The Fungal CPCR1 Protein, Which Binds Specifically to β-Lactam Biosynthesis Genes, Is Related to Human Regulatory Factor X Transcription Factors

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Here we report the isolation and characterization of a novel transcription factor from the cephalosporin C-producing fungus Acremonium chrysogenum. We have identified a protein binding site in the promoter of the β-lactam biosynthesis gene pbcC, located 418 nucleotides upstream of the translational start. Using the yeast one-hybrid system, we succeeded in isolating a cDNA clone encoding a polypeptide, which binds specifically to the pbcC promoter. The polypeptide shows significant sequence homology to human transcription factors of the regulatory factor X (RFX) family and was designated CPCR1. A high degree of CPCR1 binding specificity was observed in in vitro and in vivo experiments using mutated versions of the DNA binding site. The A. chrysogenum RFX protein CPCR1 recognizes an imperfect palindrome, which resembles binding sites of human RFX transcription factors. One- and two-hybrid experiments with truncated versions of CPCR1 showed that the protein forms a DNA binding homodimer. Nondenaturing electrophoresis revealed that the CPCR1 protein exists in vitro solely in a multimeric, probably dimeric, state. Finally, we isolated a homologue of the cpcR1 gene from the penicillin-producing fungus Penicillium chrysogenum and determined about 60% identical amino acid residues in the DNA binding domain of both fungal RFX proteins, which show an overall amino acid sequence identity of 29%.

The filamentous fungus Acremonium chrysogenum is used industrially to produce the β-lactam antibiotic cephalosporin C. The biosynthesis of cephalosporin C consists of six enzymatic steps, which are catalyzed by five different enzymes. Included among these steps are the condensation of l-cysteine and l-valine into a single tripeptide, formation of the bicyclic isopenicillin N, a ring expansion step, and the addition of an acetyl moiety (1, 2). The first two steps of the biosynthesis of cephalosporin C are catalyzed by five different enzymes. Included among these steps are the condensation of L-cysteine and L-valine into a single tripeptide, formation of the bicyclic isopenicillin N, a ring expansion step, and the addition of an acetyl moiety (1, 2). The first two steps of the biosynthesis of cephalosporin C are catalyzed by five different enzymes. Included among these steps are the condensation of L-cysteine and L-valine into a single tripeptide, formation of the bicyclic isopenicillin N, a ring expansion step, and the addition of an acetyl moiety (1, 2).

The production of cephalosporin and penicillin is influenced by a variety of parameters. These include carbon and nitrogen source, as well as ambient pH (1). The molecular analysis of penicillin production in A. nidulans and P. chrysogenum has revealed some of the genes involved in regulating penicillin biosynthesis. The most complete evidence for the transcriptional regulation of penicillin biosynthesis comes from studies with the pH regulatory transcription factor PACC. Reduced transcription activation in reporter genes located downstream of the A. nidulans pbcC promoter was achieved by mutating the PACC binding sites found in this promoter. Similarly, in vitro studies using the same promoter have identified six possible PACC binding sites in P. chrysogenum (3, 4). The NRE protein provides another example of a transcription factor regulating the expression of penicillin biosynthesis genes. This protein, which most probably mediates nitrogen repression and derepression, is able to bind to the intergenic pbAB/pcB promoter region of P. chrysogenum (5).

In contrast to A. nidulans and P. chrysogenum, no putative regulatory factors of β-lactam biosynthesis have been isolated from A. chrysogenum. However, the divergent promoter region between the pbAB/pbcC genes in A. chrysogenum contains putative binding sites for PACC and NRE homologues, suggesting the existence of similar regulatory circuits in this fungus. In addition there are three CCAAT-boxes dispersed in the 1.2-kilobase promoter region (6). Our investigations have already shown that in vitro protein binding abilities are associated with some of the putative motifs. In this investigation we provide evidence that a palindromic promoter sequence is specifically bound by a novel DNA-binding protein, which shows significant homology to human transcription factors of the RFX family. As far as we are aware, this is the first description of a putative transcription factor that recognizes promoter sequences of cephalosporin C biosynthesis genes in A. chrysogenum.

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1. R. Radicio, E. Schmitt, and U. Kück, unpublished observations.
2. The abbreviations used are: RFX, regulatory factor X; BSII, binding site II; bp, base pair(s); PCR, polymerase chain reaction; ATCC, American Type Culture Collection; 3-AT, 3-anilinotriazole; AD, activation domain; BD, DNA binding domain; EMSA, electrophoretic mobility shift assay; FPLC, fast protein liquid chromatography; PVDF, polyvinylidene difluoride; 5-methyl-dCTP, 5′-methyl-2′-deoxycytidine-5′-triphosphate.

