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Identification of a Major *cis*-Acting DNA Element Controlling the Bidirectionally Transcribed Penicillin Biosynthesis Genes *acvA* (*pcbAB*) and *ipnA* (*pcbC*) of *Aspergillus nidulans*

KATHARINA THEN BERGH, OLIVIER LITZKA, AND AXEL A. BRAKHAGE*

Lehrstuhl für Mikrobiologie, Universität München, D-80638 Munich, Germany

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The β-lactam antibiotic penicillin is produced as a secondary metabolite by some filamentous fungi. In this study, the molecular regulation of the *Aspergillus* (*Emericella*) *nidulans* penicillin biosynthesis genes *acvA* (*pcbAB*) and *ipnA* (*pcbC*) was analyzed. *acvA* and *ipnA* are divergently oriented and separated by an intergenic region of 872 bp. Translational fusions of *acvA* and *ipnA* with the two *Escherichia coli* reporter genes *lacZ* and *uidA* enabled us to measure the regulation of both genes simultaneously. A moving-window analysis of the 872-bp intergenic region indicated that the divergently oriented promoters are, at least in part, overlapping and share common regulatory elements. Removal of nucleotides −353 to −432 upstream of the *acvA* gene led to a 10-fold increase of *acvA-uidA* expression and simultaneously to a reduction of *ipnA-lacZ* expression to about 30%. Band shift assays and methyl interference analysis using partially purified protein extracts revealed that a CCAAT-containing DNA element within this region was specifically bound by a protein (complex), which we designated PENR1, for penicillin regulator. Deletion of 4 bp within the identified protein binding site caused the same contrary effects on *acvA* and *ipnA* expression as observed for all of the deletion clones which lacked nucleotides −353 to −432. The PENR1 binding site thus represents a major *cis*-acting DNA element. The intergenic regions of the corresponding genes of the β-lactam-producing fungi *Penicillium chrysogenum* and *Acremonium chrysogenum* also diluted the complex formed between the *A. nidulans* probe and PENR1 in vitro, suggesting that these β-lactam biosynthesis genes are regulated by analogous DNA elements and proteins.

Fungi produce numerous secondary metabolites. Some of these compounds have been used as antibiotics or antitumor agents (7). The analysis of the genetic regulation of their biosyntheses opens up the possibility of elucidating special cases of gene regulation in eukaryotes. Since some of the secondary metabolites have beneficial properties, e.g., as antibiotics, a better understanding of the regulation of their biosyntheses might help to improve production strains. Hence, we wished to address the question what kind of regulators and *cis*-acting DNA sequences are involved in the regulation of secondary metabolism genes in eukaryotes. Do these differ from known regulators and DNA elements involved in the regulation of genes of the primary metabolism?

As a model system for secondary metabolism, we have been studying the regulation of penicillin biosynthesis. Penicillin together with another widely used antibiotic, cephalosporin, belongs to the group of β-lactam antibiotics. Cephalosporins are produced by both some bacteria and some filamentous fungi, e.g., the fungus *Acremonium chrysogenum* (synonym, *Cephalosporium acremonium*). In contrast, penicillins are synthesized as end products by only a limited number of fungi, most notably *Penicillium chrysogenum* and *Aspergillus* (*Emericella*) *nidulans* (reviewed in references 1, 12, 18, 44, and 50).

Penicillin biosynthesis is catalyzed by three enzymes which are encoded by three genes: *acvA* (*pcbAB*), *ipnA* (*pcbC*), and *aat* (*penDE*), encoding δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase, isopenicillin N synthase, and acyl coenzyme A:6-aminopenicillanic acid acyltransferase, respectively. The genes have been cloned and sequenced. They are organized into a gene cluster in both *A. nidulans* and *P. chrysogenum* (Fig. 1) (reviewed in references 1, 12, 44, and 50). Since the biosynthetic pathways leading to penicillin and cephalosporin share the first two reactions, the fungi producing these compounds have the genes *acvA* (*pcbAB*) and *ipnA* (*pcbC*) in common (Fig. 1).

We have been studying the transcriptional regulation of the *A. nidulans* genes *acvA* and *ipnA*, which are divergently transcribed and separated by an 872-bp intergenic region (Fig. 1). Several regulatory circuits affecting the expression of these genes have been identified, suggesting that a variety of regulatory factors are involved (9–11, 20, 21).

The identification of the first regulatory protein was published by Espeso et al. (21) and Tilburn et al. (54), who showed that the *ipnA* gene is regulated by PACC, a regulatory protein which mediates pH regulation of several *A. nidulans* genes, e.g., of the alkaline phosphatase and protease genes *palD* and *prtA*, respectively (54). The intergenic region between *acvA* and *ipnA* of *A. nidulans* was found to contain at least five in vitro PACC binding sites, bound by a glutathione *S*-transferase::PACC fusion protein (54). The fusion protein was demonstrated to bind to the core consensus GCCARG (54). The physiological meaning of the involvement of PACC in the regulation of *ipnA* is not entirely understood. In addition, the identification of recessive *trans*-acting mutations in genes which are involved in the regulation of the *A. nidulans* genes *acvA* and/or *ipnA*, using genetic approaches, was reported (13, 39). Because the cloning of the corresponding genes has not been published, it is not known to which kind of putative regulatory genes these mutations correspond.

Several groups have begun to characterize the intergenic region of the corresponding *P. chrysogenum acvA* (*pcbAB*) and *ipnA* (*pcbC*) genes by band shift assays. Chu et al. (17) and Feng et al. (23) reported independently that partially purified

* Corresponding author. Mailing address: Lehrstuhl für Mikrobiologie, Universität München, Maria-Ward-Straße 1a, D-80638 Munich, Germany. Phone: 49 89 17919867. Fax: 49 89 17919862. Electronic mail address: uj44219@sunmail.LRZ-muenchen.de.

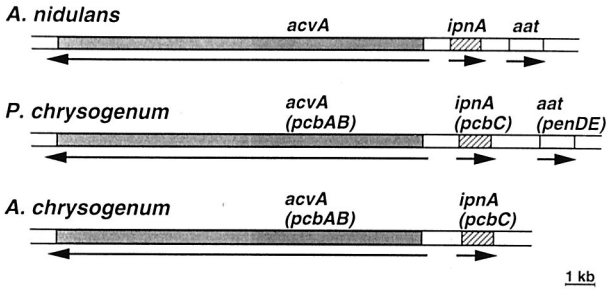

FIG. 1. β-Lactam biosynthesis gene clusters of some fungi: penicillin biosynthesis gene clusters of *A. nidulans* and *P. chrysogenum* and cephalosporin biosynthesis genes of *A. chrysogenum* (reviewed in references 1 and 12).

