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AcFKH1, a novel member of the forkhead family, associates with the RFX transcription factor CPCR1 in the cephalosporin C-producing fungus *Acremonium chrysogenum*

Esther K. Schmitt[1], Birgit Hoff, Ulrich Kück*

Lehrstuhl für Allgemeine und Molekulare Botanik, Ruhr-Universität Bochum, D-44801 Bochum, Germany

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Abstract

In the filamentous fungus *Acremonium chrysogenum*, a complex regulatory network of transcription factors controls the expression of at least seven cephalosporin C biosynthesis genes. The RFX transcription factor CPCR1 binds to regulatory sequences in the promoter region of cephalosporin C biosynthesis genes, and is involved in the transcriptional regulation of the *pcb*C gene which encodes isopenicillin N synthase. In this study, we used CPCR1 in a yeast two-hybrid screen to identify potential protein interaction partners. A cDNA was identified, encoding the C-terminal part (pos. 438–665) of the novel forkhead protein, AcFKH1. The full-length AcFKH1 amino acid sequence is 665 residues and shares between 31% and 60% identity with forkhead protein sequences in the genomes of *Aspergillus nidulans*, *Fusarium graminearum*, and *Neurospora crassa*. AcFKH1 is characterized by two conserved domains, the N-terminal forkhead-associated domain (FHA), which might be involved in phospho-protein interactions, and the C-terminal DNA-binding domain (FKH) of the winged helix/forkhead type. The two-hybrid system was also used to map the protein domains required for the interaction of transcription factors CPCR1 and AcFKH1. The observed interaction between CPCR1 and the C-terminus of AcFKH1 in the yeast system was verified in vitro in a GST pulldown assay. Using gel retardation analysis, the DNA-binding properties of the fungal forkhead protein AcFKH1 were investigated. AcFKH1 recognizes two forkhead consensus binding sites within the 1.2 kb promoter region of the divergently oriented cephalosporin biosynthesis gene pair *pcbAB–pcbC* from *A. chrysogenum*. Additionally, AcFKH1 is able to bind with high affinity to the SWI5-binding site of the yeast FKH2 protein.

Keywords: Transcription factor; RFX; Forkhead; *Acremonium chrysogenum*; β-Lactam biosynthesis; Two-hybrid system

1. Introduction

Recently, the body of evidence suggesting that the transcriptional regulation of β-lactam antibiotic biosynthesis in filamentous fungi is highly complex has been increasing. Molecular genetic and biochemical research has already uncovered a variety of regulatory mechanisms and a number of transcriptional regulators from the model organism *Aspergillus nidulans*, the major penicillin producer *Penicillium chrysogenum*, and the cephalosporin C producer *Acremonium chrysogenum* (e.g. Schmitt et al., 2004b). Cephalosporin C and derivatives thereof are penicillin-related anti-infective agents that inhibit a broad

Abbreviations: AcFKH1, forkhead transcription factor 1 from *Acremonium chrysogenum*; CPCR1, RFX transcription factor 1 from *A. chrysogenum*; FHA, forkhead-associated domain; FKH, forkhead DNA-binding domain; GST, glutathione-*S*-transferase; RFX, regulator factor X; *pcbAB*, *pcbC*, *cefD1*, *cefD2*, *cefEF*, *cefG*, cephalosporin C biosynthesis genes.

* Corresponding author. Tel.: +49 234 3226212; fax: +49 234 3214184.
E-mail address: ulrich.kueck@ruhr-uni-bochum.de (U. Kück).
[1] Current address: Novartis Pharma AG, NPU, 4002 Basel, Switzerland.

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range of both Gram-positive and -negative bacteria. However, cephalosporin C biosynthesis is not constitutive, and the level of gene expression of the biosynthesis genes greatly affects levels of antibiotic production (Litzka et al., 1999). Therefore, transcription factors are important mediators of internal and external parameters affecting β-lactam biosynthesis.

Comparative investigations with wild-type and industrial cephalosporin C production strains provided a basis to pinpoint changes in regulatory systems that had occurred during classical strain improvement. Early molecular investigations provided evidence that transcript levels of cephalosporin C biosynthesis genes are highly increased in production strains (Walz and Kück, 1993), indicating the significance of transcriptional regulators for improved β-lactam biosynthesis. This view was further supported when different regulation of CRE1 in wild-type and production strains of *A. chrysogenum* was demonstrated (Jekosch and Kück, 2000b).

In *A. chrysogenum*, three pairs of divergently oriented cephalosporin biosynthesis genes are organized in two clusters on chromosomes II and VI. These gene pairs, namely *pcbAB–pcbC*, *cefEF–cefG*, and *cefD1–cefD2*, each enclose a divergent and spatially well-defined regulatory region (Menne et al., 1994; Ullán et al., 2002). Transcription factors binding to these promoters can have an effect on the transcription of either of the two genes or on both simultaneously.

To investigate coregulation and the complexity of regulatory systems which are involved in the transcription of the adjacent cephalosporin C biosynthesis genes, novel transcriptional regulators have to be isolated and their binding sites in the promoter regions defined.

To date, three transcription factors from *A. chrysogenum* have been characterized in detail, i.e. CRE1, PACC, and CPCR1. Repressor protein CRE1 is involved in the regulation of at least two biosynthesis genes. In the presence of sufficient glucose, growth is promoted and the transcription of the *pcbC* and *cefEF* genes is repressed in the wild-type strain. However, in a semi-producer strain, only the *cefEF* gene is subject to glucose repression via CRE1 (Jekosch and Kück, 2000a,b). Transcription factor PACC mediates gene expression in response to external pH and binds four independent sites in the cephalosporin C biosynthesis gene promoters (Schmitt et al., 2001). Finally, transcription factor CPCR1 is known to recognize two binding sites in the promoter region between the *pcbAB–pcbC* genes. Additionally, CPCR1 is the first regulator factor X (RFX) transcription factor to be identified and functionally characterized in filamentous fungi (Schmitt and Kück, 2000; Schmitt et al., 2004a).

In contrast to the function of CRE1 and PACC, so far the characterization of CPCR1 has only identified its involvement in the transcriptional regulation of the cephalosporin C biosynthesis gene *pcbC* (Schmitt et al., 2004a). In knockout strains, the *pcbC* transcript levels increase in the first 36 h of cultivation and then later decrease when compared to the control strain (Schmitt and Kück, 2000). The overall production of cephalosporin C is unchanged; however, the biosynthesis level of intermediate penicillin N is reduced to less than 20%. These results therefore indicate that CPCR1 might be involved in the regulation of the early cephalosporin biosynthesis genes like *pcbC*, rather than play a major role in late biosynthesis gene expression. These data also indicate a complex regulatory circuit to be involved in the expression of cephalosporin C biosynthesis genes. CPCR1 is a large protein encompassing 830 amino acids, and is known to form a homodimer that enables binding to the DNA in this multimeric state (Schmitt and Kück, 2000). As a further step to understanding the regulatory role of CPCR1, we performed a yeast two-hybrid screen to identify the interaction partner(s) of this RFX transcription factor.