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Cloning experiments were performed with Escherichia coli strain K12 XLI-Blue MRF (Stratagene), and phage transfection experiments were performed with E. coli strain K12 K803 (7). For our investigation we used A. chrysogenum strains ATCC 14553, A32, and A1c, which is a derivative of strain A32/8 (8). A library with genomic DNA of strain A1c in λ-phae EMBL4 (9) was screened for a DNA fragment containing the cpcR1 gene by plaque hybridization with the cpcR1 cDNA as a probe. Growth conditions for A. chrysogenum were as described previously (9). The PcrFX1 gene from P. chrysogenum was isolated from a cosmid genomic library of P. chrysogenum that was custom-prepared by Stratagene.4

Isolation of the P. chrysogenum PcrFX1 Gene

Using genomic DNA from P. chrysogenum as a template, PCR was performed with primers 1115 (5′-GCTAGTTTCCCAGAATTGTTGT and 1117 (5′-CAATAGTTGATCTTTTGGACTGCC) corresponding to positions 1374–1393 and 1462–1440 of the A. chrysogenum cpcR1 gene, respectively, and resulted in a 89-bp amplification product. The DNA sequence of the 89-bp fragment was designed to design two primers for inverse PCR. Using primers 1126 (5′-CGTACGTTTTTGGGAAAGATG) and 1127 (5′-CTCCCGCTCGTGGAGTCCG), inverse PCR was performed after hydrolysis of genomic DNA with TspQI and ligation. Cloned product of the inverse PCR (490 bp) was used as a probe to screen a genomic cosmid library from P. chrysogenum. Parts of the isolated cosmid clone Pcl1 were subcloned and sequenced. The determined sequence (3916 bp) contains an open reading frame for 855 amino acids, encoding the PcrFX1 gene from P. chrysogenum.

Construction of an Activation Domain-tagged cDNA Library from A. chrysogenum by Directional Cloning

Total RNA was prepared by phenol extraction (10) from liquid cultures of A. chrysogenum strain A1c. Cultivation was undertaken over a period of 2.5 days, and poly(A) + RNA was isolated using the PolyATract mRNA isolation system (Promega). cDNA was synthesized from 8 μg of poly(A) + RNA with an oligo(dT) primer containing a BamHI recognition sequence and incorporating 5′-methyl-dCTP (11). After blunt-end treatment of cDNA, EcoRI adapters were ligated (11), and the product was digested with BamHI. After size selection using a Sizemore™ 400 spotted column (Amersham Pharmacia Biotech), the cDNA was ligated with 500 ng of pGAD424 (CLONTECH) digested with EcoRI/BamHI. E. coli transformants were obtained by electroporation using competent XL1-Blue MRF cells (Stratagene). Plasmid DNA was prepared from 4 × 106 transformants.

Construction of Reporter Plasmids and Yeast Strains for One-hybrid Screening

Complementary oligonucleotides with three copies of the BSII sequence (5′-GTTTGGCCTGCTGCTGCTGG) corresponding to positions 794–917 of the pchAB promoter (9), were cloned into plasmids pHisI and pLacZi, which had been digested with EcoRI and Smal. The resulting plasmids were designated pHisI-BSII and pLacZi-BSII. A reporter strain HISLACZ-BSII for the transformation of the cDNA library was constructed by integration of both plasmids into yeast strain YM4271. Plasmids and yeast strains were obtained from Stratagene.

Preparation of Yeast and A. chrysogenum Protein Extracts

Yeast One-hybrid and Two-hybrid Reporter Assays

To determine DNA-protein interactions of CPC1 with different binding sites (BSII, BSIII-1, BSIII-2, BSIII-3) and protein interactions between CPC1 derivatives, one- and two-hybrid reporter gene assays were performed. For the one-hybrid experiments, yeast strain YM4271, carrying integrated reporter plasmids, was transformed with derivatives of plasmid pGAD424 containing cDNA inserts and tested for growth on his' - selective media (55 mM 3-AT). To measure the β-galactosidase activity in the transformants, yeast cells were homogenized with glass beads, and cell extract was analyzed for β-galactosidase activity using the filter lift assay according to the manufacturer’s instructions (MATCHMAKER ONE-HYBRID system kit, CLONTECH). Plasmids were then transformed into yeast strain HISLACZ-BSII, and transformants were tested for growth in the presence of 55 mM 3-AT. This step was followed by transformation of plasmids conferring histidine prototrophy into a yeast reporter strain carrying the mutated BSII site upstream of the HIS3 reporter gene. If the mutation abolished protein binding in the yeast cell, transformants were unable to grow on selective media. This procedure was used to obtain transformants that carry cDNAs for DNA-binding proteins able to interact specifically with BSII sequence.