crude extracts of *P. chrysogenum* bound in vitro to the sequences TGCCAAG and GCCAAGCC, respectively, which appear to be similar to the PACC binding sites identified in *A. nidulans* (54). Haas and Marzluf (24) observed that the nitrogen regulatory protein NRE of *P. chrysogenum* bound to GATA-containing sequences in the intergenic region in vitro. In conclusion, it appears that a number of proteins bind to the region between the genes *acvA* and *ipnA*. However, experiments providing evidence for the significance of the identified DNA sequences in vivo have not been published. For PACC, however, it was shown that under alkaline conditions, the amounts of *ipnA* transcript were increased (21), which strongly supported the significance of binding of PACC in vitro (54). Furthermore, in a previous study, Pérez-Esteban et al. (40) reported a deletion analysis of the *ipnA* gene of *A. nidulans* without determining the effect of the deletions on the expression of the divergently transcribed *acvA* gene. Divergently transcribed genes provide an interesting case of regulation because single *cis*-acting sites might have the potential to regulate two genes simultaneously. To date, no data on *cis*-acting elements controlling the *acvA* gene of *A. nidulans* have been reported. Therefore, we wished to address the question of whether possible functional interactions between the promoters of *acvA* and *ipnA* exist and whether they share common *cis*-acting DNA elements coordinating the expression of both genes.

In this study, we used a reporter gene system developed by Punt et al. (43) which allowed measurement of the effects of deletions in the region between *acvA* and *ipnA* on the expression of both genes simultaneously. A major *cis*-acting DNA element which is involved in the regulation of both genes was characterized in detail by both genetic and biochemical methods.

MATERIALS AND METHODS

Strains and oligonucleotides. Bacterial and fungal strains used in this study are listed in Table 1. Vectors and plasmids were constructed and propagated in *Escherichia coli* DH5α. Oligonucleotides were synthesized by MWG Biotech (Ebersberg, Germany) and are listed in Table 2. They were designed from published sequences (28, 31, 35, 51).

Media and fermentation conditions. Fermentations were carried out in *Aspergillus* minimal medium (AMM) essentially as previously described (13, 29). Seed cultures contained 4% (wt/vol) glucose and experimental cultures contained 4% (wt/vol) lactose as the carbon source. If required, biotin (0.3 μg/ml) or L-arginine (0.2 mg/ml) was added to the flasks. Experimental cultures (20 ml of AMM in 250-ml flasks) were inoculated with 1 ml of the seed culture suspension, incubated for 48 h, and harvested.

Standard DNA techniques, PCR amplification, and DNA sequence analysis. Standard techniques for the manipulation of DNA were carried out as described by Sambrook et al. (47) or as previously described (9). PCR amplification of DNA was performed essentially as previously described (29). For DNA sequence analysis, the dideoxynucleotide chain termination procedure (48) on purified double-stranded plasmids was followed. For sequencing of the junctions of the translational fusions, primers β-GalP and β-GluP (Table 2), complementary to a part of the *E. coli lacZ* and *uidA* genes, respectively, were used. Sequence analysis to confirm the precise positions of the deletions in the intergenic region of *acvA* and *ipnA* and to exclude the introduction of base substitutions by PCR was carried out with oligonucleotides IntSeqP1 (Table 2) and the universal and reverse primer for sequencing of pUC derivatives.

Construction of the recombinant plasmids. For numbering of the nucleotides within the 872-bp intergenic region, nucleotide (nt) −1 of the *acvA* gene was designated nt 1 (Fig. 2). Plasmid pXEP7 carrying a part of the penicillin biosynthesis gene cluster was described by Brakhage et al. (9). The following plasmids were constructed by using standard procedures. To create pΔBS, plasmid pBSKS$^+$ (Stratagene, La Jolla, Calif.) was digested with *Cla*I and *Spe*I, the 5′ sticky ends were filled in with Klenow polymerase, and the vector was religated. This led to the deletion of some restriction sites (*Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I, *Sma*I, and *Spe*I) of the pBSKS$^+$ polylinker. The loss of the underlined restriction sites was useful for the cloning steps described below.

The following plasmids were all derivatives of pΔBS.

(i) pΔBSFLIRT. The region between the *A. nidulans* genes *acvA* and *ipnA* was amplified by PCR using oligonucleotides acvAP and ipnAP, both encoding *Not*I sites (Table 2), and plasmid pXEP7 as the template. The PCR product was cut with *Not*I and inserted into the unique *Not*I site of pΔBS.

(ii) pΔBSΔ1-493. The region between nt 494 and 872 was amplified by PCR using oligonucleotides 493acvAP and ipnAP, with plasmid pXEP7 as the template. The product was digested with *Not*I and inserted into the single *Not*I site of pΔBS.

(iii) pΔBSΔ428-872. The region between nt 1 and 427 was amplified by PCR using primers acvAP and 428ipnAP, with plasmid pXEP7 as the template. The product was cut with *Not*I and inserted into the *Not*I site of pΔBS.

(iv) pΔBSΔ43-182. pΔBSFLIRT was digested with *Eco*RV and *Stu*I, treated with mung bean nuclease, and religated.

TABLE 1. Bacterial and fungal strains used in this study

Strain	Relevant genotype and/or phenotype	Source or reference
E. coli DH5α	F$^-$ F80d/*lacZ* M15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17* (r_K^- m_K^+), *supE44* λ$^-$ *thi-1 gyrA96 relA1*	Bethesda Research Laboratories, Gaithersburg, Md.
A. nidulans		
R21	*pabaA1 yA2*	22
WG355	*biA1 bga0 argB2*	22 55
FLIRT	WG355; *argB2*::pFLIRT ArgB$^+$	This study
Δ428-872	WG355; *argB2*::pΔ428-872 ArgB$^+$	This study
Δ1-493	WG355; *argB2*::pΔ1-493 ArgB$^+$	This study
Δ43-182	WG355; *argB2*::pΔ43-182 ArgB$^+$	This study
Δ183-312	WG355; *argB2*::pΔ183-312 ArgB$^+$	This study
Δ183-432	WG355; *argB2*::pΔ183-432 ArgB$^+$	This study
Δ309-432	WG355; *argB2*::pΔ309-432 ArgB$^+$	This study
Δ353-569	WG355; *argB2*::pΔ353-569 ArgB$^+$	This study
ΔCCA-G	WG355; *argB2*::pΔCCA-G ArgB$^+$	This study
Acremonium chrysogenum		ATCC 11550
P. chrysogenum		NRRL 1951

TABLE 2. Oligonucleotides used in this study

Name	Sequence[a]
ipnAP	5′-CTGGCGGCCGCCATTATGAATGAGGGCAAGCAGTTG-3′
acvAP	5′-CCGGCGGCCGCCATTTTGATTCTCTTATTTCACGGAGGG-3′
428ipnAP	5′-TCAGCGGCCGCCATGTTACCCTTTTGCTATTGGCC-3′
493acvAP	5′-CCAGCGGCCGCCATCAAATTGTGGTACGAGCCAAGC-3′
IntSeqP1	5′-CGTACCACAATTTGATGAG-3′
202acvAP	5′-GATTAGGGACGTCGCTACCCGGCAATCCAG-3′
409ipnAP	5′-GTTACCCTTTT**GGATCC**TTTCCCCCAATATAGGGC-3′
409acvAP	5′-GGAAA**GGATCC**AAAAGGGTAACAAGCTTAG-3′
392ipnAP	5′-CTTTCCC*CCATGG*GCCAATATTACGGTGCAG-3′
392acvAP	5′-TATTGGC*CCATGG*GGGAAAGGGCCAATCGC-3′
AchacvAP	5′-CACGGACCGGATCCAGC-3′
AchipnAP	5′-CATGGTGACGGTTTGTCC-3′
PchacvAP	5′-CATTTTTCACACTCGAGC-3′
PchipnAP	5′-CATGGTGTCTAGAAAAATAATGG-3′
β-GalP	5′-CCTCTTCGCTATTACGCC-3′
β-GluP	5′-CCACAGTTTTCGCGATCCAG-3′

[a] *Not*I restriction sites are underlined. *Bam*HI and *Nco*I sites are indicated in boldface and italics, respectively.