Here, we describe the isolation of a cDNA clone from the two-hybrid screen, encoding the C-terminus of a novel transcription factor AcFKH1, which belongs to the family of forkhead proteins. Forkhead transcription factors and RFX proteins form subclasses of winged-helix transcription factors (Gajiwala et al., 2000). To the best of our knowledge, *Acfkh1* is the first characterized member of the forkhead gene family in filamentous fungi. The proportion of identical amino acids positions with homologs from different yeasts ranged between 18% and 22%. Additionally, the two-hybrid system was used to map the protein domains required for the interaction of the two transcription factors CPCR1 and AcFKH1. The observed interaction between CPCR1 and the C-terminus of AcFKH1 in the yeast system was verified in vitro in a GST pulldown assay. Using gel retardation analysis, FKH1 was shown to recognize two forkhead consensus binding sites within the *pcbAB–pcbC* promoter of *A. chrysogenum*, and to bind with high affinity to the SWI5-binding site of the yeast FKH2 protein.

2. Materials and methods

2.1. Strains and plasmid construction

For our investigations we used *A. chrysogenum* strains ATCC 14553 and the semi-producer strain A3/2 (Radzio and Kück, 1997). The two-hybrid plasmids used in this study are listed in Table 1. pcDNA78 was isolated from the cDNA library of *A. chrysogenum* during a two-hybrid screening. Derivatives pcDNA78Δ1/2 were obtained by deleting *Sal*I or *Nde*I–*Bam*HI restriction fragments in plasmid pcDNA78. Plasmid pADFKH1 contains an RT-PCR fragment of the *fkh1* gene and pADFHA1 contains a *Pvu*II–*Aat*II restriction fragment from the genomic copy of the *fkh1* gene which is present as a 3.6 kb fragment in plasmid pKSFKH1. Plasmids pADDIM3 and pADDIM4 contain a 0.6 kb *Apa*I–*Pst*I fragment or a 1.1 kb *Stu*I fragment from plasmid pGC1 encoding the C-terminal parts

Table 1
Plasmids used for the two-hybrid analysis

Plasmid	Protein encoded	Reference
pGAD424	GAL4 activation domain (AD)	Clontech
pGAD-C(X)	GAL4 activation domain (AD)	James et al., 1996
pGBDU-C(X)	GAL4 DNA-binding domain (BD)	James et al., 1996
pGC1	CPCR1 aa 43–830 (AD-C1)	Schmitt and Kück, 2000
pBDC1	CPCR1 aa 43–830 (BD-C1)	Schmitt and Kück, 2000
pGC1?DIM1	CPCR1 aa 43–446 (AD-DIM1)	Schmitt and Kück, 2000
pBDDIM1	CPCR1 aa 43–446 (BD-DIM1)	Schmitt and Kück, 2000
pGC1?DIM2	CPCR1 aa 43–633 (AD-DIM2)	Schmitt and Kück, 2000
pBDDIM2	CPCR1 aa 43–633 (BD-DIM2)	Schmitt and Kück, 2000
pADDIM3	CPCR1 aa 635–830 (AD-DIM3)	This work
pBDDIM3	CPCR1 aa 635–830 (BD-DIM3)	This work
pADDIM4	CPCR1 aa 447–830 (AD-DIM4)	This work
pBDDIM4	CPCR1 aa 447–830 (BD-DIM4)	This work
pcDNA78	FKH1 aa 438–665 (AD-78)	This work
pBD78	FKH1 aa 438–665 (BD-78)	This work
pcDNA78Δ1	FKH1 aa 438–485 (AD-78Δ1)	This work
pBD78Δ1	FKH1 aa 438–485 (BD-78Δ1)	This work
pcDNA78Δ2	FKH1 aa 438–550 (AD-78Δ2)	This work
pBD78Δ2	FKH1 aa 438–550 (BD-78Δ2)	This work
pcDNA78Δ3	FKH1 aa 438–626+640–665 (AD-78Δ3)	This work
pBD78?3	FKH1 aa 438–626+640–665 (BD-78Δ3)	This work
pADFKH1	FKH1 aa 298–434 (AD-FKH1)	This work
pBDFKH1	FKH1 aa 298–434 (BD-FKH1)	This work
pADFHA1	FKH1 aa 40–287 (AD-FHA1)	This work
pBDFHA1	FKH1 aa 40–287 (BD-FHA1)	This work

of the CPCR1 protein. All pBD derivatives were obtained by excision of *Eco*RI–*Bam*HI restriction fragments from the corresponding pAD constructs.

2.2. Two-hybrid screen

The cDNA library was constructed in a GAL4 activation domain vector (pGAD424) (Clontech) as detailed previously (Schmitt and Kück, 2000). The yeast host strain PJ69-4A containing the three reporter genes *ADE2*, *HIS3*, and *lacZ* (James et al., 1996) was first transformed with pBD-C1. Cells were propagated in growth medium lacking uracil, and transformed with the cDNA library using the lithium acetate protocol and carrier DNA (Becker and Lundblad, 1994). Yeast colonies were selected on agar plates lacking uracil, leucine, and adenine. An aliquot of the transformation mixture was plated on agar plates lacking only uracil and leucine to determine the total number of transformants. Colonies were picked and re-grown on plates lacking uracil, leucine, and either adenine or histidine. In the latter case, 2 mM 3-aminotriazol was added to suppress the leaky *HIS3* reporter gene activity. A quantification of β-galactosidase activity of positive colonies was performed as described previously (Schmitt and Kück, 2000). Plasmid DNA was isolated from positive yeast colonies, amplified in bacteria, and reintroduced into PJ69-4A either with or without pBD-C1 DNA. Several large-scale transformations were performed and a total of 5×10^6 yeast transformants was examined.

2.3. Protein synthesis and purification

His_6 fusion derivatives of AcFKH1 and CPCR1 were synthesized in *Escherichia coli* strain M15 [pREP4] (Qiagen). For electrophoretic mobility shift assays, a cDNA encoding amino acids 298–485 of AcFKH1 was cloned into the vector pQE32 (Qiagen) to generate plasmid pQFKH3. The resulting plasmid was transformed into *E. coli* M15 [pREP4] and expression of the *Acfkh1* gene was induced at midlog phase by adding 2 mM isopropyl β-D-thiogalactoside. Cells were harvested after 3 h of incubation at 37 °C and frozen at −80 °C until required for purification.

For pulldown assays, a cDNA fragment encoding CPCR1 (amino acids 133–830) was ligated into the *Sph*I and *Sma*I restriction sites of plasmid pQE31 (Qiagen). After transformation of the plasmid in *E. coli* M15 [pREP4], the His_6-CPCR1 fusion protein expression was induced by addition of isopropyl β-D-thiogalactoside to a final concentration of 2 mM. In this case, the cells were incubated for 2 h at 30 °C. Native purification of the His_6 fusion proteins using nickel nitrilotriacetic acid resin was realized according to the supplier's protocol (Qiagen).

