Winged Helix Transcription Factor CPCRI Is Involved in Regulation of β-Lactam Biosynthesis in the Fungus Acremonium chrysogenum

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Received 28 April 2003/Accepted 20 October 2003

Winged helix transcription factors, including members of the forkhead and the RFX subclasses, are characteristic for the eukaryotic domains in animals and fungi but seem to be missing in plants. In this study, in vitro and in vivo approaches were used to determine the functional role of the RFX transcription factor CPCRI from the filamentous fungus Acremonium chrysogenum in cephalosporin C biosynthesis. Gel retardation analyses were applied to identify new binding sites of the transcription factor in an intergenic promoter region of cephalosporin C biosynthesis genes. Here, we illustrate that CPCRI recognizes and binds at least two sequences in the intergenic region between the pcbAB and pcbC genes. The in vivo relevance of the two sequences for gene activation was demonstrated by using pcbC promoter-lacZ fusions in A. chrysogenum. The deletion of both CPCRI binding sites resulted in an extensive reduction of reporter gene activity in transgenic strains (to 12% of the activity level of the control). Furthermore, Acremonium transformants with multiple copies of the cpcR1 gene and knockout strains support the idea of CPCRI being a regulator of cephalosporin C biosynthesis gene expression. Significant differences in pcbC gene transcript levels were obtained with the knockout transformants. More-than-twofold increases in the pcbC transcript level at 24 and 36 h of cultivation were followed by a reduction to approximately 80% from 48 to 96 h in the knockout strain. The overall levels of the production of cephalosporin C were identical in transformed and nontransformed strains; however, the knockout strains showed a striking reduction in the level of the biosynthesis of intermediate penicillin N to less than 20% of that of the recipient strain. We were able to show that the complementation of the cpcR1 gene in the knockout strains reverses pcbC transcript and penicillin N amounts to levels comparable to those in the control. These results clearly indicate the involvement of CPCRI in the regulation of cephalosporin C biosynthesis. However, the complexity of the data points to a well-controlled or even functional redundant network of transcription factors, with CPCRI being only one player within this process.

The filamentous fungus Acremonium chrysogenum is exploited industrially for the production of the β-lactam antibiotic cephalosporin C. A. chrysogenum is cultured worldwide and yields around 2,500 tons of cephalosporin derivatives annually. Semisynthetic derivatives are used mainly as broad-spectrum antibiotics for the treatment of bacterial infections. In contrast to many other penicillins, cephalosporins are effective against both gram-positive and several gram-negative bacteria. β-Lactam antibiotics are products of the secondary metabolism of different fungi and bacteria and, in contrast to primary metabolites, have no direct function in cellular growth (for a review, see reference 4).

The biosynthesis of cephalosporin C consists of six enzymatic steps, which are catalyzed by six different enzymes (for an overview, see references 3 and 4). Three amino acids are fused by a peptide synthetase encoded by the pcbAB gene. Isopenicillin N synthase (IPNS), encoded by the pcbC gene, then catalyzes the formation of the bicyclic tripeptide isopenicillin N. For the subsequent epimerization step to penicillin N, at least two distinct enzymes are essential (38), whereas the conversion of penicillin N to deacetylcephalosporin is accomplished in two steps with one bifunctional enzyme with expansase and hydroxylase activity. The final step in the synthesis of cephalosporin C is completed by an acetyltransferase. The genes encoding the biosynthesis enzyme are organized as three pairs of bidirectional genes that are regulated through three intergenic promoter regions. The six genes are located in two clusters on two separate chromosomes (9, 38). The biosynthesis of β-lactam antibiotics in fungi has been optimized for industrial purposes over the past 50 years. Strain improvement has been achieved by classical mutation and selection techniques. With regard to strains of Penicillium chrysogenum that produce high titers of penicillin, an increase of penicillin biosynthesis gene copy numbers has been observed (see, e.g., reference 26). Interestingly, in A. chrysogenum, no comparable amplifications of structural genes were detected in strains with increased cephalosporin C production levels. At the molecular level, low- and high-production strains seem to be distinguishable only by their electrophoretic karyotypes (35, 40). However, substantial differences were observed at the transcriptional level when levels of mRNAs derived from cphA and cphB were followed by a reduction to approximately 80% from 48 to 96 h in the knockout strain. The overall levels of the production of cephalosporin C were identical in transformed and nontransformed strains; however, the knockout strains showed a striking reduction in the level of the biosynthesis of intermediate penicillin N to less than 20% of that of the recipient strain. We were able to show that the complementation of the cpcR1 gene in the knockout strains reverses pcbC transcript and penicillin N amounts to levels comparable to those in the control. These results clearly indicate the involvement of CPCRI in the regulation of cephalosporin C biosynthesis. However, the complexity of the data points to a well-controlled or even functional redundant network of transcription factors, with CPCRI being only one player within this process.

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genum was cultivated in CCM at 27°C strain K-12 XL1-Blue (Stratagene) was used for cloning experiments. A. chrysogenum seems likely that changes in activity or specificity of the regulatory factor X (RFX) family of eukaryotic transcription factors and are characterized by a nonconventional mode of DNA recognition. The cocrystral structure of the human RFX1 DNA-binding domain bound to its target DNA revealed a position for the so-called DNA recognition helix 3 that was different from those for other winged helix proteins. In the structure of the RFX protein, most contacts to the major groove of the DNA are made by wing 1, whereas in the structures of all other well-characterized winged helix proteins, all contacts to the major groove are made by recognition helix 3 (7).

The most prominent members of the winged helix family are the human hepatocyte nuclear factor-3 (HNF-3) and the Droso phila homeotic forkhead proteins (for a review, see reference 7). Members of the RFX class of transcription factors have been identified in a number of lower eukaryotes and mammals, and their ascribed functions are diverse. In humans, RFX1/RFX2 and RFX3 function in a tissue- and lineage-specific manner, while RFXS is part of a multiprotein complex for the regulation of major histocompatibility complex class II genes (14, 39). The Droso phila melanogaster RFX protein is involved in the development of the peripheral nervous system and brain of the embryo, and the Caenorhabditis elegans homologue is essential for cilium formation in sensory neurons (5, 37). Crt1 is the only RFX factor encoded in the yeast genome and acts in the DNA damage and replication checkpoint pathway by recruiting the general repressors Ssn6 and Tup1 to the promoters of damage-inducible genes (13). Other fungal RFX transcription factor genes have been identified in the fission yeast and in the penicillin producer P. chrysogenum; however, their target genes and molecular functions are still unknown (32, 43).