(v) pΔBSΔ183-312. pΔBSFLIRT was cut with *Stu*I and *Spe*I, treated with mung bean nuclease, and religated.

(vi) pΔBSΔ183-432. pΔBSFLIRT was cut with *Stu*I and *Hin*dIII, treated with mung bean nuclease, and religated.

(vii) pΔBSΔ309-432. pΔBSFLIRT was cut with *Spe*I and *Hin*dIII, treated with mung bean nuclease, and religated.

(viii) pΔBSΔ353-569. pΔBSFLIRT was cut with *Pst*I and religated.

(ix) pΔBSΔTAT-T. To generate specific deletions of four nucleotides within the intergenic region, the method of Higuchi et al. (26) was used. Hence, parts of the intergenic region were independently amplified by PCR using pΔBSFLIRT as the template and either primers acvAP and 392ipnAP or primers ipnAP and 392acvAP. The products were purified on an agarose gel and used as templates for a second PCR with oligonucleotides acvAP and ipnAP. The product of the second PCR was digested with *Not*I and inserted into the *Not*I site of pΔBS.

(x) pΔBSΔCCA-G. The construction was identical to that described for pΔBSΔTAT-T except for the use of the primers 409ipnAP and 409acvAP instead of 392ipnAP and 392acvAP, respectively.

Plasmids pFLIRT, pΔ1-493, pΔ428-872, pΔ43-182, pΔ183-312, pΔ183-432, pΔ309-432, pΔ353-569, and pΔCCA-G were all constructed in the following way. The corresponding pΔBS derivatives were cut with *Not*I, which in each case gave two DNA fragments consisting of the vector and the insert. The insert fragments containing the intergenic regions with and without different deletions were ligated into the unique *Not*I site of the vector pTRAN2. Each of the latter plasmids thus contained the indicated deletion within the intergenic region, except the wild-type plasmid pFLIRT, with ATG initiation codons at their ends fused in frame with the *E. coli* reporter genes *uidA* and *lacZ*.

For methyl interference assay, plasmid pMIA was constructed by ligating a *Pst*I-*Hin*dIII restriction fragment obtained from pΔBSFLIRT spanning nt 353 to 432 of the intergenic region with the *Pst*I-*Hin*dIII digested vector pBSKS$^+$.

Transformation and identification of single-copy transformants. *A. nidulans* WG355 (Table 1) was transformed to arginine prototrophy as previously described (5, 9). For transformation, plasmid DNA purified by CsCl gradient centrifugation (47) or by chromatography through anion-exchange columns (GENOMED, Bad Oeynhausen, Germany) was used. All *A. nidulans* transformant strains described in this study carried the indicated plasmids integrated in single copy at the chromosomal *argB* gene locus. Single-copy transformants were identified by Southern blot analysis essentially as previously described (13).

β-Gal and β-Glu activity assays. β-Galactosidase (β-Gal) and β-glucuronidase (β-Glu) activities were determined in mycelial extracts from cells grown in AMM essentially as previously described (9).

Determination of protein concentrations. Protein concentrations were determined by the method of Bradford (8).

Preparation of crude extracts for DNA binding assays. *A. nidulans* R21 was grown in AMM containing 2% (wt/vol) glucose, 10 mM NH_4Cl to repress the synthesis of endogenous proteases, and *p*-aminobenzoic acid (0.3 mg/100 ml) at 37°C for 24 h with shaking (250 rpm). Proteins for band shift assays derived from glucose-grown cultures because they gave higher protein concentrations. Since the use of the carbon source might have influenced the presence of DNA-binding proteins, it should be noted that the expression of *acvA* was not affected by the use of glucose instead of lactose (9). Furthermore, data essentially the same as those shown in Fig. 2 for the deletion clones grown in lactose were obtained for clones grown in glucose-containing medium (not shown).

Mycelia were harvested through Mira cloth (Calbiochem, La Jolla, Calif.), rapidly frozen with liquid nitrogen, ground with a pestle and mortar, and resuspended in extraction buffer (25 mM Tris Cl [pH 7.5], 5 mM magnesium acetate, 3 mM dithiothreitol). The samples were centrifuged at 30,000 × *g* and 4°C for 20 min. The supernatant was withdrawn and centrifuged again at 100,000 × *g* and 4°C for 120 min. Chromatography of the supernatant was performed at 4°C in a minicolumn with 5 ml of heparin-agarose resin (Bio-Rad, Richmond, Calif.) equilibrated with 15 ml of extraction buffer. After 20 to 50 mg of protein was loaded, the column was washed with extraction buffer until no protein was detected in the eluate by measuring the relative A_{280}. Proteins bound to the resin were eluted by using a series of extraction buffers containing KCl concentrations of 50, 100, 200, 300, 400, and 1,000 mM. Fractions obtained with each KCl concentration were collected, and the protein was precipitated by addition of 4 volumes of a saturated ammonium sulfate solution. The solution was centrifuged at 20,000 × *g* at 4°C for 15 min. The pellet was dissolved in extraction buffer and dialyzed against 2,000 volumes of extraction buffer at 4°C overnight. The protein-containing solution was adjusted to a glycerol concentration of 25% (vol/vol).

Band shift assays. For band shift assays, the method described in reference 4 was essentially followed. The 80-bp fragment mainly used for band shift assays as the probe was obtained by cutting the intergenic region of *acvA* and *ipnA* present in plasmid pΔBSFLIRT with *Pst*I and *Hin*dIII (see Fig. 3B). The probe was radioactively end labeled with different α-^{32}P-labeled deoxynucleoside triphosphates (dNTPs), using Klenow polymerase. Each binding assay mixture contained 0.25 ng (about 5 fM) of radioactively labeled probe, 5 μg of protein, and as unspecific competitor DNA 500 ng of poly(dI-dC). After incubation at 20°C for 5 to 15 min and addition of loading dye, the samples were run on a 5.0% (wt/vol) nondenaturing polyacrylamide gel in a minigel apparatus (Biometra, Göttingen, Germany) in 1× Tris-borate-EDTA buffer at room temperature with 55 V. The gels were transferred to blotting paper (Schleicher & Schüll, Dassel, Germany), dried in a gel drier, and subjected to autoradiography.

