A Novel Heptameric Sequence (TTAGTAA) Is the Binding Site for a Protein Required for High Level Expression of pcbAB, the First Gene of the Penicillin Biosynthesis in Penicillium chrysogenum*

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The first two genes pcbAB and pcbC of the penicillin biosynthesis pathway are expressed from a 1.01-kilobase bidirectional promoter region. A series of sequential deletions were made in the pcbAB promoter region, and the constructions with the modified promoters coupled to the lacZ reporter gene were introduced into single copies at the pyrG locus in Penicillium chrysogenum npe10. Three regions, boxes A, B, and C, produced a significant decrease in expression of the reporter gene when deleted. Protein-DNA complexes were observed by using the electrophoretic mobility shift assay with boxes A and B (complexes AG1, BG1, BG2, and BL1) but not with box C. Uracil interference assay showed that a protein in P. chrysogenum cell extracts interacts with the thymidines in a palindromic heptanucleotide TTAGTAA. Point mutations and deletion of the entire TTAGTAA sequence supported the involvement of this sequence in the binding of a transcriptional activator named penicillin transcriptional activator 1 (PTA1). In vivo studies using constructions carrying point mutations in the TTAGTAA sequence (or a deletion of the complete heptanucleotide) confirmed that this intact sequence is required for high level expression of the pcbAB gene. The TTAGTAA sequence resembles the target sequence of BAS2 (PHO2), a factor required for expression of several genes in yeasts.

High penicillin-producing strains of Penicillium chrysogenum contain increased levels of the three penicillin biosynthetic enzymes: 1-α-aminoadipyl-3-lysine-5-d-carboxamide-6-aminopenicillanic acid synthetase (I), isopenicillin N-synthase (2), and isopenicillin N-acetyltransferase (3) (reviewed in Ref. 4). This effect is due, at least in part, to the increased copy number of the three respective penicillin biosynthesis genes: pcbAB, pcbC, and penDE (5, 6), which are located in a DNA region amplified in tandem repeats (7). Nevertheless, it is likely that the level of trans-acting transcriptional activators may also be higher in penicillin overproducing strains (reviewed in Ref. 8). Knowledge of the regulatory mechanisms that control expression of the penicillin biosynthesis in P. chrysogenum is of utmost importance, because biosynthesis of the β-lactams is the best known model (both at the genetic and biochemical levels) to study secondary metabolites.

The first two genes of the penicillin pathway in P. chrysogenum are transcribed divergently from a 1.01-kilobase bidirectional promoter region (9); the same organization is found in Aspergillus nidulans (10) and Penicillium nalgiovense (11). Multiple transcriptional factors appear to bind to this bidirectional promoter region modulating the expression of both genes (reviewed in Refs. 12 and 13). In P. chrysogenum, nuclear DNA-binding proteins, which recognize the intergenic promoter region, have been reported by Feng et al. (14), Chu et al. (15), and Vichitsoonthonkul et al. (16). Wide domain regulators, such as the pH-dependent regulator PacC (17) and the nitrogen metabolite repressor NRE1 (18), bind in vitro to specific sequences of the intergenic pcbAB-pcbC region.

In P. chrysogenum, the pcbAB gene has been reported to be repressed by glucose and ammonium (19); the same occurs with the pcbC and penDE genes (20). The pcbAB gene is also under the control of extracellular pH (21), which correlates with the presence of seven PacC consensus binding sites (GCCARG) in the pcbAB-pcbC intergenic region (17). However, in P. chrysogenum the alkaline pH does not reverse the strong glucose repression of pcbAB, pcbC, and penDE (20).

Expression analysis in A. nidulans revealed that pcbC is strongly repressed by glucose, whereas pcbAB is only slightly affected by this carbon source (22, 23). However, P. chrysogenum and A. nidulans appear to be clearly different in the regulatory mechanisms of the penicillin gene cluster (13); this fact stresses the need to perform regulation studies in P. chrysogenum itself. Pérez-Esteban et al. (24) used an approach consisting of sequential and internal deletions of the pcbAB-pcbC intergenic region of A. nidulans to locate regulatory cis-acting regions involved in the expression of a pcbC::lacZ fusion; several regions involved in transcriptional activation and suppression were described. By contrast very little information exists in P. chrysogenum about cis-acting regulatory regions affecting expression of the pcbAB gene.