This report describes the first functional analysis of an RFX transcription factor from a filamentous fungus and demonstrates that the Acremonium protein is involved in the regulation of β-lactam biosynthetic gene expression.

MATERIALS AND METHODS

Strains, plasmids, and transformation of A. chrysogenum. Escherichia coli strain K-12 XL1-Blue (Stratagene) was used for cloning experiments. A. chrysogenum was cultivated in CCM at 27°C as described by Minuth et al. (23). Cultivations for time courses were started with a 5% inoculum from a 2.5-day-old preculture. Cultivation time points given in Results indicate the times following the inoculation of the main culture.

Cotransformation experiments were performed with A. chrysogenum producer strain A3/2 (27) by using vector pMW1 (hygromycin B resistance [20]) and derivatives of reporter gene plasmid pSIM9 (22) or plasmid pVIS::lacZ (33). The most prominent members of the winged helix transcription factor family are from filamentous fungi and consists of 830 amino acids. Besides the N-terminal DNA-binding domain, the protein contains a dimerization domain at its C terminus, cpcR1 gene deletion constructs and a two-hybrid analysis revealed that CPCRCR1 dimerizes and binds DNA only in a multimeric state (32). Thus, the full-length protein is required for its function as a transcription factor.

RFX proteins form a small class of winged helix transcription factor genes have been identified in a number of lower eukaryotes and mammals, and their ascribed functions are diverse. In humans, RFX1/RFX2 and RFX3 function in a tissue- and lineage-specific manner, while RFX5 is part of a multiprotein complex for the regulation of major histocompatibility complex class II genes (14, 39). The Droso phila melanogaster RFX protein is involved in the development of the peripheral nervous system and brain of the embryo, and the Caenorhabditis elegans homologue is essential for cilium formation in sensory neurons (5, 37). Crt1 is the only RFX factor encoded in the yeast genome and acts in the DNA damage and replication checkpoint pathway by recruiting the general repressors Ssn6 and Tup1 to the promoters of damage-inducible genes (13). Other fungal RFX transcription factor genes have been identified in the fission yeast and in the penicillin producer P. chrysogenum; however, their target genes and molecular functions are still unknown (32, 43).

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TABLE 1. Oligonucleotides used in this worka

| Oligonucleotide | Sequence | Specificity |
|----------------|----------|-------------|
| 5              | GGCTGTGAGACATACCTGTTGG | Pos. 134–155; frgm. 3 |
| 6              | GCAATGCTTACATACTGTTTGTATC | Pos. 223–197; frgm. 3 |
| 31             | GGAGACAGAGTTAAGTACTC | Pos. 1045–1065; frgm. 16 |
| 979            | CATTATATGGCTCCGATGCGAG | Pos. 1133–1113; frgm. 16 |
| 1010           | GTCACACCGCTTCCTGCGG | cepR1 gene |
| 1233           | CACATCGGGAAACCGGCTGCTTGC | cepR1 gene |
| 1246           | GAGGCGCTTGGCCAGGAGGCGG | cepR1 gene |
| 1336           | GGGCGGTTGCTCGCCATGAGCTCTTTCT | pchAB-pcbC promoter, BSHI primer F1 for pSIMΔ2 |
| 1348           | GGGCGGCTGTTTGGGCGCCAATTCTTGAG | pchAB-pcbC promoter, BSHI primer F2 for pSIMΔ2 |
| 1478           | GTATCAAGAATGTCGACGCGACCCGTCAGGCGG | PCR primer 1 for pSIMΔ2 |
| 1479           | GATTTATGCGACACGAGACGC | PCR primer 2 for pSIMΔ2 |
| 2804           | CCTCGGCTTGTGATACACTGTTC | Pos. 345–364; frgm. 6 |
| 2805           | GGTATGCTTTCAGCAGCCTCTGTGAAAGCGG | Pos. 150–122; frgm. 2 |
| 2806           | CGGGGCATCGGCTCTGGCGAGG | Pos. 500–478; frgm. 7 |
| 2807           | GAAGCTCTGTATATCCAAAAAGGTT | Pos. 80–57; frgm. 1 |
| 2808           | GCGGCGTTTGGAGTTACTGTTGCTGGG | Pos. 430–404; frgm. 6 |
| 2809           | GATGTCCGAAAGAAAGATGCAGCGG | Pos. 290–269; frgm. 4 |
| 2810           | CCAGGCAGTGATTCCCACGTCTTCGG | Pos. 484–508; frgm. 8 |
| 2811           | GTGAACTCTAAAGGACCGACCC | Pos. 413–440; frgm. 7 |
| 2812           | CAGTGTCTTTTGGCGACTACCGGCGG | Pos. 273–298; frgm. 5 |
| 2813           | CTGCAGAAGCAGGAGGCGACCTTGCGC | Pos. 567–541; frgm. 8 |
| 2814           | CGGAGTTCCCCAGGATTAAAGAGC | Pos. 360–336; frgm. 5 |
| 2815           | GGGATATCAGAAGCTCTATTGCGG | Pos. 65–88; frgm. 2 |
| 2816           | CACGGACGCGGCCAGGCTGACGCGG | Pos. 1–28; frgm. 1 |
| 2817           | GTATGATAGCTGTTGTGACG | Pos. 209–225; frgm. 4 |
| 2818           | CCTGATGAGTGGGCGGCTTACATGC | Pos. 834–866; frgm. 13 |
| 2819           | GCGCGCAGGAAGGCGAAATCGT | Pos. 850–830; frgm. 12 |
| 2820           | GAGTTTTGGTCTAGGAGACTGCG | Pos. 923–902; frgm. 13 |
| 2821           | ACTGTGATTTGGCTACTAAGCTTC | Pos. 626–652; frgm. 10 |
| 2822           | TCTCTGCTGTCGGAATTTAAATGTCG | Pos. 552–577; frgm. 9 |
| 2823           | CGTATGATGACTGCTGATCTACGGC | Pos. 900–894; frgm. 14 |
| 2824           | CTACATGAGTGGGCGGCTTACATGC | Pos. 909–932; frgm. 14 |
| 2825           | TACGAGTGGGCTCTACACGCGG | Pos. 766–799; frgm. 12 |
| 2826           | CATTTTCTAGTTAGGACATTCGC | Pos. 1237–1216; frgm. 17 |
| 2827           | GACGGCCGCTCCGGTATGGGACGCGG | Pos. 780–757; frgm. 11 |
| 2828           | GCAGTTACATGGATATCCCTTTGTCGG | Pos. 976–1002; frgm. 15 |
| 2829           | GGACGATGATGACTGCTGATCTACGGC | Pos. 1117–1143; frgm. 17 |
| 2830           | CTACCTCTGCTTCCCTGCGG | Pos. 1066–1040; frgm. 15 |