Methyl interference assay. The methyl interference assay was performed essentially as described in reference 4. Briefly, a 143-bp fragment was generated by cutting plasmid pMIA with *Xho*I and *Sac*I. The fragment was agarose gel purified and end labeled at the *Xho*I site with α-^{32}P-labeled dNTPs, using Klenow polymerase, resulting in a labeled top strand only. After methylation of the DNA, the

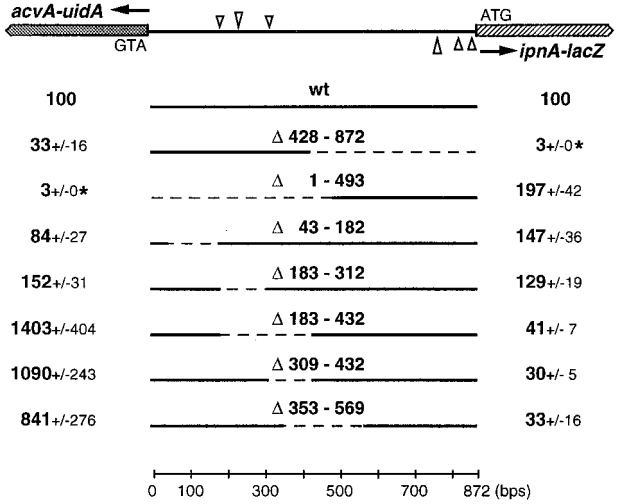

FIG. 2. Determination of *cis*-acting DNA elements involved in the expression of *acvA* and *ipnA* in vivo. Transformants of *A. nidulans* carrying the various plasmids integrated in single copy at the chromosomal *argB* gene locus were identified by Southern blot analysis (not shown; essentially as previously described [13]). Expression of *ipnA-lacZ* and *acvA-uidA* gene fusions was determined as β-Gal and β-Glu specific activities, respectively. Values are given as relative activities. The data obtained with the wild-type strain FLIRT (wt) carrying the full-length intergenic region between the reporter genes was set at 100. The relative values given for each deletion clone represent the mean and standard deviation of several complete experiments. In each of these experiments, the strains were grown in two flasks which were simultaneously harvested. Within a single experiment, the standard deviation of values, including those of the control measured from the two different flasks of the same strain, was less than 10%. Deletions are indicated by dashed lines. Transcription initiation sites of *acvA* and *ipnA* determined by MacCabe et al. (31) and Pérez-Esteban et al. (40), respectively, are labeled by triangles. The vector pTRAN2 integrated in single copy at the *argB* gene locus gave a relative activity (background) of about 1% (not shown). *, the residual β-Gal and β-Glu activities measured in clones Δ428-872 and Δ1-493, respectively, presumably resulted from the introduction of ATG start codons in front of the reporter genes, in combination with some unspecific transcription initiation.

binding reaction and separation of samples were performed as described for band shift assays except that 20 times the amount of DNA (100,000 cpm) and 5 times the amount of protein were incubated in a 5-times-larger volume. Without drying, gels were autoradiographed, and the bound and free probes were excised from the gel. After being recovered, the probes were cleaved by piperidine at the G residues as described by Maxam and Gilbert (33). The reaction products were analyzed on a 10% (wt/vol) polyacrylamide-urea sequencing gel.

RESULTS

In vivo deletion analysis indicated that multiple distinct *cis*-acting elements control the expression of *A. nidulans* penicillin biosynthesis genes *acvA* and *ipnA*. To identify *cis*-acting DNA elements, a moving-window analysis was carried out by introducing a series of deletions into the intergenic region (Fig. 2). All deletion clones contained ATG initiation codons which were fused in frame with both the *E. coli lacZ* and *uidA* reporter genes located on plasmid pTRAN2 (9, 43). DNA sequence analysis of the intergenic regions of all recombinant plasmids confirmed the precise positions of the deletions and the generation of translational fusions (not shown). Single-copy transformants of *A. nidulans* WG355 for each plasmid were named as indicated in Fig. 2. Nucleotides were numbered with nt −1 of the *acvA* gene designated nt 1. Consequently, nt 872 is the last nucleotide in front of the ATG initiation codon of the *ipnA* gene (Fig. 2). The expression of *acvA-uidA* and *ipnA-lacZ* gene fusions of the various strains was determined during a fermentation run in AMM with 4% (wt/vol) lactose as the carbon source. The results are summarized in Fig. 2.

Deletion of each half of the intergenic region had different effects on the expression of each gene fusion. Removal of nt 428 to 872 (strain Δ428-872) led to drastically reduced *acvA-uidA* expression. Deletion of nt 1 to 493 (strain Δ1-493) led to increased *ipnA-lacZ* expression (Fig. 2). These results indicated that between nt 428 and 872 there is a *cis*-acting element(s) positively influencing *acvA* expression, and between nt 1 and 493 there is an element(s) negatively affecting *ipnA* expression (Fig. 2), although each of these sites is located in the half of the intergenic region closer to the opposite gene. This finding suggests that the promoters of *acvA* and *ipnA* are, at least in part, physically overlapping. Deletion of nt 43 to 182 (strain Δ43-182) had only a slight effect on *ipnA-lacZ* expression (Fig. 2). In this clone, two of the five in vitro-determined PACC sites at nt 149 to 154 and 174 to 179 (54) were deleted. Because there were three additional PACC binding sites at nt 265 to 270, 509 to 514, and 524 to 529 (54), deletion of two sites might not be sufficient to cause a major effect on *ipnA* expression. In addition, under our experimental conditions using relatively low pH values which gave maximal penicillin titers in fermentation medium (9, 53), the influence of PACC might not be detectable.

Deletion of nt 183 to 312 led to a slightly increased expression of both *acvA* and *ipnA* (Fig. 2). Formally, this finding suggests that within this region, there is a DNA sequence(s) which negatively contributes to the expression of both genes, implying the binding of a negatively acting factor(s). For *ipnA* expression, this could be due to PACC, because a PACC site (nt 265 to 270) has been found in vitro to reside within this region (54).

The strongest effect, however, was seen in strains Δ183-432, Δ309-432, and Δ353-569 (Fig. 2). These strains showed about 10-fold-increased *acvA-uidA* expression, whereas *ipnA-lacZ* expression was simultaneously reduced to about a third of the level measured for a strain carrying the wild-type intergenic region. Furthermore, all of these strains lacked nt 353 to 432. These findings indicated that major *cis*-acting DNA element(s) resides within these 80 bp. It is noteworthy that there is no PACC site within this DNA region, (54), excluding the possibility that PACC could be involved in the effects measured on both genes.