Electrophoretic Mobility Shift Assay (EMSA)

Anealed oligonucleotides BSII were used as probes. The labeling was achieved by filling in one 3′ end with [γ-32P]dATP using Klenow polymerase. The labeled DNA was purified by gel filtration. The binding reaction consisted of 3 ng of DNA, 4–6 μg of protein, 0.5 μg of poly(dI-dC)·poly(dI-dC). 1 μl of binding buffer (1 mM NaCl, 200 mM Tris, 50 mM MgCl₂, 50% glycerol, 1% Nonidet P-40, pH 7.5) in a volume of 20 μl. For the competition experiments, nonlabeled oligonucleotides were added to a 10- to 100-fold excess. After a 20-min incubation at 24 °C, the samples were electrophoresed in a 5% polyacrylamide gel at 4 °C with Tris-glycine buffer (0.027 M Tris, 0.19 M glycine, pH 8.5).

3 M. Wizl and U. Kück, unpublished results.
4 E. Friedlin and H. Künsteiner, personal communication.
Fungal RFX Protein, Binding to β-Lactam Biosynthesis Genes

Shift-Western Blotting—Electrophoretic mobility shift assays were performed as described above. Thirty to 70 μg of cell extracts from yeast transformants were incubated with the radiolabeled DNA. After electrophoresis, the DNA-protein complexes were transferred onto nitrocellulose (Schleicher & Schuell) and PVDF (Millipore) membranes by the semidyblotting method (14). The DNA, bound to the PVDF membrane, was visualized by autoradiography, whereas the proteins were detected on the nitrocellulose membrane by immunoblotting using a chemiluminescence kit (Roche Molecular Biochemicals). The primary antibody anti-GAL4TA (Santa Cruz Biotechnology) was applied in a concentration of 0.4 μg/μL.

DNase I Footprinting Experiments—To obtain a probe, the 0.28-kilobase BgII DNA fragment (positions 724–1008) from the pcbAB/pcbC promoter was excised from the plasmid pIPNSB1 (6) and subcloned in pBluescript II KS+ (Stratagene). The resulting plasmid pPCBC5 was cleaved with either Hinfl (top strand) or PstI (for the bottom strand) and labeled with [α-32P]deoxynucleotides triphosphates using Klenow polymerase. To obtain one-end-labeled fragments, plasmid DNA was then digested with either XhoI (top strand) or HindIII (bottom strand). DNA fragments were separated by electrophoresis in a 3.75% polyacrylamide gel. DNA fragments were then eluted by incubating gel slices for 1.5 h at 37 °C in an elution buffer (0.2 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA). The eluted DNA fragments were purified by reversed-phase column chromatography using Elutip-d (Schleicher & Schuell). DNase I footprinting reactions were performed with a Core footprinting system (Promega). End-labeled DNA (10–50 ng) was mixed with either 26 μg of cell extract or 9 μg of FPLC-purified protein, 2.5 μl of binding buffer (see above) in a volume of 50 μl and incubated for 20 min on ice. Reactions were then treated with 0.05–0.4 units of DNase I for 1 min at 24 °C. The cleavage products were separated in a 10% polyacrylamide sequencing gel at 50 °C.

Native Discontinuous Electrophoresis and Ferguson Plot—Cell extracts from yeast transformants and molecular weight standards (Amersham Pharmacia Biotech) were separated in five gels with different acrylamide concentrations (5, 6.25, 7.5, 8.75, 10%) using a BIORAD Protean II xi cell. The recombinant AD-CPCR1 protein was detected by Western blotting, and the results were used to construct a molecular weight standard curve (11). Usually 120 to 150 ng of cell extracts from yeast transformants were loaded. The immunoblot on nitrocellulose membrane and the application of the primary and secondary antibodies were performed as described above.

RESULTS

Identification of DNA-Protein Complexes Binding Specifically to the pcbAB/pcbC Promoter

To identify DNA-binding proteins from A. chrysogenum, we performed in vitro studies with putative protein binding motifs derived from the divergent pcbAB/pcbC promoter region. A set of oligonucleotides was incubated with A. chrysogenum FPLC-purified protein in EMSA reactions to identify promoter sequences showing protein binding properties. Among others, the 24-bp oligonucleotide BSII (details of which are given under “Materials and Methods”) was selected as a putative binding motif. The BSII sequence, −441 to −418 relative to the translation start site of the pcbC gene, contains a CCAAT-box and an imperfect palindromic EMSA competition experiments with BSII and mutated binding sites revealed a weak but specific DNA-protein interaction (data not shown). In these assays, probes were used that consisted of complementary annealed oligonucleotides with either three copies of the 24-bp BSII motif or three copies of a mutated version of the BSII motif. Our data from the binding studies led to the decision to use the BSII sequence in a one-hybrid screen to isolate transcription factor cDNAs.