a For the double-stranded probes used in gel retardation analysis, only the sense strands are given. Positions (pos.) indicate the nucleotides within the pchAB-pcbC promoter (22). The fragment (frgm.) designation used in the promoter scanning analysis is given.

which samples were concentrated by Na acetate-ethanol precipitation. Equal mRNA amounts from the same preparation were taken either for quantitative Northern blot analysis (16) or for the labeling of targets for array-based hybridization with subsequent quantification of signals.

Membrane array preparation. Specific probes were generated by PCR, and then PCR results were verified by agarose gel electrophoresis and PCR products (between 1,000 and 1,500 bp) were purified with a QIAquick PCR purification kit according to the manufacturer’s protocol (QIAGEN, Hilden, Germany). PCR products were spotted onto positively charged Nytran membranes (7.9 by 12 cm2; Schleicher & Schuell) with a G3 arrayer (Genomic Solutions, Huntingdon, United Kingdom), with which each PCR product was separately spotted three times on each membrane. Postprinting procedures for the membranes included denaturation (10 min, 0.5 M NaOH–1.5 M NaCl), neutralization (10 min, 1.5 M NaCl–0.2 M Tris [pH 7.4]; 5 min, 2 ⋅ χ SSC [1 ⋅ SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–0.1% sodium dodecyl sulfate [SDS]), and cross-linking with UV light (Stratalinker). Membranes were dried and stored at room temperature before hybridization.

Membrane array target preparation. For membrane array hybridization, 32P-labeled targets were produced with an Omniscript RT kit (QIAGEN) following the manufacturer’s recommendations. Poly(A) RNA derived from 50 g of mycelium (after prehybridization) in 20 ml of hybridization solution (5% SSPE [1 ⋅ SSPE is 0.18 M NaCl, 10 mM Na2HPO4, and 1 mM EDTA, pH 7.7], 0.2% SDS, 5% Denhardt’s solution, 50% formamide), the appropriate labeled cDNA target was added and hybridization was carried out for 16 h at 50°C. Each membrane was washed for 5 min at 50°C in 20 ml of washing solution (5% SSPE, 0.2% SDS), dried, and exposed for 8 to 20 h to an imaging plate. Scanning was performed with a Fuji BAS1500 reader, and the analysis of pch files was done with AIDA software (Fuji). Grids were predefined and manually adjusted to ensure optimal spot recognition, and the resulting data files were further analyzed in Excel (Microsoft, Redmond, Wash.). For each sequence, the signal intensities of three spots were averaged and the mean values were normalized with respect to the gel signal intensity of the same array.

Protein purification. Western blotting, and quantification of protein levels. The fungal mycelium was ground in liquid nitrogen, and the following steps were performed with the mixture on ice. Resuspension of 1 g of mycelium in 3 ml of buffer A (0.1 M MOPS [morpholinopropansulfonic acid, pH 7.5], 0.2 mM KCl, 0.1 mM MgCl2, 1 mM EDTA, 10 mM dithiothreitol, 4.2 mM phenylmethylsulfonyl fluoride, 40% [wt/vol] glycerol) was followed by centrifugation. Protein levels in supernatants were determined by using Bradford reagent (Bio-Rad). Ten micrograms of each protein extract was separated in three independent polyacrylamide electrophoreses, and Coomassie blue-stained gels were used for the densitomet-
ric quantification of relative protein amounts as described previously (16). Western blotting and immunodetection of the pchB gene product were performed according to the method of Jekosch and Kück (17). Resulting signals were also densitometrically quantified, and IPNS amounts were calibrated using data derived from the Coomassie blue-stained gels. Relative amounts of IPNS for the different protein samples on single Western blots were calculated by using the IPNS level of strain A32/2 at day 2 as a standard (100 U).

Electrophoretic mobility shift assays and shift-Western blot analyses. The purification of protein from _Saccharomyces cerevisiae_, electrophoretic mobility shift assays, and shift-Western blot analyses were performed in principle as described previously (32). Oligonucleotides were radiolabeled with [$\alpha$-32P]dCTP. Binding reactions were conducted with 0.15 ng of a labeled DNA probe and protein crude extracts from _S. cerevisiae_ transformants synthesizing the CPR1 protein or control protein extract. For promoter scanning analysis, a series of 17 overlapping PCR fragments spanning the whole pchAB-pchC promoter region was used in gel retardation experiments. The promoter fragments were amplified with oligonucleotides given in Table 1 and radiolabeled by using a PCR mixture consisting of 50 ng of pSIM9.9 as a template, 40 ng of each primer, 4 μM dATP, 4 μM dTTP, 4 μM dGTP, 0.2 μM dCTP, 8.33 × 10^{-12} mol of [$\alpha$-32P]dCTP, and 1 U of Taq polymerase (Roche Molecular Diagnostics) in a volume of 50 μL. The samples were electrophoresed in a 5% polyacrylamide gel with 0.5 × Tris-borate-EDTA buffer to remove free nucleotides. The labeled PCR product was extracted from a single band with elution buffer (0.5 M ammonium acetate, 1 mM EDTA, 0.1% SDS) and was precipitated with ethanol. The precipitate was finally reconstituted in 100 μL of H$_2$O.

To examine the binding of CPR1 to the various promoter fragments, 1 to 3 μL of radiolabeled PCR fragments was incubated with 30 μg of crude protein extracts from _S. cerevisiae_ transformants synthesizing the CPR1 protein or with a control protein extract as well as 1 μL of binding buffer (32) and 0.8 μg of poly(dI-dC)⋅ poly(dI-dC) in a total volume of 20 μL.