The DNA region identified in vivo specifically formed a complex with a DNA-binding protein(s) in vitro. To analyze whether the DNA region of 80 bp (nt 353 to 432) identified by deletion analysis contained a recognition site for a DNA-binding protein, band shift assays were carried out. As the probe, an 80-bp fragment (nt 353 to 432) was isolated by digestion with *Pst*I and *Hin*dIII of plasmid pΔBSFLIRT and radioactively labeled (Fig. 3B). A major shifting band consisting of probe and protein was observed with the protein fraction eluted from a heparin-agarose column with 200 mM KCl (Fig. 3A). A weakly labeled band, most likely due to a complex of DNA with the same protein as in the 200 mM fraction, was also detectable with the 300 mM fraction (Fig. 3A). Under the experimental conditions applied, no DNA-binding activity was detected when fractions eluted with KCl concentrations others than 200 and 300 mM were used for band shift experiments with the 80-bp DNA fragment as the probe (Fig. 3A). Since the strongest band was observed with the 200 mM fraction, this fraction was used for the experiments described below.

To provide evidence for the specificity of the band shift detected, cross-competition experiments were carried out (Fig. 3B and C). Excess (2,000-fold in weight of the probe) unspecific poly(dI-dC) DNA, which was used in all band shift assays, did not abolish the shift, whereas a 30-fold molar excess of an unlabeled fragment spanning the whole wild-type intergenic region led to the dilution of the band shift signal (Fig. 3C, lane 4). These experiments demonstrated the specificity of DNA-protein complex formation. Additional experiments shown in Fig. 3C (lanes 5 to 10) confirmed this conclusion. The shift of the 80-bp fragment was still present when competitor DNA derived from the intergenic regions of clones Δ309-432 and Δ353-569 was used (Fig. 3B). Addition of these DNA fragments in increasing molar excess of up to 30-fold did not prevent complex formation (Fig. 3C, lanes 5 to 10), indicating that major binding sites required for the formation of the identified DNA-protein complex reside only within the identified nt 353 to 432. However, the DNA fragment derived from clone Δ309-432 led to a slight dilution of the complex when added in a molar excess of 30-fold (Fig. 3C, lane 7), implying that at least in vitro, an additional low-affinity binding site might be present in the intergenic region.

The protein-DNA contact occurred at guanosine nucleotides spanning nt 407 to 415 (GGCCAATCG). To determine which of the nucleotides within the identified DNA region are in contact with the DNA-binding protein, a methyl interference assay was used. Dimethyl sulfate treatment leads to methylation at position N-7 of guanosines of the DNA. Methylated guanosines cannot be bound by DNA-binding proteins, allowing the identification of G's important for binding, as described by Brunelle and Schleif (14).

The top strand of a DNA fragment spanning nt 353 to 432 of the intergenic region was subject to methyl interference analysis (Fig. 4). We detected a single region (nt 407 to 415) in which three G's are involved in the complex and interfere with complex formation when methylated (Fig. 4). Apparently, the G (nt 406) located just in front of the two G's important for binding (nt 407 and 408) did not show an interference, enabling a precise delimitation of the DNA sequence in contact with the protein at the 5′ site. It has not been established whether a nucleotide beyond the last G at the 3′ site (nt 415) is essential for binding of the protein. From the data shown in Fig. 4, it can be concluded, however, that at least the G at position 421 was not bound by the protein. Hence, the protein

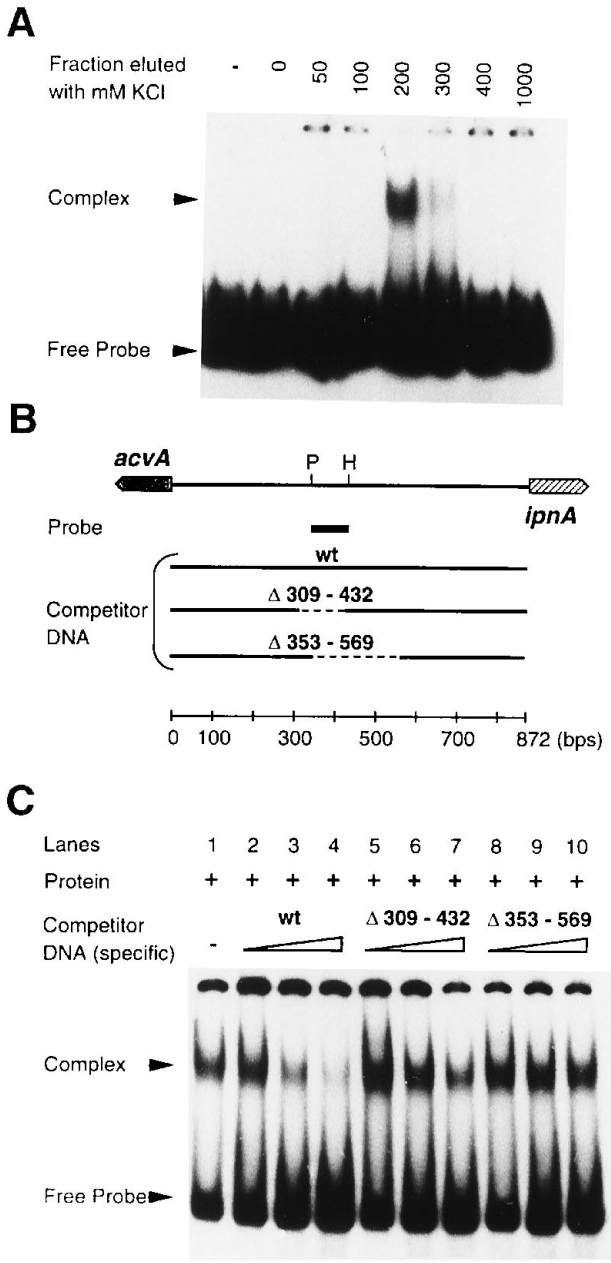

FIG. 3. Identification of protein fractions of *A. nidulans* crude extract specifically binding to an 80-bp DNA fragment spanning nt 353 to 432. (A) Band shift assays. Protein fractions of *A. nidulans* eluted from the heparin-agarose column with extraction buffers containing different KCl concentrations were incubated in the binding reaction with the probe consisting of an 80-bp DNA fragment spanning nt 353 to 432, as schematically shown in panel B. (B) Schematic diagram of the probe (bold line) and the fragments used for cross-competition experiments. Abbreviations: P, *Pst*I; H, *Hin*dIII. (C) Band shift assays. The probe was incubated with 5 μg of protein of the 200 mM KCl fraction. For cross-competition experiments, competitor DNA consisted either of the complete intergenic region (wild type [wt]) or of fragments spanning the whole intergenic region except for deletions Δ309-432 and Δ353-569, as indicated in panel B. These fragments were obtained from the intergenic regions encoded by plasmids pΔBSFLIRT, pΔBSΔ309-432, and pΔBSΔ353-569 by PCR using oligonucleotides acvAP and ipnAP (Table 2). Specific competitor DNA was added in increasing molar excesses of 3-fold (lanes 2, 5, and 8), 10-fold (lanes 3, 6, and 9), and 30-fold (lanes 4, 7, and 10) prior to addition of the protein to the binding reaction mix, as indicated at the top.