In this article we describe a sequential deletion analysis of the P. chrysogenum pcbAB-pcbC intergenic region to locate cis-acting sequences enhancing the expression of the pcbAB gene, the interaction of these sequences with DNA-binding proteins, and the in vivo effect of mutation or deletion of the main regulatory sequences on expression of the pcbAB gene.

EXPERIMENTAL PROCEDURES

Microorganisms—P. chrysogenum npe10 pyrG, used as receptor strain in transformation experiments, is a derivative of P. chrysogenum npe10, a deletion mutant lacking the complete penicillin gene cluster.

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1 The abbreviations used are: NRE, nitrogen catabolite repressor; CP, penicillin production complex; PCR, polymerase chain reaction; bp, base pair(s); EMSA, electrophoretic mobility shift assay; PTA1, penicillin transcriptional activator 1.
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(25, 26).

Escherichia coli DH5α (CLONTECH, Palo Alto, California) was used as the host strain for high frequency plasmid transformation and plasmid manipulations; single-stranded DNA was obtained from E. coli WK6. Phage M13K07 was used as helper in the infection of strain E. coli DH5α.

Culture Conditions—Seed culture medium for penicillin production contained: 20 g/l corn steep solids, 20 g/l sucrose, 5 g/l yeast extract, 5 g/l CaOAc, pH 5.7. Spore suspensions of P. chrysogenum were inoculated in 100 ml of seed medium in flasks and incubated in a rotary shaker (250 rpm) at 25 °C for 24 h. Penicillin production complex (CP) medium contained: 4 g/l phenylacetic acid, 4 g/l (NH4)2SO4, 5 g/l CaCO3, 20 g/l Pharmamedia, pH 6.1. Lactose or glucose (30 g/l) were added to each flask after autoclaving. CP medium (100 ml in 500-ml Erlenmeyer flasks) supplemented with either lactose or glucose was inoculated with 8 ml of seed medium, and cultures were incubated for 12–96 h at 25 °C in an orbital incubator at 250 rpm.

Cell Extracts and β-Galactosidase Assays—β-galactosidase activity was determined in extracts from mycelia grown in CP medium, harvested by filtration through Nytal filters, washed with 0.9% NaCl, cooled rapidly in liquid nitrogen, and stored at −20 °C. The mycelial cake was ground to a fine powder in liquid nitrogen in a mortar, and the resulting powder was stored at −80 °C until used. Frozen mycelial powder (700 mg) was homogenized in 15 ml of extraction buffer (50 mM sodium phosphate buffer, pH 7.0; 1 mM EDTA; 20 μM phenylmethylsulfonyl fluoride), and kept on ice. The extracts were centrifuged at 4000 rpm for 10 min at 4 °C, and the supernatants were removed to clean tubes, centrifuged at 14,000 rpm for 10 min at 4 °C, and stored on ice.

β-Galactosidase assays were carried out in 5–200 μl of clear extracts and O-nitrophenyl-β-D-galactopyranoside as substrate. Protein concentration in the extracts was determined with the Bradford dye reagent (Bio-Rad).

Transformation of P. chrysogenum—Protoplasts were obtained from P. chrysogenum npe10 pyrG by treatment with lysing enzymes (Sigma), and transformation was performed as described previously (27).

DNA Manipulation—Total DNA from P. chrysogenum transformants was purified by a small scale procedure, essentially as described by Fierro et al. (28). Plasmid DNA preparation, restriction enzyme digests, ligations, Southern blotting, and hybridizations were carried out by standard techniques.

Deletions to remove specific stretches of the pcbAB promoter coupled to the E. coli lacZ reporter gene were as follows: Constructions DEL0–DEL5 were made from pZ2AB (20) using the Erase-a-Base System WK6. Phage M13K07 was used as helper in the infection of strain E. coli lacZ. Deletions to remove specific stretches of the IntI or IntII region including the IntI or IntII site by digestion with exonuclease III followed by S1 nuclease treatment and ligation. Constructions DEL6 and DEL8 were made from pZ2AB by digestion with appropriate restriction enzymes (SalI, EcoRI, and BamHI, respectively) and ligation. The precise end points of all the promoter deletion mutants were determined by sequencing the DNA of each construction.