Synthesis of a recombinant CPR1 polypeptide in _E. coli_. A His$_6$ fusion derivative of CPR1 was synthesized in _E. coli_ strain M15(pREP4) (QIAGEN). For this purpose, a cDNA fragment encoding amino acids 133 to 830 of CPCR1 (C$_{\text{C}}$) was cloned into expression vector pQE31 (QIAGEN) to generate plasmid pQEC2. For purification of the His$_6$ fusion protein, M15(pREP4) cells transformed with the plasmid pQEC2 were grown in Luria-Bertani medium containing 100 μg of ampicillin/ml and 25 μg of kanamycin/ml. A 200-ml culture was induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 2 mM, and the cells were harvested by centrifugation after 2 h of incubation at 30°C. The native purification of the His$_6$-CPR1 fusion protein by use of nickel nitritotriacetic acid resin was realized according to the supplier’s protocol (QIAGEN). Two micrograms of this purified recombinant protein was used in single binding assays.

Determination of levels of penicillin N and cephalosporin C. The quantification of penicillin N and cephalosporin C was done by high-performance liquid chromatography (HPLC) analysis of crude broth from 100-ml liquid shake flask cultures. HPLC (model HP 1090 chromatograph) was performed with a Keystone BetaMax C18 acid column (5-μm inside diameter, 150 by 3 mm) by gradient elution and simultaneous wavelength detection at 210 nm (penicillin N) and 260 nm (cephalosporin C). The analysis was performed with mobile phase A (0.156 [wt/vol] NaH$_2$PO$_4$⋅2H$_2$O) and a gradient of 10 to 35% phase B (phase A-acetonitrile, 4:1 [vol/vol]) with a flow rate of 0.7 mL/min.

RESULTS

Determination of novel CPR1 binding sites in promoters of cephalosporin C biosynthesis genes. The CPR1 protein was identified through its ability to bind an imperfect palindrome sequence in the pchAB-pchC promoter region in _A. chrysogenum_ (32). The location of binding site BSII approximately 350 bp upstream of the transcriptional start site of the pchC gene suggests that the transcription factor may be involved in the regulation of this cephalosporin C biosynthetic gene. In order to analyze the function of the CPR1 protein in more detail, additional potential CPR1 binding sites were identified within the 1.2-kb intergenic pchAB-pchC promoter region (22).

A series of 17 overlapping PCR fragments spanning the whole intergenic promoter region were used in the gel retardation experiments (Fig. 1A). All amplicons were generated with oligonucleotides listed in Table 1. The binding reactions were conducted with a protein extract from an _S. cerevisiae_ transformant synthesizing a recombinant fusion protein, which consists of the GAL4 activation domain and amino acids 40 to 830 of the CPR1 transcription factor (32). For PCR fragments 7, 12, and 15, interactions with different affinities were observed. Figure 1B shows DNA-binding complexes between the CPR1 fusion protein produced in _S. cerevisiae_ (C$_\gamma$), an _E. coli_-synthesized CPR1 protein fragment (C$_\alpha$), and promoter fragments 7 and 15. The known binding site BSII is located in fragment 12, and oligonucleotides with the BSII sequence served as positive controls in the gel retardation assay. Protein purified from an _E. coli_ transformant not synthesizing the CPR1-His fusion protein was used to control binding specificity (E). As can be concluded from Fig. 1B, the interaction of CPR1 with fragment 15 seems to be weaker than that with the putative new binding site in fragment 7. Therefore, we decided to map in detail the binding sites in PCR fragment 7.

Putative binding sites for the CPR1 protein within promoter fragment 7 were identified with a FASTA program-based search (24) for sequences with similarity to the binding sites of the human RFX transcription factor RXF1 (28), the _S. cerevisiae_ RFX protein Crt1 (13), and CPR1 (32). One sequence that shares 11 out of 13 nucleotide positions with a strong Crt1 binding site was found (RNR2-Xs) (Table 2) and is located approximately 700 bp upstream of the transcriptional start of the pchB gene. This sequence was named BSIII, and corresponding oligonucleotides were used for binding reactions. Figure 1C shows the results of a gel retardation analysis with radiolabeled oligonucleotides corresponding to BSII and BSIII and recombinant CPR1 purified from _S. cerevisiae_ (C$_\gamma$) or a control expressing GAL4AD alone (Y). Specific DNA-protein complexes are indicated. Figure 1D supports the assumption of specific binding of the protein to the newly determined binding site BSIII. Increasing amounts of unlabeled oligonucleotide BSII interfere with the interaction between recombinant CPR1 and the oligonucleotide. This finding shows that both sequences are high-affinity binding sites for CPR1 and compete for binding, at least in vitro. To further substantiate these results, shift-Western blot experiments were conducted in order to confirm the presence of CPR1 in the retarded DNA-protein complex. As shown in Fig. 1E, the protein could be identified unambiguously by using an antibody against the GAL4 activation domain.

