one band was found, and it corresponded to the DNA-binding protein of GST-CTF1β(6–341) in the autoradiogram, whereas no binding was observed with proteins
from bacterial host cells containing the expression vector alone (Fig. 6).

Transactivation of Cut2 Promoter by CTF1β—We tested whether CTF1β could function as a transcriptional activator. Plasmid constructs were made to express a hybrid fusion protein in which CTF1β was fused to the DNA-binding domain (DBD) of GAL4. The GAL4(DBD)-CTF1β hybrid protein was tested for activation of transcription of the chromosomally integrated lacZ gene containing the GAL4-binding element. The transactivating capabilities were indicated by the production of active β-galactosidase that caused the formation of blue colonies on a filter containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) (data not shown). Furthermore, expression of the GAL4 DNA-binding domain alone, or the fusion of GAL4 DNA-binding domain to amino acids 72–390 of murine p53 protein, or the fusion of GAL4 DNA-binding domain to amino acids 66–230 of human lamin C, were unable to activate the transcription of the lacZ gene. These results demonstrated

Fig. 4. A, schematic representation of the clones used to obtain the complete sequence of CTF1β. B, the amino acid sequence of CTF1β deduced from the cDNA sequence. C, schematic representation showing the possible functional domains in CTF1β.

Fig. 5. Alignment of the zinc finger region of CTF1β and CTF1α with zinc finger regions of transcription factors from other organisms as described in the text. The numbers at the right show the position of the last residue.

Fig. 6. Expression of the DNA-binding domain of CTF1β in E. coli (left) and Southwestern blot showing binding to palindrome 2 of cutinase promoter (right). In both, 12.5% polyacrylamide was used. Lane 1, E. coli extract of vector control; lanes 2 and 3, two independent clones expressing 6–341 N-terminal segment of CTF1β. Experimental details are indicated under “Experimental Procedures.”

GAL4 DNA-binding domain to amino acids 72–390 of murine p53 protein, or the fusion of GAL4 DNA-binding domain to amino acids 66–230 of human lamin C, were unable to activate the transcription of the lacZ gene. These results demonstrated
that CTF1β could function as a transcriptional activator in vivo in yeast.

To assess quantitatively whether CTF1β could function as a cutinase transcriptional activator in vivo, plasmid constructs were made to express a hybrid fusion protein in which CTF1β was fused to the nuclear localization sequence of SV40. The CTF1β hybrid protein was tested for activation of transcription of the cat gene fused to the promoter of cut1 or cut2 in yeast. The transactivation was measured by the level of CAT activity. Such an approach has been used previously to demonstrate transactivation of cutinase promoters by CTF1β/H9252, CTF1β cutinase transcriptional activator of SV40 cut1 promoter; CAT activity was measured as described (5). cut1, cut2, cut2 promoter; cut1(Δpal 1), cut1 promoter in which GGATCG of palindrome 1 was replaced with ATGAGC.

FIG. 7. Transactivation of cutinase promoters by CTF1β in yeast. CAT gene was under the control of cutinase promoter, and full-length CTF1β was expressed via another plasmid under the control of SV40 promoter; CAT activity was measured as described (5). cut1, cut1 promoter; cut2, cut2 promoter; cut1(Δpal 1), cut1 promoter in which GGATCG of palindrome 1 was replaced with ATGAGC.

FIG. 8. Expression of β-glucuronidase under the control of the promoters of cutinase genes in F. solani pisi. In each case 404-bp 5'-flanking region was fused to GUS gene and used to transform F. solani pisi. Two transformants each were grown in glucose until glucose depletion and were assayed for β-glucuronidase activity at glucose depletion, after 72 starvation or after induction with cutin hydrolysate for 72 h as described under “Experimental Procedures.” Induction is expressed as the number of fold activity found after starvation or induction when compared with the activities at glucose depletion.

The first purification of fungal cutinase from the extracellular fluid of cutin-grown F. solani pisi yielded two enzymes of similar size (8). Amino acid sequence of some peptides obtained from one of these enzymes (cutinase 1) matched with that predicted from the cDNA of the induced cutinase transcript (10). The present results reveal the occurrence of other cutinase genes that could encode enzymes that are similar but not identical to cutinase 1. Comparison of the amino acid sequences that would be encoded by the three cloned genes indicates that the two newly identified genes would encode proteins that are nearly identical, differing only in three amino acid residues. Among them only one, Ala14 → Asp, would affect the ionic status of the protein if this segment is retained in the mature protein. Thus, products of cut2 and cut3 probably would be inseparable by the protein fractionation methods used, and therefore the purified cutinase 2 might have contained products of both cut2 and cut3.