PCR Amplifications—Two overlapping 86-bp fragments (designated A1 and A2) from the 131-bp SacI-TaqI region of the pcbAB promoter (box A), were generated from pZ2AB by polymerase chain reaction (PCR) reaction using plasmid pBKS1 as template DNA and oligonucleotides KA: CATC TGGG CTGC AAGC GT and KB: ACCT TTGA CGGA AAGC CT as primers. Fragment A2 was obtained by PCR digestion with appropriate restriction enzymes (SalI and EcoRI), and BamHI, respectively) and ligation. The precise end points of all the promoter deletion mutants were determined by sequencing the DNA of each construction.

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Labeling of Probes for EMSA Assays—All the DNA fragments used in binding reactions were filled with a DNA fragment containing the entire IntI region plus 100 bp of the lacZ coding region (up to the PvuII site in this gene). DNA (30 ng) was digested by filling in with total DNA in 30 μl of hybridization buffer at 54 °C for 12 h and then treated with 100 units of S1 nuclease at 45 °C for 2 h. The reaction product was precipitated with ethanol, resuspended in Tris-EDTA buffer and electrophoresed in a 5% polyacrylamide gel through a heme-persulfate-acrylamide, 40:1 in 0.5× Tris borate-EDTA buffer. Gels were run at 100 V, dried, and exposed to x-ray film.

Uracil Interference Assay—The uracil interference assay was performed essentially as described by Ausubel et al. (29). Thymine was randomly replaced by deoxyuracil in DNA fragments containing the A1 region by PCR amplification using the following oligonucleotides as primers: KD, CTGG AGAC ATTA TACG CT (positions 744–772 with respect to the pcbAB transcription start point) and KL, AAAT GCCA CGGT GGGC AC (positions 608–635). Two PCR reactions were performed (25 cycles with the following profile: 94 °C, 45 s; 50 °C, 45 s; 72 °C, 40 s; 20 s); each reaction contained a different labeled oligonucleotide in order to label each of the DNA strands separately. The labeled oligonucleotides were electrophoresed on a 1% agarose gel (in 1× Tris borate-EDTA buffer) and visualized with ethidium bromide. The labeled and nonlabeled oligonucleotides were mixed in 100 μl of PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton, 0.03 mM MgCl2) with 0.2 mM dNTP, 0.2 mM dUTP, 0.5 μg DNA template (pBKS1-AB), and 5 units Taq DNA polymerase. The amplified PCR products were separated electrophoretically in a 5% native polyacrylamide gel, recovered by electroelution, and used as probes (10× cm) for EMSA as described above; the probes were incubated separately with a filter strip of nylon membranes containing the fragment of DNA covering the entire IntI region plus 100 bp of the lacZ coding region (up to the PvuII site in this gene). DNA (30 ng) was digested by filling in with total DNA in 30 μl of hybridization buffer at 54 °C for 12 h and then treated with 100 units of S1 nuclease at 45 °C for 2 h. The reaction product was precipitated with ethanol, resuspended in Tris-EDTA buffer and electrophoresed in a 5% polyacrylamide gel. The gel (without drying) was analyzed in a phosphorimager (Instant Image, Packard) and the Protein-DNA complexes were visualized by standard methods.

S1 Nuclease Protection Studies—Total RNA was obtained by the guanidinium method (29) from mycelial methanol of the P. chrysogenum npe10 pyrG locus that contains the E. coli lacZ reporter gene (20).