Table 2 lists the CPR1 binding sites and compares them with binding sites recognized by human and _S. cerevisiae_ RFX transcription factors. The binding sites of the human RXF1 and _S. cerevisiae_ Crt1 proteins contain in the right-hand half of the site the highly conserved sequence 5’GAACCT3’ (13, 28). In addition, the consensus sequence for RXF1 binding determined by Emery et al. (6) revealed three conserved positions in the left-hand half of the site. An unexpected high degree of identity exists between the binding sites of the human RXF1 and _S. cerevisiae_ Crt1 proteins, although their DNA-binding domains show only 62% similarity (6). Surprisingly, the two binding sites BSII and BSIII of the CPR1 protein contain only one nucleotide that is the same in the right-hand half of each site. The left-hand parts of both binding sites seem to be
FIG. 1. Gel retardation analysis to determine new CPCR1 binding sites. (A) Schematic representation of the intergenic promoter region of the divergently orientated cephalosporin C biosynthesis genes pcbAB and pcbC in A. chrysogenum. The transcriptional start point (TS) of the pcbC gene is indicated by a bent arrow (36). The locations of the CPCR1 binding sites are shown in black. PCR fragments 1 to 17 were used in the promoter scanning analysis. (B) Gel retardation analysis of the CPCR1 protein together with PCR fragments 7 and 15. The recombinant GAL4AD-CPCR1 fusion protein purified from S. cerevisiae (C), 30 μg, the recombinant HIS-CPCR1 protein purified from E. coli (C), 2 μg, and an E. coli control protein (E, 2 μg) were incubated with the radiolabeled PCR fragments. Oligonucleotide BSIII was used as a positive control. (C) Gel retardation analysis with recombinant CPCR1 protein from S. cerevisiae and two binding sites from the pcbAB-pcbC promoter region. Oligonucleotides were radiolabeled and incubated with 20 μg of protein extract with the recombinant GAL4AD-CPCR1 fusion protein (C) or a control expressing GAL4AD alone (Y). (D) Competition analysis of the interaction of CPCR1 with BSIII and with oligonucleotide BSII as an unlabeled competitor. (E) Detection of specific DNA-protein complexes by shift-Western blot analysis with putative binding sites from the pcbAB-pcbC promoter region. “DNA” indicates the autoradiograph of the polyvinylidene difluoride membrane showing the free oligonucleotide and different DNA-protein complexes. “GAL4” designates the corresponding immunoblot of the nitrocellulose membrane with antibody against the activation domain of the GAL4 protein, which is part of the recombinant CPCR1 protein. Arrowheads in panels B to E point to specific DNA-protein complexes.
more conserved and match the consensus sequence for RFX1 reasonably well.

**Generation of fungal transformants for the functional analysis of CPCRI.** To test the regulatory function of CPCRI in β-lactam biosynthesis gene expression, we pursued two different approaches. One was to construct strains with multiple copies of the cpcRI gene, and the other was to generate strains with a gene disruption.

For the first approach, cotransformation experiments using plasmid pMW1 together with plasmid pKSC1 carrying the cpcRI locus were performed. Transformants were analyzed for the integration of plasmid DNA using Southern hybridization (Fig. 2). Cotransformants with multiple copies of the cpcRI gene were selected for further analysis. Transformant T11mc carries at least 5 to 10 copies of plasmid pKSC1, integrated ectopically into different genomic loci. For the second approach, plasmid pKOC1 was constructed in order to disrupt the cpcRI gene. An hph cassette was integrated into the unique AatII restriction site of the cpcRI gene, disrupting the open reading frame 1,112 bp downstream of the ATG (Fig. 2A). Even if a putative truncated protein encompassing the N-terminal 335 amino acids or the C terminus of CPCRI could be generated from the disrupted cpcRI gene, it would be functionally inactive as a transcription factor. Only a full-length CPCRI protein can dimerize and bind DNA, as was shown in one- and two-hybrid analyses of C-terminal deletions of CPCRI (32).

**Transcript analysis of transformants.** The effect of a disrupted or multicopy cpcRI gene on gene expression in A. chrysoogenum was investigated at the transcription level. Total RNA was isolated from recipient strain A3/2, knockout strain T572Δ, transformant TΔ+58 with the complemented cpcRI gene, and multicopy transformant T11mc over a period of 24 to 96 h. The mRNA isolated from 50 μg of total RNA of each sample was analyzed by using Northern hybridization. Figure 3A shows autoradiographs obtained from a Northern blot hybridized with different probes.

The probe corresponding to the open reading frame of the cpcRI gene revealed the presence of multiple transcripts of the cpcRI gene in some of the samples. The full-length cpcRI transcript of about 3.5 kb was detected in the RNA of all A3/2 samples. The strongest signal was obtained for the RNA from the sample taken at 48 h.

In transformant T11mc, the full-length transcript was detected in all samples. The transcript level for transformant T11mc was not significantly increased in comparison to that for strain A3/2. In addition to the transcript with the expected size of 3.5 kb, two other bands with lower molecular weights were detected after 36 h.

**Knockout strain T572Δ displayed a different pattern of hybridizing bands.** A transcript of about 3.5 kb was detected in the RNA of all A3/2 samples. The strongest signal was obtained for the RNA from the sample taken at 48 h.

In transformant T11mc, the full-length transcript was detected in all samples. The transcript level for transformant T11mc was not significantly increased in comparison to that for strain A3/2. In addition to the transcript with the expected size of 3.5 kb, two other bands with lower molecular weights were detected after 36 h.

Knockout strain T572Δ displayed a different pattern of hybridizing bands. A transcript of the same size as the wild-type cpcRI gene could not be detected. The signal with a size greater than 3.5 kb resulted from the integration of the 1.8-kb hph cassette into the cpcRI locus. This transcript cannot be translated into a wild-type CPCRI protein. As with the samples of transformant T11mc, additional bands were present in the RNA from knockout strain T572Δ after 36 h of cultivation. To characterize the transcript patterns of the knockout strain

| Protein | Binding site or sequence | Nucleotide sequence* | Degree of recognition | Reference |
|---------|--------------------------|----------------------|----------------------|-----------|
| RFX1    | X box                    | T T C C C C T A G C A A C | Binding              | 28        |
|         | HBV                      | T T G C C C G G G A A C | Binding              | 28        |
| Crt1    | RNR2-Xs                  | T C G C C A T G C A A C | Binding              | 13        |
|         | RNR2-Xw                  | T C T C T G T G C A A C | Weak binding         | 13        |
| RFX1    | Consensus                | T N R C C N N R G Y A A C | Binding              | 6         |
| CPCRI   | BSII                     | T T G C C G G G C A A T | Binding              | 32        |
|         | BSIII                    | T C G C C A T G A T A C | Binding              | TW5       |
|         | BSIIIm1                  | T T G C C G G G g t t a | No binding           | 32        |
|         | BSIIIm2                  | T T G C C G G G g t A T | Weak binding         | 32        |
|         | BSIIIm3                  | a a c C C G G G C A A T | No binding           | 32        |

* Boldface indicates conserved position, and lowercase letters indicate mutated positions.

b-TW, this work.

HBV, hepatitis B virus.