Yeast One-hybrid Cloning of cDNAs for Polypeptides That Bind BSII

We used the yeast one-hybrid system for the isolation of a transcription factor interacting with the binding site BSII. This site was cloned into reporter gene plasmids pHISi and pLacZi, and the corresponding recombinant plasmids were integrated into the genome of yeast strain YM4271, generating reporter strain HISLACZ-BSII. This strain served as a host for the transformation of the A. chrysogenum cDNA library. The cDNA library was constructed in plasmid pGAD424 by directional cloning to facilitate the expression of hybrid proteins (with the GAL4 activation domain) in yeast. More than 5 × 10⁶ yeast transformants were plated on media lacking histidine. During a period of 5 to 21 days after DNA-mediated transformation, 2000 putative positives were isolated, re-grown, and assayed for β-galactosidase activity. This selection resulted in 10 positive yeast transformants, which were analyzed further. To verify the dependence of reporter gene activation on plasmid DNAs, plasmids were isolated and re-transformed into strain HISLACZ-BSII. Only two plasmids resulted in histidine prototrophic yeast transformants. To further confirm the specificity of the observed DNA-protein interaction, a strain with a mutated BSII in the sequence located upstream of the HIS3 gene was tested. The mutation in BSII was introduced by substituting a CCAAT for a GGTTA, thereby destroying the putative CCAAT-Box. The two plasmids failed to rescue the HIS+ phenotype of cells in the reporter strain HIS-BSII1, thus providing evidence for binding specificity. Our results indicate that the isolated cDNAs encode DNA-binding proteins with specificity for the BSII sequence from the pcbC promoter.

The identified cDNAs Encode a Novel Polypeptide Related to RFX Transcription Factors

Restriction and hybridization analyses revealed that the two plasmids described above contained similar cDNA inserts with identical restriction sites for different enzymes. The data indicate that the two selected cDNAs encode identical polypeptides from A. chrysogenum with binding specificity for BSII. Nucleotide sequence analysis of the cDNA insert in the corresponding plasmid (pGC1) revealed an open reading frame for 788 amino acids. As expected, the insert was in-frame with the GAL4 activation domain. Analysis of genomic DNA extended the open reading frame to 830 amino acids and revealed the positions of two short introns encompassing 52 and 53 nucleotides (see Fig. 1). The gene was named cpr1 (for cephalosporin C regulator 1). Southern analysis of genomic DNA isolated from the wild type A. chrysogenum strain ATCC 14553 as well as producer strain A3/2 with a cpr1 gene probe detected only one single hybridization band (data not shown).

The predicted CPCR1 protein sequence shows significant amino acid sequence homology with several polypeptides present in GenBank® databases. All these polypeptides belong to the family of RFX transcription factors, which are characterized by a typical DNA binding and dimerization domain (15). Among all the RFX transcription factors identified from different organisms, these two domains show the highest degree of sequence homology. Fig. 2 shows an amino acid sequence alignment of the DNA binding domains (75 to 77 amino acids) from selected RFX proteins. More than 50% of the amino acid residues from the DNA binding domains of the CPCR1 protein and the SAK1 protein from the fission yeast Schizosaccharomyces pombe are identical. In the dimerization domain, a homology of 31.8% was observed between the two proteins, and the overall homology between CPCR1 and SAK1 was still 28%. In comparison, the DNA binding domain of the human members of the RFX protein family, RFX1/2/3 and 5, each shared approximately 40% homology with the DNA binding domain of CPCR1.

Another novel RFX protein was isolated from P. chrysogenum through sequence homology to CPCR1. Using PCR, a small DNA fragment of 89 bp, corresponding to the conserved COOH part of the DNA binding domain, was amplified from P. chrysogenum genomic DNA for DNA sequencing. Using another pair of oligonucleotide primers for inverse PCR, a 490-bp
Fig. 1. The nucleotide sequence and predicted protein sequence of the cpeR1 gene. The co-ordinates are given in the left-hand margin. The translation initiation codon was predicted from the genomic sequence. The first and last nucleotide residue present in the cloned cDNA are marked by a dot. The main transcriptional start point (arrow) was determined using the primer extension method. Polyadenylation followed nucleotide 3308. Introns are shown in lowercase letters. 5' and 3' splice junctions, potential sequences for lariat formation, and a putative polyadenylation signal are underlined. The DNA binding domain is highlighted in gray, and the dimerization domain is framed.
A fragment was obtained, that was used to screen a cosmid library of *P. chrysogenum* genomic DNA. About 3.9 kilobases of a positive cosmid clone were sequenced, identifying an open reading frame for 855 amino acids. The encoded proteins *PcRFX1* and *CPCR1* from *A. chrysogenum* show an overall homology of 29%. However, nearly 60% of the amino acid residues from their DNA binding domains are identical, and the two proteins are more closely related to each other than to any other RFX protein.