EMSA of DNA-binding Proteins—P. chrysogenum npe10 was grown for 12–72 h in CP medium with 3% lactose or 3% glucose as a carbon source. The mycelium was harvested by filtration through Nytal filters, washed with 0.9% NaCl, frozen in liquid nitrogen, and stored at −75 °C. The mycelium was ground to a fine powder with a mortar cooled with liquid nitrogen and resuspended in 1 volume of extraction buffer (20 mM HEPES, pH 7.9; 1.5 mM MgCl2, 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 1.25 mM phenylmethylsulfonyl fluoride) kept on ice. The suspension was centrifuged at 4000 rpm for 5 min at 4 °C, and the supernatant was removed to clean tubes and centrifuged at 14,000 rpm for 10 min at 4 °C. The clean supernatant (crude extract) was precipitated with (NH4)2SO4 (final concentration 60%) and resuspended in a half volume of sample buffer (15 mM Tris-HCl, pH 7.5; 5 mM MgCl2, 0.1 mM EDTA; 50 mM NaCl; 1 mM dithiothreitol; 10% glycerol). The protein extract was desalted by Sephadex G-25 gel filtration and kept in glycerol (40% final concentration) at −20 °C until used for the binding reaction.

The binding reaction mixture (20-μl final volume) contained: 0.1 pg of labeled probe DNA, 2 μg of nonspecific competitor poly(dI-dC) (Roche Molecular Biochemicals), and different amounts of protein extracts in binding buffer (20 mM HEPES, pH 7.9; 4 mM Tris-HCl, pH 7.4; 50 mM KCl, 1 mM EDTA; 10% glycerol). Binding reactions were incubated for 30 min at 25 °C. The reactions were then mixed with loading buffer (40% glycerol, 0.25% bromphenol blue, 0.25% xylene cyanol) and electrophoresed in 5% native polyacrylamide gels (acylamide: bisacrylamide, 40:1) in 0.5× Tris borate-EDTA buffer. Gels were run at 100 V, dried, and exposed to x-ray film.
RESULTS

Expression of the pcbAB: lacZ Gene Fusion in P. chrysogenum—To study the expression of the pcbAB gene in P. chrysogenum, a 1161-bp NcoI fragment containing the intergenic pcbAB-pcbC region was coupled to the complete E. coli lacZ gene (3069 bp) as an in-frame translational fusion that resulted in a hybrid protein with 49 additional amino acids corresponding to the amino-terminal region of the L-α-aminoacyl-p-L-cysteinyl-d-valine synthetase protein. This promoter-reporter gene fusion was introduced in a vector designed for targeted integration at the pyrG locus in P. chrysogenum npe10, a mutant that lacks the penicillin gene cluster due to a deletion of the entire pen region (thus avoiding interference by the endogenous copy of the pen cluster) (20, 31). P. chrysogenum npe10 transformants that had incorporated a single copy of the vector carrying the pcbAB-lacZ fusion at the pyrG locus were selected by their restriction pattern, and expression from the pcbAB promoter under different metabolic conditions was tested by assaying the reporter β-galactosidase activity in the collected mycelia. Untransformed controls show a very low endogenous β-galactosidase activity that was subtracted from the values determined.

As shown in Fig. 1, when 3% lactose was used as carbon source, expression of the reporter gene occurred early during the fermentation. β-Galactosidase activity in transformant cells was high between 24 and 48 h and then decreased sharply at 72 h (Fig. 1B). This pattern of expression is consistent with the early expression of the pcbAB gene as measured by Northern blot analysis (20). In medium containing 3% glucose the expression of the reporter gene was much lower than in medium with lactose. In glucose-based medium the glucose content in the broth decreased to 2% in the first 24 h, to 1.5% at 36 h, and to 0.7% at 48 h (Fig. 1C), and the β-galactosidase activity increased slowly from the beginning of fermentation (Fig. 1B). Comparatively, the β-Galactosidase activity was 16-fold higher in cells grown in lactose than in cells grown in glucose medium at 24 h and 8-fold higher at 48 h. A small increase of enzyme activity in glucose-supplemented medium occurred after 48 h of cultivation, when the residual glucose decreased to a 0.7% or lower levels; these results suggest that glucose exerts a repression of the pcbAB promoter. The pH in glucose-supplemented cultures ranged between 0.5 and 1.4 units below the pH values in lactose cultures throughout the fermentation (Fig. 1A).