**Table 2. Comparison of RFX-binding sites**

Degree of recognition

| Protein | Binding site or sequence | Nucleotide sequence* | Degree of recognition | Reference |
|---------|--------------------------|----------------------|----------------------|-----------|
| RFX1    | X box                    | T T C C C C T A G C A A C | Binding              | 28        |
|         | HBV                      | T T G C C C G G G A A C | Binding              | 28        |
| Crt1    | RNR2-Xs                  | T C G C C A T G C A A C | Binding              | 13        |
|         | RNR2-Xw                  | T C T C T G T G C A A C | Weak binding         | 13        |
| RFX1    | Consensus                | T N R C C N N R G Y A A C | Binding              | 6         |
| CPCRI   | BSII                     | T T G C C G G G C A A T | Binding              | 32        |
|         | BSIII                    | T C G C C A T G A T A C | Binding              | TW5       |
|         | BSIIIm1                  | T T G C C G G G g t t a | No binding           | 32        |
|         | BSIIIm2                  | T T G C C G G G g t A T | Weak binding         | 32        |
|         | BSIIIm3                  | a a c C C G G G C A A T | No binding           | 32        |
FIG. 2. Disruption of the \textit{cpeR1} gene in \textit{A. chrysogenum}. (A) Schematic representation of homologous integration at the \textit{cpeR1} locus. In plasmid pKOC1, the \textit{cpeR1} gene is disrupted by an \textit{hph} cassette. Double crossover at the flanking homologous DNA regions with the genomic DNA results in a disrupted \textit{cpeR1} gene. 

\begin{itemize}
  \item E, \textit{EcoRI};
  \item Sac, \textit{SacI};
  \item P, \textit{PstI};
  \item B, \textit{BamHI};
  \item St, \textit{StuI};
  \item Sal, \textit{SalI};
  \item Xh, \textit{XhoI};
  \item N, \textit{NcoI};
  \item A, \textit{AatII}.
\end{itemize}

(B) PCR analysis using genomic DNA of fungal transformants. PCR A resulted in a 1.3-kb amplicon from the \textit{cpeR1} gene in recipient strain A3/2 and transformants with ectopic copies of pKOC1 (T). Primers for PCR B are located in the \textit{hph} cassette and upstream of the \textit{cpeR1} gene; the 2-kb amplicon can be generated only after the homologous integration of pKOC1 into genomic DNA. (C) Southern analysis of multicopy strains (left), knockout strains (middle), and derivatives of knockout strain T572Δ (right), which was transformed with a full-length copy of the \textit{cpeR1} gene. All genomic DNA from recipient and transformed strains was restricted as indicated and hybridized with a fragment containing the \textit{cpeR1} gene. The arrowheads mark fragments which carry the nondisrupted \textit{cpeR1} gene.
were calculated with the gpd change in of this assay are shown in Fig. 3C and D. The most dramatic (were quantified and used to hybridize the membranes. Resulting signals were highly increased. bands corresponding to transcripts of low molecular weight hybrized with a probe which represents the 3/H11032 and the multicopy strain T11mc more precisely, mRNA was

Transformant TΔ+58 has one ectopically integrated cpeR1 gene, and consequently the full-length cpeR1 transcript could be observed at all time points. In addition, the intensities of bands corresponding to transcripts of low molecular weight were highly increased.

(Fig. 3B). As can be seen in Fig. 3B, the multicopy strain shows a full-size transcript for the cpeR1 gene, while a transcript larger than 3.5 kb is detectable in the knockout strain. From these data it can be concluded that multiple truncated transcripts comprising the 5' part of the cpeR1 gene are generated in addition to the full-size mRNA in the transgenic strains.

To allow more precise quantification of transcript levels, a macroarray assay was applied. Small amounts of PCR fragments (1,000 to 1,500 bp) corresponding to the pcbC, beta-tubulin, and gpd genes were spotted onto a nylon membrane. At five time points between 24 and 96 h of cultivation, mRNA was prepared from strain A3/2 and transformants T11mc, T572Δ, and TΔ+58. cDNA was synthesized with 32P-nucleotides and used to hybridize the membranes. Resulting signals were quantified, and transcript levels for pcbC and beta-tubulin were calculated with the gpd signal as the standard. The results of this assay are shown in Fig. 2C and D. The most dramatic change in pcbC transcript levels was observed during 24 and 48 h of cultivation. Strain A3/2 had a maximum after 36 h (~180 U) and showed relatively constant levels from 48 to 96 h (± 130 U). Knockout strain T572Δ had two to three times more pcbC transcript at 24 and 36 h (285 to 300 U), but its transcript level dropped to below that of strain A3/2 at 48 h (115 U). From 48 to 96 h, the knockout strain showed the lowest pcbC transcript levels of all of the investigated strains. The strain with the complemented cpeR1 gene (TΔ+58) showed a transcript level time course similar to that of A3/2 (e.g., a maximum at 36 h). However, slightly higher levels can be seen at all time points. At 24 h, the pcbC transcript level in the strain with the complemented cpeR1 gene is more similar to that of A3/2 than to that of the knockout strain. The pcbC transcript level for multicopy transformant T11mc differs from that for A3/2 only during the first 36 h. At 24 h, it shows nearly twice the amount of pcbC transcript, and then its level drops at 36 h. As a control, the time course of another transcript was monitored in all strains. Figure 3D shows the transcript levels of the beta-tubulin gene relative to those of the gpd gene. The deviations between the strains are only marginal, indicating that the observed differences in pcbC transcript levels do not echo general changes in transcription rates.

Quantitative analysis of IPNS levels. The different effects on protein levels in the fungal transformants were studied by measuring the relative amounts of IPNS. IPNS is encoded by the pcbC gene and catalyzes the formation of isopenicillin N, the first active antimicrobial compound of the pathway. Figure 4A illustrates the relative amounts of IPNS after 24 to 96 h of cultivation in recipient strain A3/2, knockout strain T572Δ, and multicopy transformant T11mc. The amount of IPNS was determined by Western blotting with antibodies against the enzyme and protein crude extracts obtained from fungal mycelia. Relative amounts were determined as averages of amounts from 10 individual blots. The blot signals were calibrated by using data from parallel loaded and run Coomassie blue-stained gels. The largest amount of IPNS in strain A3/2 was used as a standard. Figure 4B gives an example of a Western
blot in which we used an antibody against IPNS with proteins from strain A3/2, knockout strain T572Δ, and multicopy transformant T11mc.