GST and GST fusion proteins were prepared from *E. coli* BL21 (DE3) cells (Stratagene). For this purpose, the C-terminal coding fragment of *Acfkh1* (residues 437–665) was ligated in-frame to the coding sequence of GST by using the *Bam*HI and *Eco*RI restriction sites of pGEX-4T1 (Amersham Biosciences) and the plasmid was transformed into the *E. coli* expression host BL21 (DE3). Expression of the gene was induced at midlog phase by adding 2 mM isopropyl β-D-thiogalactoside and the cells were incubated at 25 °C for 3 h. GST and GST fusion proteins were purified by affinity chromatography using a 50% slurry of glutathione Sepharose beads according to the supplier's instructions (Amersham Biosciences). Purified proteins were stored at −80 °C until used for pulldown assays.

2.4. GST pulldown assays and Western blotting

Pulldown assays were conducted by adding 5 μg of purified GST or GST-AcFKH1c fusion protein to the His-tagged recombinant protein (5 μg) in a final volume of 300 μl binding buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol). Following a 2-h incubation at 4 °C, 15 μl of a 50% slurry of glutathione 4B-Sepharose (Amersham Biosciences) was

added and the mixture was incubated for 3 h at 4 °C on a rocking platform. The resin was washed three times with 1 ml binding buffer. Bound proteins were eluted into sample buffer, resolved by SDS-PAGE and transferred to PVDF membrane.

For Western blot analysis, PVDF membranes were incubated for 1 h at RT with either anti-RGS-His HRP-conjugated antibody (1:1000) diluted in blocking puffer (Qiagen) or anti-GST HRP-conjugated antibody (1:5000) (Amersham Biosciences) in phosphate-buffered saline–Tween (PBST). Following incubation, membranes were washed for 30 min in PBST. Detection of proteins was done with a BM chemiluminescence Western blotting kit according to the supplier's recommendations (Roche Diagnostics).

2.5. Gel retardation analysis

Annealed oligonucleotides (top strand) SWI5 (5′ GGGCTAGTTAACCTGTTAGGAAAAAG**GTAAACGTAAACA**ATAAC3′ , Pic et al., 2000), FKHI (5′ GGGCCTTTGAT**ATCAACA**TGACTATC3′), and FKHII (5′ GGGAGCCGCCCGT**ATAAACA**GCTTCAAGA3′) were used as probes. The labeling was achieved by filling both ends with [α-^{32}P]dCTP using Klenow polymerase. The binding reaction consisted of 3 ng DNA, 0.5–1 μg protein, 4 mM EDTA, 1 μl binding buffer (Schmitt and Kück, 2000) in a volume of 20 μl. After a 15-min incubation at 24 °C and 15 min on ice, the samples were electrophoresed in a 7.5% polyacrylamide gel at 4 °C with Tris/glycine buffer.

2.6. Accession number

The nucleotide sequence of the *fkh1* gene from *A. chrysogenum* has been deposited in the GenBank/EBI Data Bank under accession no. AY196786.

3. Results

3.1. Two-hybrid screen to identify cDNAs encoding CPCR1-interacting proteins

To identify CPCR1-interacting proteins, a two-hybrid screen was performed using the CPCR1 protein (pos. 43–830) as bait. The yeast host PJ69-4A harboring plasmid pBD-C1, encoding the bait protein, was grown in selective media and transformed with an *A. chrysogenum* cDNA library constructed in the GAL4 activation domain vector pGAD424. Four independent large-scale transformation experiments using a total of 332 μg library DNA generated 5×10^6 yeast transformants. All transformants were plated on media lacking adenine to select for *ADE2* reporter gene activation. Next, 94 positive colonies were transferred to appropriate fresh selection plates and analyzed for both the *ADE2* and *HIS3* reporter genes. Colonies were quantified for β-galactosidase activity, and the plasmid DNA of 19 different colonies was isolated and reintroduced by transformation into the host strain with and without bait plasmid. Of these, 10 showed the expected activation of reporter genes in the strain harboring the bait plasmid, indicating a correlation between the plasmid-encoded protein and reporter gene activity. However, only yeast transformants with pcDNA78-DNA showed reproducible high *lacZ* activity. Sequencing of the cDNA of clone 78 (cDNA78) revealed an open reading frame for 228 amino acids corresponding to the C-terminus of a putative protein of unknown size. The amino acid sequence showed no significant homology to any known protein.

3.2. Acfkh1 encodes a protein with similarity to forkhead transcription factors

Using cDNA78 as probe, a *A. chrysogenum* lambda-genomic library was screened for the full-length gene. A 3.6 kb *Eco*RI fragment was subcloned from the DNA of a positive phage and sequenced. The DNA fragment contains an open reading frame which is interrupted by three introns and encodes a polypeptide of 665 amino acids (see Fig. 1). Splice sites of the introns were confirmed by sequencing appropriate RT-PCR products. Intron 2 is located in the region encoding the DNA-binding domain. The novel gene was designated *Acfkh1* due to its similarity to the corresponding yeast genes (see below). Southern analysis confirmed that it is a single copy gene (data not shown).

A comparison of the polypeptide sequence with databases revealed significant similarities to a number of proteins from different organisms. All matching proteins are members of the family of forkhead transcription factors, which have a structurally well-defined DNA-binding domain that characterizes them as members of the eukaryotic winged-helix proteins. Although several members of this family have already been characterized in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, AcFKH1 is the first forkhead protein functionally identified in filamentous fungi. A similarity search with the AcFKH1 protein sequence in the public genome databases of filamentous fungi revealed some further homologos. Fig. 2 gives a sequence comparison of selected forkhead proteins. AcFKH1 shares 42%, 31%, and 60% identical amino acid positions with its closed homologs from *Neurospora crassa*, *A. nidulans*, and *Fusarium graminearum*, respectively, and between 18% and 22% identity exist with the forkhead proteins from *S. cerevisiae*, *S. pombe*, and *Candida albicans*.

All forkhead proteins show highly conserved amino acid stretches in the DNA-binding forkhead domain. Additional conserved positions are located in the N-terminal forkhead-associated domain. The *S. cerevisiae* homolog ScFKH2 is the longest of the polypeptides with

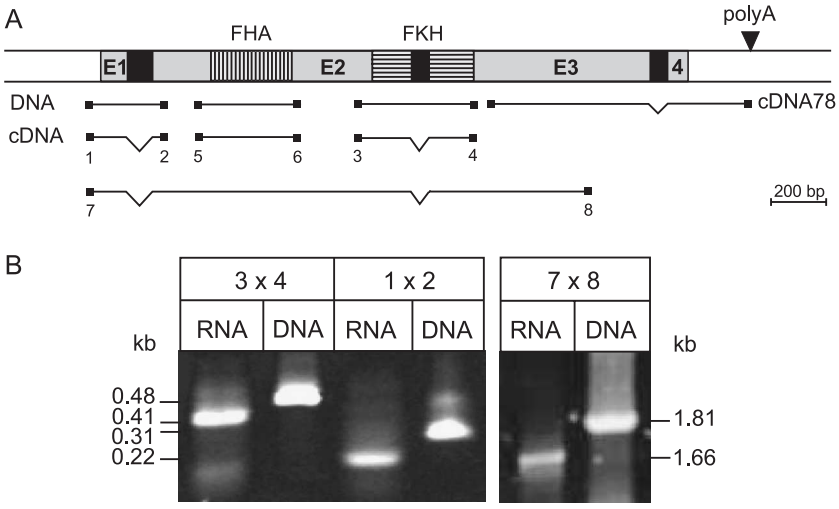


Fig. 1. Structure and transcriptional expression of the *Acfkh1* gene from *A. chrysogenum*. (A) Schematic representation of the gene locus with exonic (grey) and intronic (black) regions; amplicons, which were obtained from PCR reactions with DNA or cDNA as template are given below the gene locus. (B) Electrophoretic separation of amplicons which were obtained by PCR or RT-PCR analysis. Oligonucleotide primers were used as indicated. Abbreviations: FHA, forkhead-associated domain; FKH, forkhead domain.