Transcription Start Point in Transformants with Deleted Promoter Regions—To define important regions for regulation of pcbAB gene expression, progressive deletions of the promoter region of the pcbAB gene were made (Fig. 2). Plasmids with the different deleted regions were introduced by transformation into P. chrysogenum npe10 pyrG at the pyrG locus, and single copy transformants were selected as described previously (20). The transformants were named from DEL0 (no deletion, complete promoter) to DEL8 (intergenic region completely deleted).

Transcription initiation of the pcbAB gene occurs at nucleotide 933 of the intergenic region (numbering starting from the NcoI site at the pcbC start codon3); the transcriptional start point has been labeled +1 in Fig. 2.

To establish how the sequential deletions influenced the transcription ability of the pcbAB gene, a S1 nuclease low resolution analysis was performed by hybridizing a 1.35-kilobase PvuII-SmaI probe to the total RNA isolated from the transformants. Results (Fig. 2D) showed that the size of the DNA fragments resistant to S1 nuclease activity was the same

\(^3\) In all transformants from DEL0 to DEL4 (approximately 350 bp) covering the sequence between the transcriptional start point of the pcbAB gene and the restriction site PvuII in the lacZ gene. This indicates that no abnormal transcription start points appear in the deleted promoter regions. In transformants DEL5, DEL6, DEL7, and DEL8 no hybridization signal appears indicating either lack of transcription from those short promoter regions or a level of transcription that is below the detection threshold of the technique (see “Discussion”).

Locatin of cis-Acting Regions Involved in Transcriptional Regulation—cis-acting sequences involved in regulation of the pcbAB gene expression were studied by growing the eight transformants with the sequentially deleted promoter regions shown in Fig. 2 in the same medium with either glucose or lactose as sole carbon sources. The level of expression was monitored by measuring the β-galactosidase activity of mycelium collected at 48 h of fermentation.

Transformant DEL0 represents the activity of the intact promoter. Transformant DEL8 carried a 5′-promoter deletion, which removed sequences up to +259 (including the transcriptional start point of pcbAB gene); it was therefore used as a

\(^2\) B. Díez, and J. F. Martín, unpublished results.
control to check the endogenous β-galactosidase activity plus the possible background expression of the fusion (dotted line in Fig. 2C). In all transformants, activities in glucose-grown cells were clearly lower than in lactose-grown mycelia; this effect of glucose repression was also observed on the endogenous activity in an untransformed strain.

Four regions appeared to be important in regulation of the pcbAB gene expression. Deletion of the most distal region of the pcbAB promoter (DEL2, position −933 to −789 relative to the transcriptional start point) results in an increase in pcbAB gene expression in lactose medium. Though we cannot exclude the possibility that regulatory sites exist in this region it is also possible that its deletion leads to a reporter activity increase in lactose-grown mycelium simply because this is the region to which basal RNA polymerase factors for expression of the pcbC gene are expected to bind, which might interfere with binding of transcription factors important for pcbAB expression.

The results obtained for transformants DEL2 to DEL6 indicate that there are three other important regions (named A, B, and C) that produce a sharp decrease in gene expression when deleted both in cultures with lactose or glucose. A decrease in the reporter activity (about 41%) was observed between transformants DEL2 (−789) and DEL3 (−665) (box A) and also between transformants DEL4 (−543) and DEL5 (−329) (box C) (59% of the activity) in lactose medium. Similar percentages of reduction were observed when the same constructions were grown in glucose-based medium.

Transformant DEL5 (deleted up to the position −329) kept a 24% activity with respect to the complete promoter in lactose medium, whereas transformant DEL6 (position −178) lost al-

coupled to the reporter lacZ gene. A, scheme of the 1161-bp Ncol fragment containing the bidirectional pcbAB-pcbC promoter region (1013 bp) indicating the positions of boxes A, B, and C (see “Results” for details). B, promoter regions in the different deleted mutants DEL2 to DEL8. Strain DEL0 contains the entire promoter region. C, β-galactosidase activity of the reporter gene from single copy constructions containing the different promoter regions, integrated at the pyrG locus of P. chrysogenum npe10 in cultures with lactose (3%) or glucose (3%) as carbon source. The dotted line corresponds to the basal level of β-galactosidase in nontransformed P. chrysogenum npe10. The level of expression was monitored by measuring the β-galactosidase activity of mycelia collected after 48 h of fermentation. D, identification by low resolution S1 mapping of the lacZ transcript expressed from the different promoters with deleted regions (DEL0 to DEL8). The estimated size of the protected transcript is 350 bp (arrow). Note that the transcript size is the same in transformants DEL0 to DEL4 and that there is no detectable transcript in transformants DEL5 to DEL8.
most all activity, retaining only 1.7% of the activity of the entire promoter. These observations indicate an important role of box B in pcbAB gene expression. The activity of transformant DEL8 (no promoter) is identical to the endogenous activity found in the untransformed strain P. chrysogenum npe10 (dotted line in Fig. 2C), indicating that no residual expression is produced in this fusion.