The protein IPNS levels were highest at 48 h (100%) in strain A3/2 and decreased thereafter to about 60%. The IPNS level in the knockout strain was only about 50% of that in strain A3/2 after 24 h. However, IPNS accumulated over time to higher levels. T11mc is a transformant with additional copies
of the cpcR1 gene and had increased levels of IPNS compared to those of strain A3/2 after 24 h of cultivation. Again, this effect did not last. After 48 h, the level of IPNS in T11mc was reduced to a value comparable to that for strain A3/2. These data have been confirmed by other knockout and multicopy transformants, which were generated independently. Due to the comparable levels of IPNS after 48 h in all strains, we abstained from analyzing the retransformant of the knockout strain.

Analysis of reporter gene activity from the pcbC promoter and internal deletion derivatives. To determine the in vivo significance of the two CPCR1 binding sites for pcbC gene expression, reporter gene fusions were constructed. pcbC promoter derivatives with nucleotide deletions in binding site BSII, BSIII, or both were fused to the lacZ gene. A. chrysogenum transformants harboring these constructs were analyzed for β-galactosidase activity. Fungal mycelia used for the reporter gene studies were grown for 7 days on CCM solid medium. The latest time point was chosen to determine the long-term effects of the internal promoter deletions on pcbC transcriptional activity. For comparison, the wild-type promoter sequence was used in parallel.

The integration of the plasmids into the fungal genomic DNA was tested by Southern hybridization. Each transformant showed an individual pattern of ectopically integrated plasmid DNA, indicating that no common area of integration exists. An average of three to four plasmid copies was determined (data...
not shown). Despite carrying identical reporter gene constructs, independent transformants showed various β-galactosidase activities owing to the ectopic integration pattern and varied copy numbers of the plasmids in the genomic DNA (22). Therefore, the β-galactosidase activities of 10 independent transformants of each construct were repeatedly measured in order to calculate a statistically significant average value and standard deviation. The levels of these activities are shown in Fig. 5. These data allow the conclusion that binding site BSII, which is closer to the pcbC gene, is important for the transcription of the gene. Its deletion reduces the reporter gene activity to 20% of that of the control (pSIM9.9). The effect of binding site BSIII, which is located approximately 700 bp upstream of the transcription start point, seems to grow weaker as the reporter gene activity decreases to only approximately 70%. The deletion of both sites results in a decrease in reporter gene activity to 12%.

**Cephalosporin C production in fungal transformants of strain A3/2.** Finally, we investigated modifications in the biosynthesis of cephalosporin C. The amounts of penicillin N and cephalosporin C were determined by HPLC analysis of the culture broth. Samples were obtained from batch cultures of the intermediate. The amounts of penicillin N even decreases from 36 to 26 mg/liter from 48 to 96 h. This decrease results in accumulation of less than 20% of the penicillin N produced by strain A3/2 at 96 h.

**FIG. 5.** Effect of a promoter mutation on pcbC-lacZ expression. Shown are a schematic representation of reporter gene plasmids and the results of quantitative analyses of resulting reporter gene expression in *A. chrysogenum* transformants. β-Galactosidase activity was measured fluorometrically in protein extracts of fungal transformants harboring the reporter gene plasmid pSIM9.9 or its derivatives. Results correspond to at least four independent measurements from 10 transformants for each plasmid. Mycelia of transformants were harvested from solid cultures after 7 days. Standard deviations indicate the deviations between single transformants. TS indicates the transcription start site for the pcbC gene. The presence of binding site BSII or BSIII is indicated in black.

**DISCUSSION**

A *cpcR1* knockout has significant effects on the biosynthesis of cephalosporin. The *pcbC* transcript level was greatly increased at 24 and 36 h in the knockout strain and slightly reduced after 48 h, whereas the amount of intermediate penicillin N decreased between 48 and 96 h. These effects were reversed in a significant manner in the strain carrying the complemented *cpcR1* gene. This finding shows that the transcription factor is indeed responsible for the observed changes. The importance of CPCR1 for regulated *pcbC* transcription is substantiated by a decrease in reporter gene activity in *A. chrysogenum* transformants harboring *pcbC* promoter derivatives with binding sites BSII and BSIII deleted.

Nevertheless, the effects of transcript and protein levels did not have a great impact on the production of cephalosporin C. Levels of the biosynthesis of intermediate penicillin N were reduced to 20% of the usual level in the knockout strain, but cephalosporin production as such did not change significantly. This finding is consistent with the fact that IPNS activity is not the rate-limiting step in the biosynthesis (8, 34). One possible explanation for the observed decrease in penicillin N levels is that in the knockout strain, less IPNS is present. However, the enzyme level may be sufficient to feed the subsequent biosynthesis steps. The two other intermediates occurring in the following steps of cephalosporin biosynthesis, desacetoxycephalosporin and deacetycephalosporin, never accumulate in amounts similar to those of penicillin N or cephalosporin C. Their levels are low (approximately 2 mg/liter) and very similar to those in all other strains (data not shown). If the assumption of reduced IPNS activity in the knockout strain holds true, it is likely that CPCR1 is not involved in the regulation of the late genes of the biosynthesis. Otherwise, a knockout would have a more severe effect on cephalosporin production.

CPCR1 is probably part of a complex regulatory network acting on cephalosporin C biosynthesis gene expression. The CPCR1 knockout yields a short-term increase and a long-term decrease in *pcbC* transcript levels. Consequently, it is difficult to assign an activation or repression role to the CPCR1 transcription factor. It is obvious that the transcription of the *pcbC* gene can be initiated by other transcription factors. The proteins might work synergistically with CPCR1 so that some degree of functional redundancy is reached.