**Binding Specificity of CPCR1**

**One-hybrid**—The *cpcR1* gene cDNA was isolated through a one-hybrid screen using the BSII binding site. This binding site contains an imperfect palindrome and a CCAAT-box, which overlaps with the right half of the palindrome. We created three mutated binding sites to determine which parts of the BSII sequence play an important or essential role in CPCR1 recognition. All sequences were used to generate yeast reporter strains containing HIS3 and *lacZ* reporter genes. Reporter strain transformants carrying the plasmid pGC1, which harbors the *cpcR1* cDNA, were analyzed for reporter gene activity (Fig. 3). For both *BSIIm1* and *BSIIm3*, no binding of CPCR1 was observed. Both sequences contain substitutions either in the right-hand or in the left-hand part of the palindrome. Yeast cells carrying these mutated binding sites show a dramatic decrease in reporter gene activity. In contrast, the substitution of two nucleotides, which are part of the CCAAT-box, and the right half of the palindrome does not lead to a total loss of CPCR1 binding. The quantification of β-galactosidase activity revealed a reduction to less than one-fourth that of the control. These data suggest that in vivo the CPCR1 protein binds to an imperfect palindromic sequence and that the integrity of both halves is essential for protein binding.

**EMSA**—To further characterize the binding specificity of the CPCR1 protein, we performed in vitro binding experiments. As mentioned above, a specific interaction of proteins from *A. chrysogenum* with BSII has already been demonstrated. The availability of yeast transformants with ADH1 promoter-driven *cpcR1* gene expression makes in vitro binding assays with an increased amount of specific protein feasible. FPLC-purified protein from yeast transformants was used in EMSA, and the formation of a DNA-protein complex with BSII was observed. In competition analyses with BSII, the formation of the complex could be prevented by a 100-fold molar excess, whereas the same excess of BSIIm3 did not result in the complete reduction of complex formation (Fig. 4). This confirms the binding specificity of the CPCR1 protein with the palindromic sequence, as previously indicated by the results from the one-hybrid experiments. To exclude the involvement of yeast proteins, we have tested protein extracts from a control yeast transformant in EMSA. This strain synthesized only the GAL4 AD, and protein extracts from the strain produced DNA-protein complexes clearly different from those above.

The binding specificity of the recombinant fusion protein AD-CPCR1 with BSII was further analyzed using shift-Western blotting (14). In this method the proteins are transferred onto nitrocellulose membrane, whereas the DNA is blotted on a second membrane (PVDF). Using an antibody against the yeast GAL4 AD, which is part of the recombinant protein AD-CPCR1, we were able to detect protein components of the BSII DNA-protein complex (Fig. 5). The same band also appeared by loading protein without DNA (lane 5, Fig. 5), indicating that the migration of the retarded complex in the gel mainly depends on the size and charge of the protein component. Results obtained from protein extracts of the control yeast transformant synthesizing only the GAL4 AD underline the specificity of the retarded complex (Fig. 5, lanes 6 and 7). In conclusion, the electrophoretic mobility shift assays with protein extracts from recombinant yeast transformants underscore the binding specificity of the CPCR1 protein in vitro.
DNase I Footprinting—Both one-hybrid experiments and EMSA were performed with synthetic oligonucleotides containing three copies of the analyzed binding site. In DNase I footprinting experiments, we used a DNA fragment from the pcbC promoter together with protein from either A. chrysogenum strain Ac1 or yeast transformants to detect a DNA-protein interaction close to the BSII sequence. In Fig. 6, data from DNase I footprinting experiments are shown with protection patterns of A. chrysogenum cell crude extract as well as FPLC-purified protein from a yeast transformant producing a recombinant AD-CPCR1 protein. Using the bottom strand, inhibition of DNase I cleavage was observed with both protein extracts in the range of -2444 to -2409 with respect to the translational start of the pcbC gene. The A. chrysogenum cell extract seems to inhibit DNase I cleavage even beyond position -2444, implying the possibility of additional DNA-protein interactions in this promoter region. It was found that protection patterns in the top strand appeared between positions -2401 to -2435. In this case, a difference in pattern was recognized with the A. chrysogenum extract and the AD-CPCR1 protein. As for the bottom strand, additional sites of DNase I cleavage have been detected with the A. chrysogenum protein. In conclusion, with regard to the pcbC promoter, the BSII sequence is recognized and bound by proteins from both A. chrysogenum and the recombinant yeast strains. The comparison of protection patterns also suggests that the inhibition of DNase I cleavage in the region -401 to -444 is due to binding of CPCR1.