Therefore, putative transcriptional activators should bind to box A (stretching out from −789 to −665), box C (from −543 to −329), and box B (from −329 to −178). Several EMSA assays were carried out with DNA probes covering each of these regions. No retardation complexes were detected with fragment C, but they were observed with DNA fragments A and B (see below).

**Protein-DNA Complexes Formed with Boxes A and B**—To identify transcriptional factors that participate in regulation with the pcbAB gene, crude extracts were obtained from P. chrysogenum npe10 pyrG grown either with glucose or lactose as carbon source. These protein extracts were partially purified (see “Experimental Procedures”) and tested in mobility shift assays with a 130-bp SacI-Taq1 DNA probe corresponding to box A, and with a 156-bp TaqI-SalI probe corresponding to box B.

Using probe A, a single retardation complex (named AG1) was formed with extracts obtained at different times of fermentation from glucose-grown mycelia (Fig. 3A). The AG1 retardation complex was clearly observed in extracts from glucose- and lactose-grown cells. These protein extracts were partially purified and tested in mobility shift assays with a 130-bp SacI-Taq1 DNA probe corresponding to box A, and with a 156-bp TaqI-SalI probe corresponding to box B.

Using probe B, two major complexes of different sizes appear in extracts obtained from glucose-grown mycelium. They were named BG1 and BG2, and both showed a concentration peak at 24 h of fermentation, decreasing at 48 h and were not formed with extracts of cells grown for 72 h. The protein that forms this complex is later named PTA1 (penicillin transcriptional activator 1). Using extracts of lactose-grown cell extracts to locate more precisely the protein binding site, box A was divided into two smaller probes named A1 and A2. Protein extracts from glucose-grown cells were incubated with the labeled probe B and an excess of unlabeled fragments A or B. As shown in Fig. 4 A1 gave a very weak retardation signal, whereas with probe A2 a clear effect of EDTA (25 mM) on in vitro formation of the protein-DNA complexes AG1, BG1, BG2, and BL1. Note that formation of AG1, BG1, and BL1 (but not BG2) is prevented by EDTA.

Extracts from lactose-grown cells.

**Specificity and Properties of the Protein-DNA Complexes Formed with Boxes A and B**—Cross-competition experiments were carried out with 150-fold molar excess of either the cold target fragment or a DNA fragment corresponding to the other box (A or B) to determine the specificity of the retardation complexes (Fig. 4). Results showed that excess unlabeled box B did not affect the retardation AG1 complex formed with probe A, whereas the unlabeled box A diluted the formation of this complex (Fig. 4A). Therefore, complex AG1 is specific to box A.

Similarly, protein extracts from glucose- and lactose-grown cells were incubated with the labeled probe B and an excess of unlabeled fragments A or B. As shown in Fig. 4B none of the box B complexes (BG1, BG2, and BL1) were affected by the presence of molar excess of unlabeled probe A, but the three complexes disappeared when unlabeled probe B is added in excess. These results show that BG1, BG2, and BL1 complexes are specific to box B.

Several regulatory proteins in fungi (e.g. PacC, Gal4) contain zinc-finger domains, thus requiring the presence of Zn$^{2+}$ cations to be able to bind to DNA. To check if the protein(s) present in a retardation complex belongs to this class of DNA-binding proteins EDTA was used to trap divalent cations. The addition of 25 mM EDTA to the binding reaction of box A with glucose-grown cell extracts prevented the formation of the AG1 complex (Fig. 5A). Similarly, EDTA prevented the formation of complexes BG1 and BL1 in box B, but it did not affect complex BG2 (Fig. 5B). These data indicate that whereas proteins involved in the formation of complexes AG1, BG1, and BL1 require the presence of divalent cations (presumably Zn$^{2+}$) for binding to DNA, the protein present in complex BG2 does not.