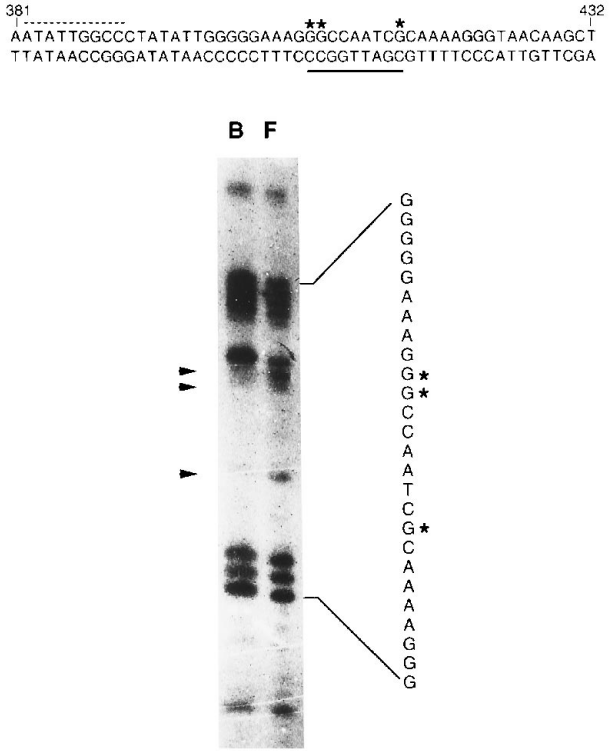

FIG. 4. Methyl interference assay to identify the guanosine residues in contact with the protein (complex). The double-stranded 143-bp *Xho*I-*Sac*I restriction fragment which included nt 353 to 432 of the intergenic region was obtained from plasmid pMIA. The DNA fragment was labeled on the top strand and subsequently methylated. The DNA fragment was then incubated with the 200 mM KCl fraction. Complexed and uncomplexed probes were separated on a nondenaturing polyacrylamide gel, then recovered, and subjected to the degradation reaction according to the Maxam-Gilbert procedure (G reaction) (33). The DNA fragments were separated on a 10% (wt/vol) sequencing gel. Arrows indicate the guanosine nucleotides missing in the bound probe. The nucleotide sequence is shown at the top. The guanosine nucleotides that, when methylated, interfere with binding of the protein are marked with asterisks. Numbers indicate the positions of the nucleotides in the intergenic region. The sequence flanked by identified G's and the divergently oriented sequence showing some degree of identity are indicated by bold and dashed lines, respectively. Abbreviations: F, free probe; B, bound probe.

binding site can be expected to extend maximally up to nucleotide 420.

Adjacent to the identified region, a sequence (GGC CAATAT) with a high degree of homology to the identified protein binding site is divergently oriented (Fig. 4, indicated by a dashed line). Methyl interference analysis, however, did not reveal any interaction of the binding protein with this sequence, suggesting that the core GGCCAAT is not sufficient for specific binding in vitro.

To further characterize the DNA binding sequence defined by methyl interference analysis, DNA fragments containing deletions in the binding site were analyzed in cross-competition experiments. Four nucleotides of the identified binding site were simultaneously deleted in order to get a clear result with respect to the functionality of the protein binding site in vitro. The results are shown in Fig. 5.

When nt CCA-G within the GGCCAATCG sequence were deleted (Fig. 5A), the capacity of such a DNA fragment to prevent complex formation was lost when added in a molar excess of up to 30-fold (Fig. 5B, lanes 9 to 11). This finding confirmed that these nucleotides are essential for the binding of the protein factor. As a negative control, the TAT-T se-

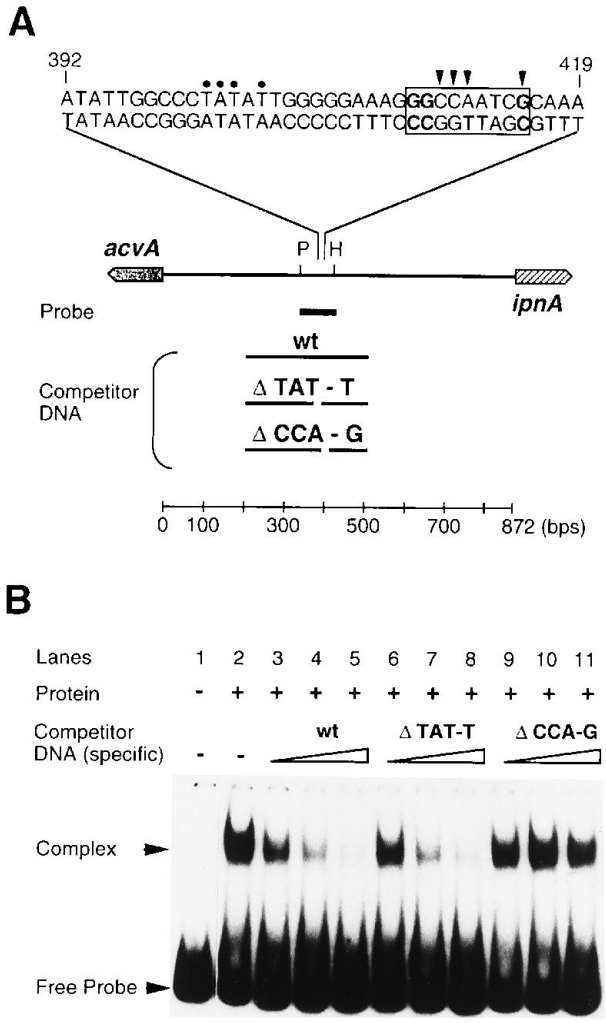

FIG. 5. Cross-competition experiments (band shift assays) using a DNA fragment carrying a deletion of four nucleotides within the identified protein binding site. (A) Schematic diagram of the probe and the PCR fragments used as specific competitor DNA. Parts of the intergenic region from nt 192 to 507 were amplified by using oligonucleotides IntSeqP1 and 202acvAP (Table 2). As the templates, plasmids pΔBSFLIRT, pΔBSΔTAT-T, and pΔBSΔCCA-G were used. wt, the wild-type fragment. Dots and arrowheads above the sequence indicate the deleted nucleotides in fragments ΔTAT-T and ΔCCA-G, respectively. The identified binding site is boxed. Abbreviations: P, *Pst*I; H, *Hin*dIII. (B) Band shift assays using the 80-bp fragment as the probe (A). Specific competitor DNA, shown in panel A, was added in increasing molar excesses of 3-fold (lanes 3, 6, and 9), 10-fold (lanes 4, 7, and 10), and 30-fold (lanes 5, 8, and 11) prior to addition of the protein to the binding reaction mix, as indicated at the top.

quence adjacent to the identified DNA sequence was deleted (Fig. 5A). Addition of a DNA fragment containing this deletion in a molar excess of 30-fold did compete and abolished the band shift (Fig. 5B, lanes 6 to 8), indicating that the deleted TAT-T nucleotides were not essential for formation of the DNA-protein complex in vitro.