Homodimerization of CPCR1

Two-hybrid—The existence of a putative dimerization domain in the CPCR1 protein implies homodimerization of CPCR1. To test this assumption, we performed two-hybrid experiments. Recombinant plasmids encoding fusion proteins composed of CPCR1 or truncated versions with either the DNA binding (BD) or the AD from GAL4 were constructed. The truncated DIM1 protein composes the NH2-terminal half of CPCR1 with the DNA binding domain but does not include the putative dimerization domain. As can be seen from the schematic representation in Fig. 8, the DIM2 protein is COOH-terminus-truncated; the deletion is smaller than in DIM1, thus preserving the NH2-terminal half of the dimerization domain. All three plasmids carrying the DNA binding domain were each separately combined in different yeast transformants with the corresponding plasmids carrying either an AD-CPCR1 fusion or, as controls the AD (pGAD424) or CPCR1, without a fusion (pC1). All resulting yeast transformants were tested for growth in the presence of 3-AT to determine the expression level of the HIS3 reporter gene (see Fig. 7). The combination of BD-CPCR1/AD-CPCR1 leads to good growth of the transformant on selective media, indicating a strong interaction between the two proteins in the yeast cell. A weaker interaction can be recognized with the combination BD-DIM2/AD-DIM2, and no interaction and growth at all was observed with BD-DIM1/AD-DIM1. In addition, these two-hybrid experiments show that CPCR1 is able to make protein-protein interactions with both...
truncated versions DIM1 and DIM2, albeit to a lesser extent, as can be seen from weak growth of the CPCR1/DIM1 combination. None of the controls enables the corresponding yeast transformants to grow on selective media. The two-hybrid experiments suggest that CPCR1 forms a homodimer and also that the dimerization mainly depends on the integrity of the dimerization domain.

One-hybrid—The next step was to investigate whether the DNA binding ability of CPCR1 is influenced by the reduced dimerization properties of the truncated versions. To test this DNA binding ability, we introduced the AD fusion plasmids into the one-hybrid reporter strain HISLACZ-BSII and analyzed the activity of reporter genes in yeast transformants. Whether the complete CPCR1 protein or truncated versions of it were produced in the yeast cells had a significant effect on the levels of $\beta$-galactosidase activity (see Fig. 8). Only the complete AD-CPCR1 fusion protein resulted in high reporter activities, indicating a strong DNA-protein interaction. A reduction to 5% of AD-CPCR1 activity was observed with AD-DIM2, and no specific activity could be detected for AD-DIM1. In conclusion, CPCR1 forms a homodimer and binds DNA only in this conformation. The destruction of the dimerization domain also leads to a loss of DNA binding ability. Thus the two CPCR1 protein domains for DNA binding and dimerization are not functionally independent.

Nondenaturating Electrophoresis—In nondenaturating gels, the native size and subunit structure of a protein can be analyzed. In the shift-Western analysis, using cell extracts of recombinant yeast strains and an antibody against the AD-CPCR1 protein, only one specific protein band has been detected (Fig. 5). This indicates that in vitro only one native state of the protein exists in detectable amounts. From the one- and two-hybrid data we conclude that even in vitro the AD-CPCR1 protein binds BSII DNA as a dimer or multimer. To determine the native size of the presumptive complex, we performed a series of native discontinuous gels with varying acrylamide concentrations to conduct Ferguson plots (data not shown). Cell extracts of recombinant yeasts synthesizing the AD-CPCR1 protein and native molecular weight standards were separated on 5 to 10% gels. The log (relative mobility) of the proteins was plotted against the acrylamide gel concentration using linear regression, and the slope ($=retardation coefficient$) was determined. By plotting the negative slope versus the molecular weight of the standards we generated a molecular weight standard curve and estimated the approximate native size of AD-CPCR1 to be around 280,000. With this native molecular weight of the protein it can be excluded that a CPCR1 monomer (100,000) was detected. The combination of in

Fig. 4. BSII-specific binding of yeast-synthesized CPCR1 protein. A, oligonucleotides used for binding and competition contain three copies of either the BSII site or the mutated BSIIm3 site. B, an EMSA of FPLC-purified protein from a yeast transformant synthesizing the AD-CPCR1 fusion protein. Protein (5.4 $\mu$g) was mixed with $^{32}$P-BSII, except the control (–). As competitors, BSII and BSIIm3 were added in 10-, 50-, and 100-fold molar excess. C, comparison of DNA binding activity by EMSA of proteins from two yeast transformants. Besides yeast transformant carrying plasmid pGC1 used in B, a control that produced only the AD is shown. In both cases, 3.8 $\mu$g of protein were incubated with $^{32}$P-BSII. As a competitor, a 10-, 50-, and 100-fold molar excess of BSII was added.