**Nucleotide Sequence in Box A That Binds to the Regulatory Protein PTA1**—Further studies were concentrated on the characterization of the sequence that binds the PTA1 protein to form the AG1 complex. To locate more precisely the protein binding site, box A was divided into two smaller probes named A1 and A2. Protein extracts from glucose-grown cells were incubated with both probes. As shown in Fig. 6, probe A1 gave a very weak retardation signal, whereas with probe A2 a clear
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A retardation complex was observed, indicating that PTA1 binds specifically to probe A2. The sequence involved in PTA1 binding was determined by the uracil interference assay. This method identifies thymines involved in protein-DNA interactions. Using a DNA fragment spanning from nucleotide −744 to −608 of the pcbAB-pcbC intergenic region, uracil interference assays detected a region inside box A (−679 to −673) in which four thymines (two in each DNA strand) contributed strongly to the binding of the PTA1 protein (Fig. 7). The region identified as the target for PTA1 binding was a 7-bp sequence TTAGTAA with diad symmetry centered at the GC pair. Thymines at positions 1 and 5 of this heptanucleotide, in both strands, showed a strong affinity for PTA1, whereas the thymine at position 2 showed weaker interaction with PTA1 (Fig. 7).

Directed Mutation and Deletion of the TTAGTAA Box Prevented Formation of the AG1 Complex—To examine how changes in the TTAGTAA sequence affected the binding affinity of PTA1 to its target site, different nucleotides of this sequence were changed by in vitro mutagenesis and the mutated fragments were tested by EMSA (Fig. 8). All the nucleotide changes tested decreased the intensity of the retardation complex with respect to the intensity with the unchanged sequence TTAGTAA. The T → C substitution at position 1 (MUT2) had a high disturbing effect on the affinity of PTA1 for its target site and the same occurs with the T → C substitution at position 5 (MUT3). Substitution of the G at position 4 (the center of the palindrome) by an A (MUT4) produced a smaller effect on the binding of PTA1 as shown by the intensity of the complex. Simultaneous substitution of both Ts at positions 1 and 5 by a C (MUT5) completely abolished the formation of the AG1 complex. Finally no retardation complex was formed when the seven nucleotides were removed from the probe (MUT1) (Fig. 8). These results confirmed the specificity of the PTA1 protein for the TTAGTAA sequence as well as the involvement of thymines at positions 1 and 5 in the protein-DNA interaction.

In Vivo Effect of Point Mutations and Deletion of the TTAGTAA Sequence on the Promoter Activity—The seven nucleotides that form the target site for binding of PTA1 were deleted from the sequence of the entire promoter as indicated under "Experimental Procedures." The mutated (MUT2 and MUT5) and deleted promoters (MUT1) were fused to the E. coli lacZ gene, and two to four monocopy transformants of each construction were selected. Results of the fermentations in glucose-based medium with monocopy transformants of each construction (MUT1, MUT2, and MUT5), and transformants DEL0 and DEL8 (as controls) are shown in Fig. 9. Point mutations in the TTAGTAA sequence resulted in reduced in vivo expression of the reporter gene but were still able to express the gene to a different extent. Transformants MUT2 lost 35% of the β-galactosidase activity, whereas transformant MUT5 lost 46% of the activity. The two transformants (MUT1) having the heptameric sequence TTAGTAA deleted showed only the endogenous activity. This result indicates that the TTAGTAA sequence is strictly necessary for expression of the pcbAB gene. To confirm that no other alteration in the construction was responsible for the loss of expression, the com-

FIG. 6. Formation of the complex AG1 with the two moieties of the A box. Note that the complex is formed only with the A2 moiety.
Complete promoter and the fusion between the pcbAB promoter and lacZ, including the first 180 nucleotides of the lacZ gene, were PCR-amplified from total DNA of transformant MUT1 and sequenced. The nucleotide sequence confirmed that no other base change was observed with regard to the original pcbAB promoter and lacZ gene. Hybridization results further confirmed that integration of the construction occurred at the pyrG locus as a single copy without rearrangement of the construction.