862 amino acids, while forkhead proteins from filamentous fungi encompass 665–710 amino acids. The C-terminal part of the proteins shows no significant conservation. However, most interestingly, this part of the *A. chrysogenum* protein FKH1 was identified in the two-hybrid screen to interact with the bait protein, the RFX-type transcription factor CPCR1. These 228 C-terminal amino acids of AcFKH1 contain 29 (12.7%) proline residues and 26 (11.4%) acid amino acids. Additionally, there is a cluster of 12 glutamate and aspartate residues between positions 627 and 639. The proline residues are more dispersed and the most C-terminal proline (pos. 618) is located before the cluster of acid amino acids.

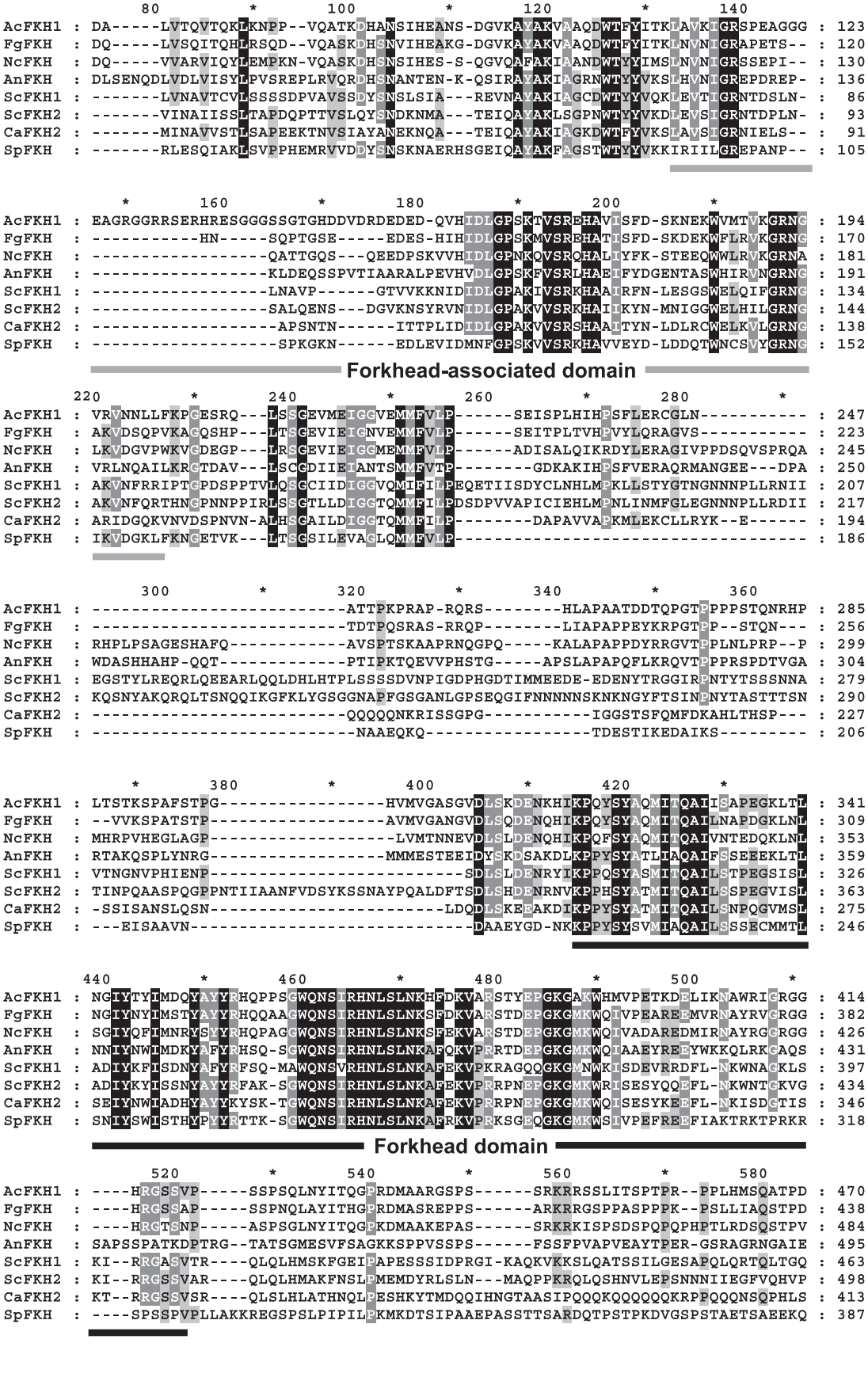
3.3. Mapping of protein domains required for CPCR1–AcFKH1 interaction

The two-hybrid screen revealed that the C-terminus of the FKH1 protein encoded by cDNA78 associates with the full-length CPCR1 protein. To further characterize domains of both proteins involved in this interaction, a detailed two-hybrid analysis was performed. Five or six different protein fragments were synthesized for CPCR1 or AcFKH1, respectively, in the appropriate yeast host as fusion proteins with the GAL4 activation (AD) or DNA-binding domain (BD) (see Fig. 3). All protein combinations of the proteins were tested for activation of reporter genes *HIS3* and *lacZ* in yeast (Table 2). Under the conditions tested, the β-galactosidase background activity of the yeast strain is approximately 5 U/mg protein, and loss of growth on histidine selection plates correlated with a β-galactosidase activity <10 U/mg protein (data not shown).

Different CPCR1 fragments encompassing the full-length protein C1 or the truncated proteins designated DIM1/2/3/4 were combined with polypeptide 78 representing the C-terminus of AcFKH1 encoded by cDNA78. The obtained β-galactosidase activities indicate that AcFKH1 requires a CPCR1 dimer together with the C-terminus of CPCR1 for interaction (see C1+78 and DIM3+78). The CPCR1-derivative DIM2 was able to form a dimer, but lacks the C-terminus, and therefore no interaction with AcFKH1 was observed. DIM4 did not form a dimer, although the corresponding construct encodes the complete dimerization domain. The absence of dimerization can be explained by incorrect folding of the truncated fusion protein in yeast.

A major difference in β-galactosidase activity can be observed when polypeptide 78 is fused to the GAL4 activation domain (AD) or the DNA-binding domain (BD). The BD-78 fusions always resulted in much higher activities. Controls show that the BD-78 fusion alone caused an increased activity of about 18 U/mg protein. This activity results from transcriptional activation properties in yeast (see below). Thus, the data with BD-78 do not correspond to the investigated protein interaction, but rather reflect a complex network of interactions with the yeast transcriptional machinery. Therefore, data with BD-78 have not been considered further.