Fig. 5. Detection of AD-CPCR1 protein and DNA components by shift-Western analysis. Increasing amounts of cell extracts from a yeast transformant synthesizing the AD-CPCR1 fusion protein were incubated with $^{32}$P-BSII, separated in a gel, and transferred to nitrocellulose and PVDF membranes. As a control, cell extracts from strains synthesizing only the GAL4 AD were used. A, autoradiography of the PVDF membrane visualizing the free oligonucleotide and the DNA-protein complex. B, immunoblot of the nitrocellulose membrane with antibody against the activation domain of the GAL4 protein (AD), which is part of the recombinant AD-CPCR1 protein. The arrow indicates the position of the detected protein.
vitro shift-Western and native electrophoresis experiments supports the in vivo result that CPCR1 binds DNA solely in a dimeric state.

DISCUSSION

The biosynthesis of β-lactam antibiotics in fungi has been optimized for industrial purposes over the last 50 years. Strain improvement has been achieved by classical mutation and selection techniques (16). In the case of high titer penicillin producing P. chrysogenum strains, an increase in the number of copies of penicillin biosynthesis genes has been observed (17). Furthermore, increases in the steady-state levels of mRNA transcribed from these genes indicate increased levels of gene expression, suggesting the involvement of other mechanisms besides gene dose. The fact that no promoter alterations have been reported indicates that the difference is most likely due to changes in transcription factors (16). Interestingly, in A. chrysogenum, no comparable amplifications of structural genes were detected in strains with increased production of cephalosporin C. At the molecular level, low and high production strains seem only to be differentiated by their electrophoretic karyotypes (18). Therefore changes in activity or specificity at the transcription-factor level seem to be of major importance for strain improvement. The analysis of transcription factors opens the possibility of understanding the regulatory mechanisms controlling β-lactam biosynthesis in fungi.

To identify transcription factors and their binding sites, we have analyzed the common promoter region of the pcbAB and pcbC genes from A. chrysogenum. The BSII sequence was used as a bait in the yeast one-hybrid system. BSII contains a putative CCAAT box, which has already been characterized as a protein binding site in promoters from other fungi (19, 20). The one-hybrid system is an accepted method to clone cDNAs of transcription factors, whose protein binding sites have been characterized in biochemical studies (21, 22). A screening of yeast transformants resulted in the isolation of a cDNA from A. chrysogenum, which encodes a polypeptide with binding specificity for BSII and also shows significant homology to transcription factor genes of the RFX family. Members of this

FIG. 6. DNase I footprint analysis of the pcbC promoter. Top (B) and bottom (A) strands of PCBC5 DNA fragments were end-labeled and incubated with either 26 μg of A. chrysogenum cell extracts (Ac1) or 9 μg of FPLC-purified protein of a yeast transformant (AD-CPCR1) partially digested with DNase I and electrophoresed in a 10% polyacrylamide DNA sequencing gel. A slash indicates reaction mixtures containing no protein. Protected DNA sequences are represented by open boxes. Coordinates given are with respect to the pcbC translational start. G-reacted PCBC5 fragment (G) was used as a position marker. C, schematic representation of protection patterns determined for top and bottom strand of the pcbC promoter (gray box). The location of BSII sequence is framed, and nucleotides that are part of the imperfect palindrome are underlined.

FIG. 7. Two-hybrid reporter gene assay of full-length and truncated forms of CPCR1. Transformants with BD and AD plasmids were examined for growth in the presence of 3-AT. A, distribution of AD fusion plasmids in the three strains. Schematic representation of the resulting proteins see Fig. 8. Transformants with the binding domain fused to the CPCR1 protein (B), to the truncated DIM1 protein (C), and the truncated DIM2 protein (D) are shown.

FIG. 8. One-hybrid reporter gene assay of full-length and truncated forms of CPCR1. The deletion constructs are shown schematically. The GAL4-AD is hatched. Besides the DNA binding domain (DBD) and the dimerization domain (DIM) there are two conserved regions of unknown function (B and C). Regions rich in proline (P) and glutamine (Q) or acidic amino acids (DE) are indicated. U, units.
Fungal RFX Protein, Binding to \(\beta\)-Lactam Biosynthesis Genes