Induction of the cut1, cut2, or cut3 Promoter by Starvation and Cutin Monomers in F. solani pisi—Because the transcripts of the three cutinase genes are very similar, it is difficult to quantitate their transcripts individually. Therefore, the individual cut promoters were fused to gus gene as a reporter and introduced into the genome of F. solani pisi. After screening several stable transformants for each promoter, two randomly selected transformants for each promoter were used for quantitative measurements of the degree of expression of gus under the control of the three cutinase promoters under various conditions (Fig. 8). The results showed that cut1 and cut3 promoter did not allow high level expression of the gus gene upon glucose depletion or subsequently during a 3-day starvation period; cut2 promoter was induced by starvation (Fig. 8). Cutin monomers prepared by alkaline hydrolysis of cutin highly induced cut1 promoter and moderately induced cut3 (Fig. 8). This result confirms that cut1 promoter is responsible for a major part of the induced expression of cutinase activity. cut2 promoter, on the other hand, allowed some expression of gus gene upon glucose depletion, and moderate induction of gus gene was observed upon starvation for a 72-h period. These results strongly suggest that cut2 is probably responsible for the low constitutive levels of cutinase activity.

DISCUSSION

The first purification of fungal cutinase from the extracellular fluid of cutin-grown F. solani pisi yielded two enzymes of similar size (8). Amino acid sequence of some peptides obtained from one of these enzymes (cutinase 1) matched with that predicted from the cDNA of the induced cutinase transcript (10). The present results reveal the occurrence of other cutinase genes that could encode enzymes that are similar but not identical to cutinase 1. Comparison of the amino acid sequences that would be encoded by the three cloned genes indicates that the two newly identified genes would encode proteins that are nearly identical, differing only in three amino acid residues. Among them only one, Ala14 → Asp, would affect the ionic status of the protein if this segment is retained in the mature protein. Thus, products of cut2 and cut3 probably would be inseparable by the protein fractionation methods used, and therefore the purified cutinase 2 might have contained products of both cut2 and cut3. If cut3 is not expressed at significant levels either constitutively or under the induction conditions used, the isolated cutinases 1 and 2 might be products of cut1 and cut2, respectively. cut1, although similar, does not share the same degree of identity, suggesting that cut2 and cut3 probably originated from a more recent gene duplication when compared with the earlier divergence between the constitutive (cut2/3) and inducible (cut1) cutinase genes. This conclusion is consistent with our finding that cut2 and cut3 have exactly the same two nucleotide substitutions in the palindrome 1 in the promoter and with the finding that the size and sequence of cut2 and cut3 intron are identical but distinctly different from those of cut1 intron.
CTF1β shows characteristics of a transcription factor. The presence of putative nuclear localization signals suggested that CTF1β may be a nuclear protein. Binding to the palindromic DNA element in the cutinase promoter by the expressed segment of CTF1β is demonstrated. The N-terminal Cys6Zn(II)2 binuclear cluster motif found in CTF1β is probably involved in the binding to the palindrome. Such DNA-binding motifs are characteristic of other regulatory proteins such as GAL4 (29), ARGRII (30), PPR1 (31), PDR1 (32), PUT3 (33), HAP1 (34), and UGA3 (35) of Saccharomyces cerevisiae; LAC9 of Kluyveromyces lactis (36); MAL63 of Saccharomyces carlsbergensis (37); NIT4 of Neurospora crassa (38); NIRA (39), UAY (40), QUTA (41), and AMDR (42) of Aspergillus nidulans; and AFLR of Aspergillus flavus (43). However, CTF1β does not share homology to any other regions of those factors. Functionally, GAL4 is a positive activator that regulates the transcription of the galactose-inducible genes gal1, gal2, gal7, gal10, and mel1 (29). PPR1 positively regulates transcription of the genes ura1, ura3, and ura4 involved in controlling pyrimidine levels (31).