This combinatorial control of β-lactam gene expression by several transcription factors is not surprising, as many internal and external parameters influence the biosynthesis of cephalosporin C. Primary candidates from *Acremonium* are the glucose repressor CRE1 and the PACC protein that mediates transcriptional activation in response to ambient alkaline pH (16, 33). A comparable situation has been described for the regulation of penicillin biosynthesis in *Aspergillus nidulans* and *P. chrysogenum*, in which a number of transcription factors contribute to coordinated gene expression (for a review, see reference 21). The principal parameters influencing β-lactam gene expression are alkaline pH, glucose, and ammonium, but their effects on the transcription rates of biosynthesis genes are not equal in the different producers. We are far from understanding how the interaction of transcription factors contributes to differences in regulation and production levels of the antibiotics.
In contrast to the complex regulation of β-lactam biosynthesis, the production of aflatoxins in aspergilli depends mainly on a single transcription factor, the strong transcriptional activator AfIR (for a review, see reference 42). Similarly, the zinc finger transcription factor TRI6 from *Fusarium sporotrichioides* is a pathway-specific regulator of the biosynthesis of the secondary metabolite trichothecene (10). A pathway-specific regulation role is also suggested for MLCR, a Cys6 zinc finger protein that is thought to activate the biosynthesis of ML-236B (compactin) in *Penicillium citrinum* (1). Another example of a probable pathway-specific regulator is the TOXE transcription factor, which is essential for the production of HC toxin, a cyclic tetrapeptide, in plant-pathogenic strains of *Cochliobolus carbonum* (2). TOXE is the prototype of a new family of transcription factors and is characterized by a DNA-binding domain with a basic region and four ankyrin repeats (25).

**RFX proteins can be activators and repressors.** The CPCR1 protein binds at least two sequences in the 1.2-kb intergenic region between the divergently orientated biosynthetic genes *pcbAB* and *pcbC*. Using reporter gene fusions, we were able to show that both binding sites contribute to the transcriptional activation of the *pcbC* gene. These binding sites are located approximately 350 and 700 bp upstream of the transcriptional start site of the gene. Only a few data on the analysis of promoters from target genes for RFX transcription factors are presently available. The *S. cerevisiae* Crl1 protein, a member of the RFX transcription factor family, binds to promoters of the damage-inducible RNR2, -3, and -4 genes. Each promoter

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**FIG. 6.** Determination of cephalosporin production in strain A3/2 and transformants T11mc, T572Δ, and TΔ+58. Shown are the results of quantitative HPLC analyses of culture broth for the biosynthesis of intermediate penicillin N and the final product, cephalosporin C, after 24, 48, 72, and 96 h of cultivation in shaking flasks.
contains two or three binding sites for Crt1. However, both the orientation of the binding site and the distance from the transcriptional start site vary. Only the promoter of the RN4 gene shows a situation comparable to that for the pcbC promoter from A. chrysogenum. Its Crt1 binding sites are located at positions −400 and −760 (13). The human interleukin-5 receptor α gene is the first cellular target identified as being regulated by RFX1, RFX2, and RFX3 via a cis element at position −415 (14). In contrast, functional binding sites for the Caenorhabditis elegans DAF-19 protein were recognized in five different gene promoters between positions −79 and −130 (37).

The fivefold reduction in reporter gene activity upon the deletion of the single binding site BSI in the pcbC promoter indicates the importance of the CPC1 protein for pcbC gene activation on a long-term basis. Similarly, human and C. elegans RFX proteins were found to activate gene expression upon binding to the promoter sequences (14, 37). In contrast, the S. cerevisiae RFX factor recruits the general repressor complex Tup1-SS6 to promoters of the RN4 genes, and subsequently, mutations in binding sites for Crt1 can activate transcription up to 17-fold (13).

In contrast to the long-term decrease in transcriptional activity indicated by the reporter gene experiments, the quantification of pcbC transcript levels revealed a more complex situation. Strong increases in the pcbC transcript level in the knockout strain at early time points are succeeded by decreased levels starting at 48 h of cultivation. These results do not encourage the proposal for a simple role, like the activation or repression of pcbC transcription, for CPC1. The strong increase in transcript levels at the beginning of cultivation in a knockout background points to a repression function of CPC1, but the long-term decrease of transcript levels and reporter gene activity rather suggests an activation role for CPC1. Full-length CPC1 showed no significant transcriptional activity in an S. cerevisiae one-hybrid system (32). In this context, it is worth mentioning that for human RFX1, both a repressor and an activator function have been proposed. Katan et al. (18) suggest that RFX1 acts as a dual-function regulator via its activation and repression domains in a context-dependent manner. The conserved C terminus mediates the formation of two alternative homodimeric DNA-protein complexes, one of which has been linked to transcriptional repression in human RFX1 and SAK1 from the yeast Schizosaccharomyces pombe (19). Allosteric conformational changes and different levels of transcriptional activity can be induced by the multisite phosphorylation of transcription factors (for a review, see reference 12).

**Distribution of winged helix proteins in genomes of lower eukaryotes.** Winged helix proteins in fungi have so far been studied only in the unicellular yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe. In both organisms, one member of the RFX subclass of winged helix proteins and four winged helix proteins of the forhead type have been identified. While the Schizosaccharomyces pombe RFX factor SAK1 is involved in the exit from the mitotic cell cycle (43), the forhead proteins regulate hyphal growth and the cell cycle in both yeasts. Mutations in these genes interfere with septation, thus leading to a pseudohyphal morphology of the usually unicellular yeast cells (11, 29, 44).

A comparison of transcription factors encoded by the whole genomes from organisms of all three domains of eukaryotic life revealed a remarkable distribution of winged helix proteins. One RFX gene exists in each genome of the lower eukaryotes S. cerevisiae and C. elegans and of Drosophila, but none exists in the genome of the plant Arabidopsis thaliana. In addition, forhead proteins are represented by 4 (S. cerevisiae and S. pombe) to 18 (Drosophila) copies, and again, no member of this family was found in the Arabidopsis genome (30). A search for CPC1 homologues in the genomes of the yeast Candida albicans and the filamentous fungus Neurospora crassa also identified only one RFX gene (E. K. Schmitt, unpublished data). In contrast, gene duplication events evolved five RFX genes in humans and mice.

**ACKNOWLEDGMENTS**

We express our thanks to Kerstin Kalkreuter and Andrea Wimbart for their excellent technical assistance. Sequence data for C. albicans was obtained from the Stanford Genome Technology Center website at http://www-sequence.standford.edu/group/candida. Sequence data for N. crassa were obtained from the Neurospora crassa sequencing project at the Whitehead Institute/MIT Center for Genome Research (www-genome.wi.mit.edu).

Sequencing of Candida albicans was accomplished with the support of the NIDR and the Burroughs Wellcome Fund. This investigation received financial support from Biochemie GmbH, Austria.

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