Truncation of polypeptide 78 resulting in 78Δ1/2 when combined with BD-C1, reduced the β-galactosidase activity to 5–6 U/mg protein, which is background level. These results suggest that amino acids 551–665, which are lacking in both constructs, are sufficient to enable interaction with CPCR1. Within this protein region of AcFKH1, a cluster of acid amino acids is located (see Fig. 2). This cluster has been deleted to generate derivative 78Δ3. Deletion of these 13 amino acids (pos. 626–640) in protein 78 resulted in a decrease of the β-galactosidase activity to approximately 30% in combination with BD-C1, indicating only a weak interaction. Hence, we conclude that this acid region in the


Forkhead-associated domain
Forkhead domain

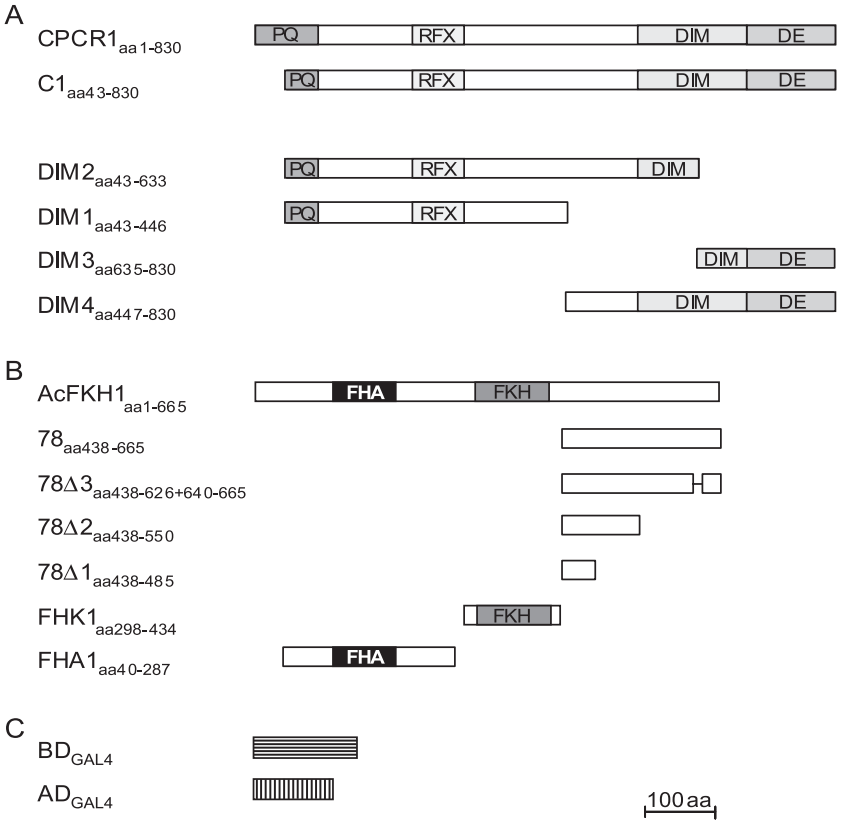


Fig. 3. Constructs used in the yeast two-hybrid system to investigate the interaction of the RFX transcription factor CPCR1 and the forkhead protein FKH1 from *A. chrysogenum*. (A) Derivatives of *cpcR1*. (B) Derivatives of *Acfkh1*. (C) GAL4 domains BDgal4 and ADgal4 were used for the two-hybrid analysis as controls. Besides the DNA-binding domain (RFX) and the dimerization domain (DIM) of CPCR1, there are two regions rich in proline and glutamine (PQ) or acidic amino acids (DE). In AcFKH1, the DNA-binding domain of the forkhead-type (FKH) and the forkhead-associated domain (FHA) are indicated.

C-terminal part of AcFKH1 mediates most of the protein–protein interaction with CPCR1.

Another interesting result of the two-hybrid tests is that the CPCR1–AcFKH1 protein interaction was restricted to the C-terminus of AcFKH1 (protein 78) and did not involve the N-terminal forkhead-associated domain (FHA). Similarly, the central region of AcFKH1, the DNA-binding domain present in derivative FKH1, did not mediate any protein–protein interaction with CPCR1 (see Table 2).

3.4. Localization of the transcriptional activation domain of AcFKH1

The differences in β-galactosidase activity obtained with AD-78 and BD-78 fusions suggested that the C-terminus of FKH1 not only interacts with CPCR1, but also mediates transcriptional activation in yeast. To further address this question and to ascertain the precise location of the activation domain of AcFKH1, yeast transformants with the BD fusions were analyzed for β-galactosidase activity. Polypeptide 78 induced a reporter gene activity of 18 U/mg protein. Two truncations 78Δ1/2, however, resulted in about 3 U/mg protein. This indicates that the activation domain like the interaction domain with CPCR1 is localized in the most C-terminal part of AcFKH1 encompassing amino acid positions 550–665. The results with derivative 78Δ3 show that the cluster of acid amino acids between positions 625 and 640 is not involved in transcriptional activation. Thus, this stretch of amino acids is most probably specific for the interaction with the CPCR1 transcription factor.

Of all the CPCR1 protein fragments, only fragment C1 stimulated slight reporter gene activation, suggesting a weak transcriptional activity for the full-length CPCR1 transcription factor (see Table 2).

Fig. 2. Amino acid sequence alignment of forkhead proteins from *A. chrysogenum* (AcFKH1; AY196786), *A. nidulans* (AnFKH), *C. albicans* (CaFKH2; orf6.8625), *F. graminearum* (FgFKH), *N. crassa* (NcFKH), *S. cerevisiae* (ScFKH1; L38848; ScFKH2; P41813), and *S. pombe* (SpFKH; gi19113556). The shown amino acid residues represent about 60 % of the complete protein sequences. Invariant residues are marked in black, and positions that are conserved in at least three of the proteins are highlighted in gray. The N-terminal forkhead-associated domain and the DNA-binding forkhead domain are underlined. Protein sequences from NcFKH, AnFKH, and FgFKH were annotated from genome sequence data (for details see text).