protein family possess a unique DNA binding and dimerization domain. Although at least 11 proteins of this family have been identified in 5 different organisms, only limited information is available about the function of RFX proteins (15). It was shown that SAK1, the RFX protein from \(S.\) \(pombe\) is an essential regulatory factor in the life cycle of this yeast, although DNA binding properties and the target genes of SAK1 are unknown (23). To date, RFX proteins from mammals provide some of the best-characterized examples. Special interest in mammalian RFX proteins comes from the important role they play in disease, notably virus infections. Human RFX5, for example, is essential for expression of major histocompatibility complex II genes; mutations that disrupt RFX5 genes lead to immunodeficiency (24). Another example is the human RFX1, which is used by the pathogenic hepatitis B virus as a cellular transactivator (25). RFX1 is expressed ubiquitously in all cell types and forms homodimers as well as heterodimers with RFX2 or RFX3 (26). Protein complexes containing RFX1-3 bind a variety of DNA sequences, with the highest affinity being observed with imperfect palindrome sequences (26). Such an imperfect palindrome forms part of the BSII sequence, which is similar to the consensus binding site of human RFX1 (27). This indicates that the palindromic features of the BSII sequence are important for binding of the \(A.\) chrysogenum RFX protein CPC1 (this was confirmed in our binding assays, see Figs. 3 and 4). Substitutions in the right-hand or the left-hand half of the palindrome (mutated BSIIm1 and BSIIm3 sequences) abolish \textit{in vivo} and \textit{in vitro} binding of CPC1. A similar observation was described for RFX1, in which binding of RFX1 to one-half of a palindrome is unstable (28). One-hybrid experiments with CPC1 not only revealed the importance of an intact binding site but also that truncated versions of CPC1 have reduced ability to bind DNA \textit{in vivo}, although the DNA binding domain remains intact. Surprisingly, the NH\textsubscript{2}-terminal half of the CPC1 dimerization domain is sufficient to mediate some degree of dimerization and DNA binding (see Figs. 7 and 8). These results are in line with observations by another group, who defined an “extended dimerization domain” for RFX1 (29), which comprises the conserved regions B and C (Fig. 8). Their analysis indicates that the dimerization domain is required, but not sufficient, for efficient dimerization in cellular extracts. Interestingly, only one state of the CPC1 protein was observed in shift-Western blotting and native electrophoresis. The shift-Western also showed that this state was able to bind DNA (Fig. 5). Using Ferguson plots, we estimated the native protein size to be around 280 kDa, with a supposed size of a AD-CPC1 dimer being 200 kDa. Our data suggest that homodimerization of CPC1 is very stable, a fact that was also reported from other RFX proteins that form dimers as the predominant state in cellular extracts (28, 29). Dimerization can also help to prevent inactivation of a protein (30), whereas monomers are more unstable or might not even evolve during inactivation or folding of the dimer (31). There are two possibilities to inactivate a dimeric or multimeric protein. A dimer can dissociate in two folded monomers, followed by denaturing the monomers (three-state model), or a dimeric protein undergoes a transition to a partially unfolded state before the dissociation of the monomers (two-state model) (32, 33). According to the latter model, no folded monomers can be observed. Taken together the \textit{in vivo} DNA binding and dimerization experiments confirm that the fungal CPC1 protein is a typical transcription factor of the RFX family.

RFX transcription factors have been found in many different organisms, including mammals, nematodes, and fungi. However, the physiological roles and target genes of most RFX proteins have not yet been clearly determined. This is the case even for the biochemically well characterized human RFX1 (29). Recently, a one-hybrid screening using an enhancer-like element from the interleukin-5 receptor promoter revealed the binding of RFX2 to this cis element. Further analysis showed that different RFX proteins bind and contribute to the lineage-specific expression of the receptor gene (34). CPC1RFX was identified through its ability to bind sequences of the \(A.\) chrysogenum \(pcbAB\)/\(\textit{pcbC}\) promoter. Therefore the cephalosporin C biosynthesis genes are potential target genes of the novel transcription factor. The binding site for CPC1RFX is located about 350 nucleotides upstream of the transcriptional start of the \(\textit{pcbC}\) gene, indicating that CPC1RFX is most likely not part of the basal transcription machinery but an accessory transcription factor modulating the expression of the \(\textit{pcbC}\) gene. In yeast, binding sites for activator proteins are typically found about 100–250 bp upstream of the gene (Ref. 35 and references therein). It remains to be tested whether CPC1RFX is involved in the activation or repression of \(\textit{pcbC}\) gene expression. The human RFX5 is an essential activator of major histocompatibility complex class II genes, and RFX1 is used as a trans-activator by viruses; thus, CPC1RFX might act as an activator of cephalosporin C genes. Nevertheless, recent investigations revealed that RFX1 is a context-dependent regulator and contains activation and repression domains (34, 36). But if one takes the relatively modest overall homology of RFX proteins outside the conserved domains into consideration, different properties of individual RFX proteins can be assumed.

The CPC1RFX protein is the first RFX transcription factor to be identified and characterized in filamentous fungi. With members of this protein family previously detected in the yeasts \(S.\) \(pombe\) and \(S.\) \(cerevisiae\), it is likely that RFX transcription factors are present in many, if not all, filamentous fungi. The presence of a RFX gene in the penicillin-producing fungus \(P.\) chrysogenum indicates the wide distribution of this type of transcription factor in fungi, and it remains to be tested whether their regulatory functions are restricted to \(\beta\)-lactam biosynthesis.

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