Some of these protein factors recognize a DNA sequence with ura3, and ura1. CTF1 positively regulates transcription of the genes ura1, ura3, and ura4 involved in controlling pyrimidine levels (31). The presence of putative nuclear localization signals suggested that CTF1β may be a nuclear protein. Binding to the palindromic DNA element in the cutinase promoter by the expressed segment of CTF1β is demonstrated. The N-terminal Cys6Zn(II)2 binuclear cluster motif found in CTF1β is probably involved in the binding to the palindrome. Such DNA-binding motifs are characteristic of other regulatory proteins such as GAL4 (29), ARGRII (30), PPR1 (31), PDR1 (32), PUT3 (33), HAP1 (34), and UGA3 (35) of Saccharomyces cerevisiae; LAC9 of Kluyveromyces lactis (36); MAL63 of Saccharomyces carlsbergensis (37); NIT4 of Neurospora crassa (38); NIRA (39), UAY (40), QUTA (41), and AMDR (42) of Aspergillus nidulans; and AFLR of Aspergillus flavus (43). However, CTF1β does not share homology to any other regions of those factors. Functionally, GAL4 is a positive activator that regulates the transcription of the galactose-inducible genes gal1, gal2, gal7, gal10, and mel1 (29). PPR1 positively regulates transcription of the genes ura1, ura3, and ura4 involved in controlling pyrimidine levels (31).

Some of these protein factors recognize a DNA sequence with ura3, and ura1. CTF1 positively regulates transcription of the genes ura1, ura3, and ura4 involved in controlling pyrimidine levels (31). The presence of putative nuclear localization signals suggested that CTF1β may be a nuclear protein. Binding to the palindromic DNA element in the cutinase promoter by the expressed segment of CTF1β is demonstrated. The N-terminal Cys6Zn(II)2 binuclear cluster motif found in CTF1β is probably involved in the binding to the palindrome. Such DNA-binding motifs are characteristic of other regulatory proteins such as GAL4 (29), ARGRII (30), PPR1 (31), PDR1 (32), PUT3 (33), HAP1 (34), and UGA3 (35) of Saccharomyces cerevisiae; LAC9 of Kluyveromyces lactis (36); MAL63 of Saccharomyces carlsbergensis (37); NIT4 of Neurospora crassa (38); NIRA (39), UAY (40), QUTA (41), and AMDR (42) of Aspergillus nidulans; and AFLR of Aspergillus flavus (43). However, CTF1β does not share homology to any other regions of those factors. Functionally, GAL4 is a positive activator that regulates the transcription of the galactose-inducible genes gal1, gal2, gal7, gal10, and mel1 (29). PPR1 positively regulates transcription of the genes ura1, ura3, and ura4 involved in controlling pyrimidine levels (31).

The transcription factor CTF1α binds palindrome 2 of cut1 promoter that is known to be essential for cutinase induction by cutin monomers. In vivo CTF1α, in fact, transactivates cut1 promoter in yeast. However, another transcription factor, CTF1β that has now been cloned as described here, does not transactivate cut1 promoter but it transactivates cut2 promoter. The chief difference between the promoters of cut1 gene and cut2/3 genes in the palindromic promoter region is that there are two nucleotide substitutions in palindrome 1. Here we show that PBP binds palindrome 1 of cut1, whereas it does not bind palindrom 1 of cut2/3. If palindrome 1 of cut1 is occupied by PBP, then CTF1α may not be able to bind palindrome 2 of the promoter because of steric hindrance and thus would not induce the transcription of cut1 gene. On the other hand, CTF1β may bind palindrome 2 of cut2/3 because palindrome 1 of this gene cannot bind PBP because of the nucleotide substitutions in this palindrome. The hypothesis that PBP may function as a repressor of cut1 is consistent with our previous finding that mutations in palindrome 1 of cut1 promoter fused to cat reporter gene increased the inducibility of this promoter 2-fold when assayed in F. solani pisi transformants generated with this promoter fusion construct (5). The transactivation of cat gene expressed under the control of cutinase gene promoters by CTF1β in yeast is consistent with this hypothesis.