Table 2
Results of the quantitative β-galactosidase reporter gene assays with yeast transformants harboring different plasmid combinations in the yeast two-hybrid system

		CPCR1					FKH1						
		BD-C1	BD-DIM2	BD-DIM1	BD-DIM3	BD-DIM4	BD-78	BD-78Δ3	BD-78Δ2	BD-78Δ1	BD-FKH1	BD-FHA1	BD
CPCR1	AD-C1	15.8 ±4.4					48.7 ±10.6	28.2 ±4.2	7.0 ±2.7	5.5 ±1.0	5.7 ±2.2	4.5 ±14	
	AD-DIM2		10.8 ±1.8				33.1 ±6.5						
	AD-DIM1			4.6 ±1.3			30.2 ±5.9						
	AD-DIM3				10.6 ±4.9		29.9 ±7.3						
	AD-DIM4					4.0 ±1.0	37.2 ±6.2						
FKH1	AD-78	36.7 ±6.9	4.2 ±1.8	4.3 ±1.4	9.2 ±2.2	4.0 ±0.4	24.5 ±6.2						
	AD-78Δ3	12.1 ±3.3						20.7 ±2.1					
	AD-78Δ2	4.8 ±1.0							4.6 ±1.5				
	AD-78Δ1	6.6 ±1.7								2.8 ±0.7			
	AD-FKH1	5.9 ±1.6									4.1 ±1.4		
	AD-FHA1	7.7 ±2.0										4.8 ±0.8	
	AD	7.7 ±2.6	4.4 ±1.5	2.4 ±0.3	5.2 ±1.0	3.8 ±0.5	18.5 ±4.0	18.9 ±5.3	3.2 ±1.1	3.1 ±0.9	3.3 ±1.4	3.1 ±0.9	4.8 ±1.3

: Homodimerization
: Interaction between CPCR1 and AcFKH1
: Transcriptional activation in yeast
: Results can not be interpreted due to overlapping of protein-protein-interaction and transcriptional activation

3.5. AcFKH1 interacts with CPCR1 in vitro

The specificity of the interaction between AcFKH1 and CPCR1 was biochemically investigated by a GST pulldown assay with immobilized GST-AcFKH1c. Recombinant His_6-tagged CPCR1 protein was incubated with either GST or GST-AcFKH1c. Immunoblotting with anti-RGS-His or anti-GST antibodies demonstrated that the purified 80 kDa His_6-CPCR1 fusion protein interacts with the GST-fused AcFKH1c protein (52 kDa), but not with GST (27 kDa) alone (see Fig. 4). The specificity of binding between AcFKH1 and CPCR1 was further proved when recombinant His_6-tagged CRE1 (46 kDa) and CEFEF (38 kDa) from *A. chrysogenum* were incubated with either GST or the GST-AcFKH1c protein. CRE1 is a glucose-dependent repressor from *A. chrysogenum*, whereas the CEFEF polypeptide catalyzes the expandase/hydroxylase reaction in cephalosporin C biosynthesis. As demonstrated in Fig. 4, both proteins could not be identified by immunoblotting using the anti-RGS-His antibody. Consequently, they showed no specific binding with the forkhead transcription factor AcFKH1 from *A. chrysogenum*. Thus, our data confirm that there is a specific and direct physical interaction between CPCR1 and the C-terminal region of AcFKH1.

3.6. DNA-binding specificity

To address the question of DNA-binding specificity of the forkhead domain present in AcFKH1, a protein fragment corresponding to this region was synthesized in *E. coli* as a His_6-tagged fusion protein. After native preparation of protein extracts and partial purification using affinity chromatography, the protein was used in gel retardation experiments. Many forkhead proteins from a variety of organisms recognize highly similar binding sites; thus, consensus binding sites have been determined. Kaufmann et al. (1995) determined a consensus including seven core nucleotides 5′ G/A T/C C/A A A T/C A 3′ from 17 different sequences. The 1.2 kb intergenic region between the *pcbAB* and *pcbC* genes was analyzed for sequences fitting the consensus. Two putative forkhead-binding sites (FKHI and FKHII) were identified and chosen for in vitro binding studies. A high affinity binding site (SWI5) of the yeast ScFKH2 protein was selected as a further probe for gel retardation analysis (Pic et al., 2000). Fig. 5A shows such an analysis using the three above-described probes. Obviously, only the binding site from the promoter of the yeast *SWI5* gene is recognized with high affinity. Nevertheless, a weak interaction between the forkhead domain of AcFKH1 and

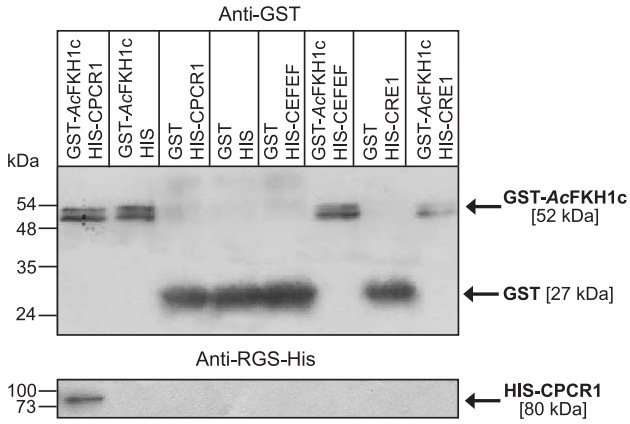


Fig. 4. In vitro interactions of CPCR1 and AcFKH1 in a GST pulldown assay. Purified His_6-CPCR1 fusion protein was incubated with glutathione 4B-Sepharose immobilized GST-AcFKH1c or GST (control). Retained proteins were analyzed by SDS-PAGE and immunoblotting with anti-RGS-His or anti-GST antibodies. As negative controls, His_6-tagged CEFEF and CRE1 polypeptides were incubated with either GST-AcFKH1 or GST alone as indicated. A strong and specific retention was only observed in the case of His_6-CPCR1 while no binding reactions were detected using the His_6-CEFEF and His_6-CRE1 fusion proteins.

the putative binding sites from the *pcbAB–pcbC* promoter could be detected when a 50-fold molar excess of AcFKH1 to DNA is used.

Similar to a previous study (Schmitt et al., 2004a), the whole intergenic promoter region was analyzed in order to determine all existent AcFKH1-binding sites. For these purposes, a series of 17 overlapping PCR fragments spanning the whole promoter region were radiolabeled and used in a gel retardation experiment (see Fig. 5B). From all 17 tested fragments, two amplicons of 88 bp (numbers 3 and 16 in Fig. 5C) were identified carrying either the FKHI- (pos. 185–207) or FKHII- (pos. 1087–1112) binding site. With both, the control protein HIS resulted in a retarded band which must come from unspecific *E. coli* proteins present in the extract. This unspecific interaction is not equally prominent in the lanes with AcFKH1 fusion protein. On the contrary, there is an additional retarded band that could be due to a specific AcFKH1/DNA protein complex. The stronger interaction of AcFKH1 with the promoter fragments might be due to adjacent sequences which are lacking in the oligonucleotides. No specific DNA-binding complexes could be observed for all other tested amplicons, as is shown for fragment 7 in Fig. 5C.

4. Discussion

4.1. Protein interactions of winged-helix transcription factors

Only very limited information is available on protein interaction partners of RFX and forkhead proteins in organisms other than humans and yeast. In humans, RFX1–3 form heterodimers that enable cell-type and organ-specific gene expression (Reith et al., 1994). The interaction between RFX1 and MIBP1 is thought to be involved in regulating the EP element in the enhancer I of hepatitis B virus and in down-regulating cellular c-myc levels by binding to the intron I element of the c-myc gene (Blake et al., 1996; Zajac-Kaye et al., 2000). Concerning forkhead proteins, several interactions have been reported. One example is the human forkhead protein FKHR, which associates with different hormone receptors to act as either a coactivator or a corepressor depending on the receptor type (Zhao et al., 2001). In contrast, the estrogen receptor-α interacts with at least three different forkhead proteins, but only in the presence of the ligand β-estradiol (Schuur et al., 2001). For the FKHR protein, an additional protein interaction was described: interaction with the CCAAT/enhancer-binding protein beta mediates the differentiation of human endometrial stromal cells (Christian et al., 2002).