Specific deletion of nucleotides in the identified protein binding site led to the same phenotype in vivo as observed for the deletion clones. To provide evidence that the DNA sequence defined in vitro is functional in vivo, an *A. nidulans* strain carrying the same CCA-G deletion as present in the DNA fragment (CCA-G) used for cross-competition experiments (Fig. 5) was analyzed. For this analysis, the recombinant plasmid pΔCCA-G was generated. It contained the complete intergenic region with specific deletions of the nucleotides indicated, fused to the reporter genes *uidA* and *lacZ*, thus yielding translational *acvA-uidA* and *ipnA-lacZ* gene fusions (Fig. 6). The resulting strain of *A. nidulans* (strain ΔCCA-G) was analyzed in a fermentation run. The results are summarized in Fig. 6.

Deletion of nt CCA-G led to a ninefold increase of *acvA-uidA* expression and to a reduction to about 30% of *ipnA-lacZ* expression. This phenotype was thus almost identical to that of the deletion clones Δ183-432, Δ309-432, and Δ353-569 (Fig. 2 and 6). The in vivo analysis also agreed well with the cross-competition experiments shown in Fig. 5. Hence, these results suggest that the deletion of the identified DNA site is, at least in part, responsible for the effects detected in vivo. The identified *cis*-acting site thus represents a DNA element bound by a major regulatory factor which we designated PENR1, for penicillin regulator. It has not been clarified whether PENR1 is a single protein or if it consists of a complex of different proteins.

DNA-protein (PENR1) complex formation was prevented by the use of DNA fragments spanning the corresponding intergenic regions between *acvA* (*pcbAB*) and *ipnA* (*pcbC*) of *P. chrysogenum* and *A. chrysogenum*. To analyze whether the identified *cis*-acting DNA element of *A. nidulans* is conserved among other β-lactam-producing fungi, DNA fragments spanning the intergenic regions of the corresponding *P. chrysogenum* and *A. chrysogenum* genes were used for cross-competition experiments (Fig. 1 and 7). Addition of these DNA fragments in a molar excess of up to 30-fold led to a reduction of complex formation, indicating that these fragments were capable of competing specifically with the *A. nidulans* fragment for binding of the *A. nidulans* DNA-binding protein PENR1 (Fig. 7). This result strongly suggests that both *P. chrysogenum* and *A. chrysogenum* are regulated by a protein which recognizes DNA sites similar to the identified *A. nidulans* site.

By computer analysis, such DNA elements were found in the intergenic regions of both *P. chrysogenum* and *A. chrysogenum* (Fig. 8). In the intergenic region of *P. chrysogenum*, there are two sites of extensive sequence identity (Fig. 8). This homology may explain why a DNA fragment spanning this region diluted the complex even when added in a molar excess of only 10-fold (Fig. 7, lane 5). However, at least the *A. chrysogenum* site and one of the two assumed *P. chrysogenum* sites lack the 3′ flanking G of the sequence which, for *A. nidulans*, was identified to be in contact with PENR1 (Fig. 4) (see Discussion). Therefore, the functionality of the heterologously identified sites has to be verified yet in the corresponding homologous systems.

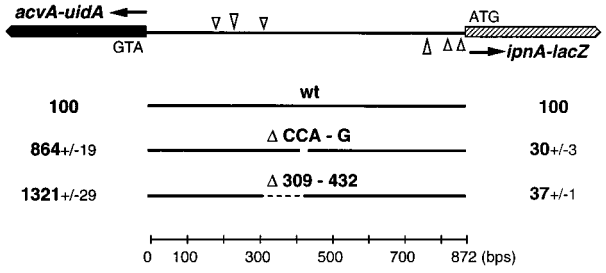

FIG. 6. In vivo analysis of an *A. nidulans* strain carrying a specific deletion in the identified protein binding site. A single-copy transformant for plasmid pΔCCA-G (strain ΔCCA-G) was fermented as described in the legend to Fig. 2. Values for β-Gal and β-Glu activities are given as relative activities. The relative values for each deletion clone represent the mean and standard deviation of several complete experiments (see the legend to Fig. 2). Abbreviations: wt, wild-type strain FLIRT; ΔCCA-G and Δ309-432, strains carrying the indicated deleted nucleotides in the intergenic region between their reporter genes.

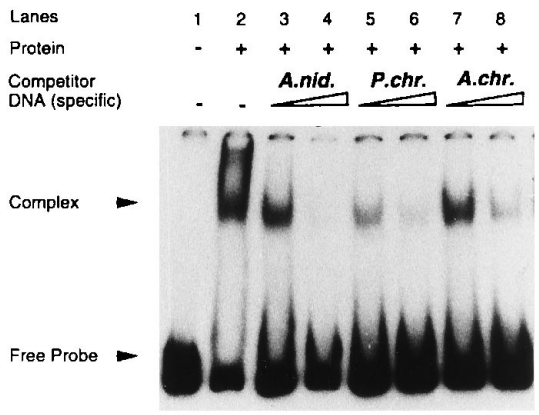

FIG. 7. Cross-competition experiments using DNA fragments spanning the intergenic regions of the corresponding genes of *P. chrysogenum* and *A. chrysogenum*. The intergenic regions (shown in Fig. 1) were amplified by PCR using plasmid pΔBSFLIRT for *A. nidulans* and chromosomal DNA for both *P. chrysogenum* and *A. chrysogenum* as the templates and the primer pairs acvAP and ipnAP for *A. nidulans*, PchacvAP and PchipnAP for *P. chrysogenum*, and AchacvAP and AchipnAP for *A. chrysogenum*. For the binding reaction, the 80-bp DNA fragment (nt 353 to 432) of *A. nidulans* was incubated with crude protein extracts from *A. nidulans* eluted from the heparin-agarose column with 200 mM KCl in the extraction buffer. Specific competitor DNA was added to the binding reaction mix prior to the protein in molar excesses of 10-fold (lanes 3, 5, and 7) and 30-fold (lanes 4, 6, and 8), as indicated at the top.

DISCUSSION

Structural organization of the bidirectionally oriented promoter regions between *acvA* and *ipnA*. The moving-window analysis of the intergenic region between *acvA* and *ipnA* revealed that this region contains several distinct *cis*-acting DNA elements. We did not find any element which affected the expression of one gene only. All deletion clones with the exception of clones Δ428-872 and Δ1-493, which carried deletions right to the ATG initiation codons of one of the reporter genes and thus could give data for only a single reporter gene fusion, showed effects on the expression of both genes simultaneously. This finding suggests that the promoters of both genes are, at least in part, physically overlapping and share common *cis*-acting elements. Since we have measured both increased and reduced expression of *acvA* and *ipnA* gene fusions in the various deletion constructs, we conclude that the genes are regulated by distinct positively and negatively acting factors. This conclusion is supported by the identification and characterization of a major *cis*-acting site reported here which negatively affected *acvA* expression and positively affected *ipnA* expression and by the work of Pérez-Esteban et al. (40), who concluded from a deletion analysis of the *ipnA* promoter that the gene is regulated by activator and repressor proteins. Furthermore, the identification of recessive *trans*-acting mutations (13, 39) also suggested the existence of positively acting factors in the regulation of both genes. For the expression of the *ipnA* gene, this has been demonstrated by the finding that at alkaline pH values, *ipnA* is positively regulated by PACC (21, 54). Taken together, the present data are thus consistent with a model in which the activities of the promoters of the secondary metabolism genes *acvA* and *ipnA* are controlled by the interplay between positively and negatively acting factors.