The results presented here and previously published results suggest the following mechanism by which a Cys6Zn(II)2 transcription factor causes constitutive expression of one cutinase gene whose product generates an inducer from cutin in the environment, and this inducer uses a different Cys6Zn(II)2 transcription factor to cause induction of high levels of cutinase (Fig. 9). (The speculative part of the hypothesis is indicated by dotted lines and lowercase letters.) Cutinase gene promoter contains two overlapping palindromic elements. Proteins that bind the two palindromes regulate the transcription of cutinase genes. CTF1α and CTF1β both bind palindrome 2. In the absence of PBP binding to palindrome 1 of cut2, CTF1β can bind palindrome 2 and activate cut2 gene transcription, albeit at low levels, explaining the constitutive production of low levels of cutinase. Palindrome 1 of cut1 binds PBP, and the bound PBP interferes with CTF1α binding to palindrome 2. We postulate that the presence of high levels of CTF1α may allow it to overcome the interference from PBP and thus bind palindrome 2 of cut1 and transcriptionally activate it and thus cause production of high levels of cutinase. From the amino acid sequence it is clear that the major induced protein is in fact encoded by cut1. CTF1α promoter does contain an element that binds a transcription factor that has been cloned.3 We postulate that cutin monomer-dependent phosphorylation of cutin-responsive element-binding protein and the role of this phosphorylation in CTF1α transcription do not have direct experimental evidence and are therefore indicated by dashed lines and lowercase lettering.

3 D. Li and P. E. Kolattukudy, unpublished observations.
any detectable change in growth rate in cultures, although the decreased level of cutinase resulting from the CTF1α disruption was not adequate to explain the loss of virulence, as supplementation with cutinase did not restore pathogenicity.\textsuperscript{3} Thus, CTF1α may participate also in the regulation of other genes essential for pathogenesis. Obviously, CTF1β cannot substitute for CTF1α in this function, further illustrating the differential functions of these zinc finger proteins.

In the field, the constitutively expressed low levels of cutinase from fungi would release cutin monomers from cutin found in their immediate environment, and these monomers would in turn induce the synthesis of high levels of the enzyme. Such a mechanism could allow saprophytic growth or growth into the host during infection, as in both cases the fungal conidia with their low level of cutinase contact cutin, presenting basically the same situation as far as induction is concerned. This molecular strategy is probably used widely by microbes to utilize insoluble polymers they find in their environment. The low levels of polymer-degrading enzymes secreted upon depletion of readily utilisable soluble nutrients generates soluble products (monomers or oligomers) that in turn induce high levels of the degrading enzymes that generate soluble nutrients from the insoluble polymer in the medium for growth of the microbe. We present evidence that constitutively expressed and induced cutinase genes use their own unique transcription factors. Whether the mechanism similar to that described here for cutinase is used for the constitutive and induced expression of the other polymer-degrading enzymes in general is not known.

Acknowledgment—We thank Usha Raman for technical help.

REFERENCES
1. Kolattukudy, P. E., Rogers, L. M., Li, D., Hwang, C. H., and Flaishman, M. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4080–4087
2. Kolattukudy, P. E. (2001) in Advances in Biochemical Engineering/Biotechnology: Biopolymesters (Babel, W., and Steinbücker, A., eds) pp. 1–49, Springer-Verlag, Heidelberg
3. Köller, N., Allan, C. R., and Kolattukudy, P. E. (1982) Physiol. Plant Pathol. 20, 47–60
4. Woloshuk, C. P., and Kolattukudy, P. E. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 1704–1708
5. Kamper, J. T., Kamper, U., Rogers, L. M., and Kolattukudy, P. E. (1994) J. Biol. Chem. 269, 9195–9204
6. Li, D., and Kolattukudy, P. E. (1995) J. Biol. Chem. 270, 11753–11756
7. Li, D., and Kolattukudy, P. E. (1997) J. Biol. Chem. 272, 124462–124467
8. Purdy, R. E., and Kolattukudy, P. E. (1975) Biochemistry 14, 2824–2831
Regulation of Constitutively Expressed and Induced Cutinase Genes by Different Zinc Finger Transcription Factors in Fusarium solani f. sp. pisi (Nectria haematococca)

Daoxin Li, Tatiana Sirakova, Linda Rogers, William F. Ettinger and P.E. Kolattukudy

J. Biol. Chem. 2002, 277:7905-7912.
doi: 10.1074/jbc.M108799200 originally published online December 26, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M108799200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 40 references, 22 of which can be accessed free at http://www.jbc.org/content/277/10/7905.full.html#ref-list-1