In yeast, a well-studied protein complex involving a forkhead protein is the transcriptional module MCM1–FKH2–NDD1. This complex consists of three transcription factors and activates approximately 33 genes of the CLB2 cluster during the G2-to-M-phase transition in the cell cycle. MCM1 recruits distinct coregulatory proteins to promoters in order to regulate diverse processes. During the cell cycle, FKH2 cooperatively binds with MCM1 to promoters of the CLB2 cluster. Recently, the binding of FKH2 to DNA was reported to require prior binding by MCM1, and that the C-terminus of FKH2 is not required for the formation of this ternary complex (Boros et al., 2003). The permanent protein–DNA complex consisting of MCM1 and FKH2 then recruits the positive regulator NDD1 to the CLB2 promoter regions. According to a recent model, NDD1 is phosphorylated at position T319 by Cdc28-Clb kinase activity, and the FHA domain of FKH2 is necessary for recruitment of NDD1 into the DNA–MCM1–FKH2–NDD1 complex (Reynolds et al., 2003). Most interestingly, although NDD1 is an essential gene in yeast, it was found to be dispensable in cells expressing a truncated form of FKH2 which lacks its C-terminus (Reynolds et al., 2003). Additionally, it was suggested that FKH2 itself might have a negative regulatory role in the transcription of the CLB2 cluster genes; hence, without the activator NDD1, the cluster cannot be expressed.

These examples clearly illustrate that forkhead proteins are involved in higher order transcriptional complexes and have multiple interaction partners. Moreover, both DNA-binding and recruiting of additional proteins in a preassembled complex often depend on other proteins.

4.2. FKH1 associates with a CPCR1 dimer

The two-hybrid experiments showed that the exact mapping of an interaction domain in the CPCR1 protein is not possible. However, the results indicate that the C-terminus of CPCR1 is important. The C-terminal trunca-

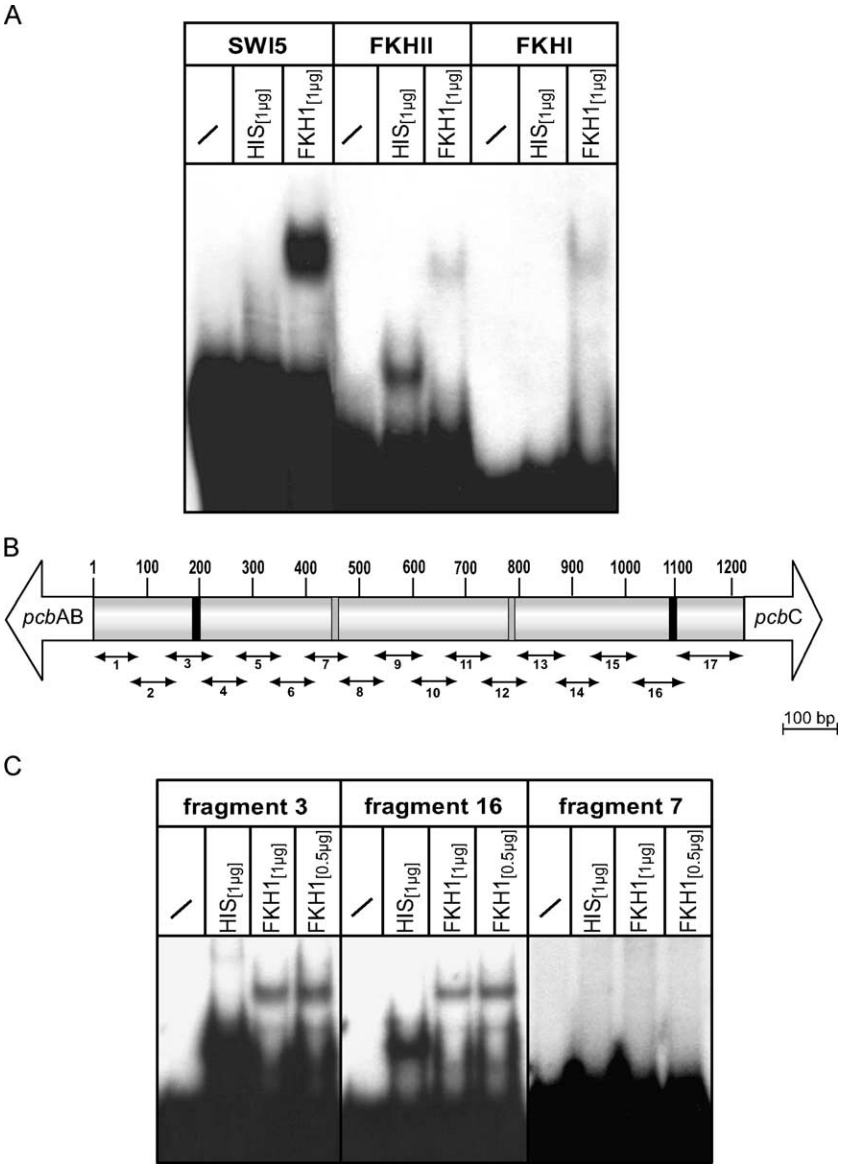


Fig. 5. Identification of forkhead protein-binding sites in the *pcbAB–pcbC* promoter. (A) Gel retardation analysis using 1 µg of recombinant AcFKH1 protein (FKH1) and control protein (HIS) with two different oligonucleotide probes, FKHI/FKHII representing the AcFKH1-binding sequences. The ScFKH2-binding site from the yeast *SWI5* gene was used as a control. (B) Schematic representation of the promoter region with CPCR1- (grey) and FKH1- (black) binding sites; 17 overlapping PCR fragments used in a promoter scanning analysis are indicated by double-headed arrows and numerals. (C) Gel retardation analysis using 0.5–1 µg of recombinant AcFKH1 protein (FKH1) and a control (HIS) with three different DNA fragments (numbers 3, 16, and 7) from the *pcbAB–pcbC* intergenic region in *A. chrysogenum*.

tion of CPCR1, protein DIM1, inhibited both the dimerization and the interaction with FKH1, whereas the protein DIM2 forms a homodimer, but did not interact with FKH1 (see Table 2). Both proteins lack the most C-terminal part of CPCR1. With DIM3, which represents solely the C-terminus, a homodimer was formed that interacted with FKH1. These results strongly imply that the interaction interface may be generated through the tertiary or quartiary structure of CPCR1 via the surface of the dimer. Dimerization of CPCR1 is strong, and a monomer cannot be detected in biochemical studies under native conditions (Schmitt and Kück, 2000). From the two-hybrid studies here, we cannot conclude whether a single molecule of AcFKH1 or two molecules interact with a CPCR1 dimer.

4.3. Is CPCR1 the only protein interacting with AcFKH1?

The novel transcription factor AcFKH1 contains the DNA-binding forkhead domain and an N-terminal FHA domain. FHA domains mediate phosphoprotein interactions not only in transcription factors but also in different types of proteins, e.g. kinases (Li et al., 2000). Their presence indicates that the FHA-containing protein will interact with a protein partner in a process regulated by reversible protein phosphorylation. Recently, their interactions were shown not

to be restricted to phospho-peptides as phospho-independent interaction is important for the function of the FHA domain in the tumor suppressor kinase Chk2 (Li et al., 2000).