Deletion of the PENR1 binding site had contrary effects on *acvA* and *ipnA* expression. There are some examples of dual-function regulatory proteins which are either activators or repressors. Their mode of activity as repressor or activator depends on the location of their binding sites (42). Here, deletion of a protein binding site showed contrary effects on two genes simultaneously. Although the mechanism mediating these contrary effects has not been elucidated, it is conceivable that the distance between the PENR1 binding site and the transcriptional start sites of both structural genes is important. Furthermore, additional regulatory proteins which affect PENR1 by protein-protein contacts could be required. Alternatively, the ability of PENR1 to control bidirectionally expression of two genes might be altered by factors bound to nearby sites. Other possibilities that could be envisaged involve small sensor molecules binding to the regulatory protein, as proposed for the action of the bacterial transcription factor MERR, which regulates the bidirectionally transcribed genes *merR* and *merTPAD*. MERR can mediate repression as well as activation through stereospecific modulation of DNA structure (2). It is tempting to speculate that PENR1 could be involved in a differential regulation of *acvA* and *ipnA*. Hence, it will be interesting to elucidate the physiological signals influencing PENR1 activity.

PENR1 binding site and CCAAT-binding proteins. The binding site of PENR1 contains the core motif CCAAT, suggesting that PENR1 belongs to the class of CCAAT-binding proteins. Although there are other CCAAT-containing sequences within the intergenic region of *acvA* and *ipnA*, PENR1 appeared to bind with high affinity to a single site. The presence of the sequence CCAAT is apparently not sufficient for specific binding of PENR1.

DNA fragments spanning the intergenic region of the corresponding genes of *P. chrysogenum* and *A. chrysogenum* competed against the probe for binding of PENR1. Sequences with a high degree of identity with the *A. nidulans* site were identified (Fig. 8), although in the *A. chrysogenum* sequence, the last G identified at the 3′ site of the *A. nidulans* motif is missing. An independent promoter analysis of the *aat* gene of *A. nidulans* indicated that PENR1 also binds to this promoter (30). The identified sequence contains the identical nucleotides GCC AATCNC. Although it has not been verified whether PENR1 bound to the different CCAAT-containing sequences with the same affinity, on the basis of the experimental data reported here, the data on the *aat* promoter region (30), and the computer analysis of the other promoter regions shown in Fig. 8, the nucleotides GCCAATCNC were found in almost all of these sequences (Fig. 8). Further experiments, however, must be carried out to define precisely all of the nucleotides in contact with PENR1 and to show whether exactly the same nucleotides in the different promoters are bound by PENR1.

CCAAT boxes have been found in the promoter and enhancer regions of a large number of genes in eukaryotes. Different distinct transcription factors have been reported to bind to CCAAT boxes, either with exquisite specificity or with a loose specificity encompassing the CCAAT motif (reviewed in references 27 and 36). The multiplicity of factors may explain

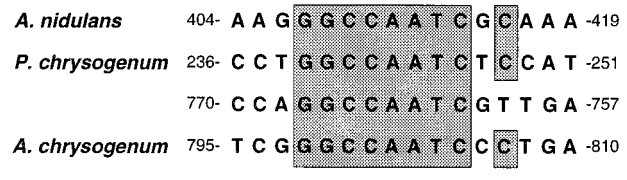

FIG. 8. Inspection by computer analysis of the regions between *acvA* and *ipnA* of *P. chrysogenum* and *A. chrysogenum* for the presence of PENR1 binding sites. As reported here, the functionality of the *A. nidulans* site was proved experimentally. Identical nucleotides in most of the CCAAT-containing sequences are boxed.

the variety of functions that have been attributed to the CCAAT sequences in the modulation of transcript levels in eukaryotic cells (6, 15, 19, 32, 34, 37, 41, 49, 52). In general, CCAAT-binding proteins appear to bind to their target sequences with highest affinity as complexes composed of heteromers (16, 25, 45). We do not know whether PENR1 represents a single protein or a complex of proteins. In *A. nidulans*, functional CCAAT-containing *cis*-acting DNA elements have been found. On the basis of deletion analysis and band shift assays, the involvement of a CCAAT-binding factor, designated AnCF, in the regulation of the *A. nidulans* acetamidase gene (*amdS*) was observed (56). The *amdS* gene product is required for the use of acetamide as the nitrogen and carbon source by *A. nidulans*. Furthermore, the region between the bidirectionally transcribed genes *lamA* and *lamB* (needed for utilization of lactams) and the promoter region of *gatA* (γ-amino butyric acid transaminase) contain CCAAT sequences which are bound by a DNA-binding factor(s) (46).

Nagata et al. (38) reported that two CCAAT-binding factors (AnCP1 and AnCP2) of *A. nidulans* bound in vitro to the promoter of the Taka-amylase gene of *A. oryzae*. A CCAAT-binding factor was also suggested to be involved in the developmentally regulated *yA* gene of *A. nidulans* (3). Deletion of the corresponding CCAAT motif led to increased expression of an *yA-lacZ* gene fusion (3).

It is not yet known how many different CCAAT-binding proteins exist in filamentous fungi and which of these CCAAT-binding proteins are involved in the expression of genes with such different functions as *yA*, *amdS*, *lamA*, *lamB*, *gatA*, *acvA*, and *ipnA*. Since none of the CCAAT-binding proteins of *A. nidulans* has been purified, nor have the corresponding genes been cloned, it is not known whether some of them are identical. Further analysis will show what kind of CCAAT-binding factor PENR1 represents and whether PENR1 is a general regulator or a specific gene regulator for secondary metabolism genes in fungi.

The PENR1 binding site appears to be conserved among other β-lactam-producing fungi. DNA fragments spanning the intergenic regions between *acvA* and *ipnA* of *P. chrysogenum* and *A. chrysogenum* were able to compete against the *A. nidulans* probe for binding of the *A. nidulans* PENR1 protein (complex). This observation suggests that PENR1-analogous proteins exist in both industrially important fungi, suggesting that PENR1 represents a major regulatory protein controlling the expression of the secondary metabolism genes *acvA* and *ipnA*. In addition, the *aat* genes of both *A. nidulans* and *P. chrysogenum* seem to be bound by PENR1 as well (30).

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