The two-hybrid results show that CPCR1 does not interact with the FHA domain of FKH1. This implies the probability of additional interaction partners of AcFKH1 in *A. chrysogenum* and a possible link to the signal transduction via reversible phosphorylation (see Fig. 6).

We are highly aware that results obtained from the yeast two-hybrid system can give false negatives. For example, CPCR1 may be phosphorylated in *A. chrysogenum*; however, this signal is missing in yeast, and thus no interaction can be observed. The CPCR1 homologue of yeast, the DNA-damage check-point regulator CRT1, is phosphorylated and its activity is regulated via this phosphorylation (Huang et al., 1998). Nevertheless, it is possible to detect interactions involving FHA domains in yeast. At least two heterologous proteins have been identified in a two-hybrid screen and shown to interact with the FHA-domain containing fragment of the human KI-67 (Sueishi et al., 2000; Takagi et al., 2001).

4.4. FKH1 acts as a transcriptional activator in yeast

CPCR1 itself has only very weak activation properties in yeast (see Table 2). The mode in which CPCR1 acts in transcriptional regulation in *A. chrysogenum* is still a matter of debate. In a *lacZ* reporter experiment, we found that the deletion of two CPCR1-binding sites in the *pcbC* promoter results in a reduced transcriptional activation of about 12% (reduction of 88%) after 7 days of cultivation. However, quantitative measurements of transcript levels using macroarrays indicated a more complex picture. The *pcbC* transcript increased for up to 1.5 days of cultivation, after which transcript levels reduced (Schmitt et al., 2004a). As FKH1 activates transcription in yeast, it might also function as a transcriptional activator in *A. chrysogenum*. From this, we propose that AcFKH1 could be the mediator, which allows transcriptional activation through a CPCR1–FKH1 complex in *A. chrysogenum*. However, this hypothesis has to be proven by further experiments with *A. chrysogenum*.

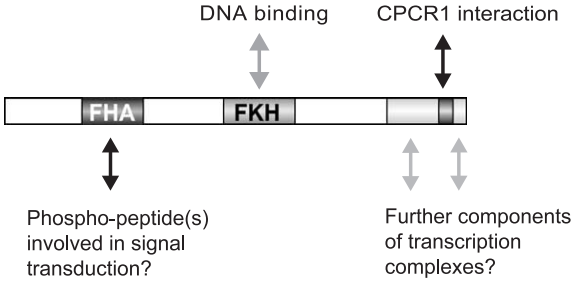


Fig. 6. Schematic representation of AcFKH1 with binding sites involved in DNA or protein interactions. Arrows indicate the corresponding interaction partners of the highlighted AcFKH1 regions. The DNA-binding and the CPCR1 interaction have been clearly proved experimentally, whereas the interaction with components from the transcription machinery was deduced from the transcriptional activity of AcFKH1 in yeast in vivo experiments. The interaction with phospho-proteins mediated by the FHA domain was suggested by literature.

The interaction with CPCR1 was assigned to a large stretch of acid amino acids in the C-terminus of FKH1 (pos. 626–640), but these residues are not involved in transcriptional activation. Similar to protein 78, the protein 78Δ3, which lacks this stretch of amino acids, activates transcription in yeast (see Table 2). Thus, the interaction with proteins from the yeast transcription apparatus is mediated by residues 551–626 and/or 640–665 (see Fig. 6). Amino acid positions before residue 551 are not involved as could be shown with proteins 78Δ1 and 78Δ2.

The C-terminus of the forkhead protein Mei4+ from *S. pombe* also acts as an activation domain (pos. 343–517) (Horie et al., 1998). Forkhead proteins often contain several activating regions and these can be found in any location relative to the DNA-binding domain. In addition, there is an almost total lack of similarity between activation domains in different forkhead proteins (Carlsson and Mahlapuu, 2002).

In general, acid amino acids present on a protein surface are often involved in protein–protein interaction as their negative charge allows high binding affinities. Another characteristic of protein–protein interaction surfaces is their flexible secondary structure. It has been suggested that a certain degree of flexibility is required to allow an interaction and that the partially unfolded domain takes shape when proteins interact with their proper binding partner The high abundance of proline residues in the C-terminal part of FKH1 probably hinders the formation of a rigid secondary structure and might favor flexibility which can result in interaction.

4.5. Abundance and role of FKH proteins in filamentous fungi

The two yeasts *S. cerevisiae* and *S. pombe* each have four forkhead proteins with overlapping and distinct functions and DNA-binding specificities. The *N. crassa* genome sequence contains only one region with significant identity to the forkhead DNA-binding domain. A comparison of the protein sequence from AcFKH1 with the *N. crassa* sequences results in an overlap of 711 amino acids with greater than 40% identity. Due to an array of genome defense mechanisms, *N. crassa* has a low proportion of genes in multigene families (Galagan et al., 2003), and therefore the single forkhead gene copy might not be representative for other filamentous fungi. At this experimental stage, whether further forkhead genes exist in *A. chrysogenum* is still to be determined.

The yeast ScFKH2 and ScFKH1 are involved in cell-cycle control via their role in activating the CLB2 cluster and the separate aspect of pseudohyphal growth (Zhu et al., 2000). Forkhead proteins are also associated with cell cycle and death control in humans as well as *Caenorhabditis elegans* and *Drosophila melanogaster* (Burgering and Kops, 2002). In eukaryotes, forkhead proteins regulate a

diverse range of developmental processes, and alterations in forkhead gene dosage have been connected with human diseases. Recently, an additional role of forkhead proteins in the regulation of gene expression in *S. cerevisiae* was discovered; this strengthens the importance of forkhead proteins for gene expression in general. ScFKH1 and ScFKH2 coordinate early transcription elongation and pre-mRNA processing in yeast (Morillon et al., 2003).

To the best of our knowledge, the *A. chrysogenum* AcFKH1 protein is the first forkhead transcription factor from filamentous fungi with known target genes and a proposed function. The FHA domain of AcFKH1 might link signal transduction cascades to transcriptional regulation of cephalosporin C biosynthesis. DNA-binding analysis with FKH1 identified two forkhead-binding sites containing the core consensus in the *pcbAB–pcbC* promoter region. However, oligonucleotides corresponding to the sequences are bound by AcFKH1 only with low affinity in vitro. It is possible that other proteins are required to form a stable DNA–protein complex. For example, in yeast, FKH2–MCM1 increases FKH2–DNA-binding affinity in vitro even in the absence of MCM1-binding sites (Hollenhorst et al., 2001). Whether CPCR1 influences AcFKH1-binding affinity is currently not known. The binding sites of CPCR1 and FKH1 are not directly adjacent to each other (see Fig. 5). However, forkhead proteins can induce strong bending of a DNA molecule, which might result in a loop in the promoter. Further in vitro and in vivo work will be directed towards the elucidation of AcFKH1 function in *A. chrysogenum*, especially in respect to cephalosporin C biosynthesis.

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