**DISCUSSION**

In *P. chrysogenum* expression of the first two genes of the penicillin biosynthetic pathway, pcbAB and pcbC, must be coordinately expressed as both show a similar pattern of temporal expression and regulation (13, 20). Both genes are transcribed from a common intergenic region of 1013 bp between the translational start codons (9). Analysis of the DNA sequence of this intergenic region reveals the presence of multiple consensus cis-acting regulatory sequences that have been described in *A. nidulans* or in other related fungi as targets for binding of transcriptional regulators. A total of seven PacC, six CreA, six NRE, and six CCAAT consensus sequences are distributed throughout the bidirectional promoter region (Fig. 10); some of these sites may be involved in regulation of either one or both genes (pcbAB and pcbC) simultaneously.

Our results showed that at least three sequences of the pcbAB promoter are important in the control of gene expression. Two of these regions containing cis-acting sequences (boxes A and B) gave clear DNA-protein complexes with cell-free extracts. Box B contains two GATA motifs (NRE2 and NRE3) situated close to its borders. It seems unlikely that these GATA boxes participate in regulation with the expression, because only very weak binding activity was detected when DNA fragments containing these GATA motifs were incubated with a fusion protein containing the zinc-finger region of NRE (18), in contrast to the strong binding activity detected with a DNA fragment containing sites NRE4 and NRE5. In box B there are also three putative recognition sites for PacC (PacC2, PacC3, and PacC4) located close to each other and a consensus CreA binding site located between sites PacC2 and PacC3 (CreA1). Our results indicate that removal of box B from the promoter causes a sharp decrease of transcriptional activity both in media with glucose and with lactose (Fig. 2C). Three retardation complexes were formed between box B and *P. chrysogenum* cell extracts. One of them (BL1) appeared only in extracts of lactose-grown mycelium; this complex was sensitive to the

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**Figure 8.** Formation of the AG1 complex in a deletion mutant MUT1 (lacking the seven nucleotide target sequence) or in the point mutants MUT2, MUT3, MUT4, and MUT5. Lane AB corresponds to the control (nonmutated) box A. Note that the deletion (MUT1) and point mutations in MUT2, MUT3, and MUT5 (and to a lesser extent in MUT4) prevent formation of the AG1 complex. At the top of the figure, the sequences of oligonucleotides used for in vitro mutations are shown.

**Figure 9.** *In vivo* expression of promoters in single copy transformants containing the mutant promoters MUT1, MUT2, and MUT5. The β-galactosidase levels in cultures containing the entire promoter DEL0 and the fully deleted promoter (DEL8) are shown for comparative purposes.

**Figure 10.** Scheme showing the location of the TATA and CCAAT boxes and the PacC, CreA, and NRE consensus sequence in the bidirectional promoter region. The regions corresponding to box A and box B are shaded. The PTA1 protein is shown bound to box A. The transcripts of pcbAB and pcbC are indicated by wavy lines.
presence of 25 mM EDTA suggesting that the DNA-binding protein may contain a zinc-finger. This protein profile fits well with that of PacC; the BL1 complex appeared only with lactose when the pH of the medium is higher (after 24 h of fermentation). In extracts from glucose-grown cells two other retardation complexes of slower mobility than the previous one were detected: BG1 and BG2. Formation of complex BG1 was sensitive to EDTA (i.e., it involves a putative zinc-finger DNA-binding protein), whereas BG2 was not. Formation of the BG1 and BG2 complexes respond to the presence of glucose in the medium, indicating that these two DNA-binding proteins might be involved in glucose regulation of penicillin biosynthesis. The possible interaction of several proteins with the box B is the subject of a separate work.

The expression enhancing role of box A (stretching out from −789 to −665) has been studied in detail. This region contains four putative CreA binding sites: CreA3, CreA4, CreA5, and CreA6. All these sites have the consensus sequence SYGGRG reported by Cubero and Scazzocchio (32) as the target for CreA binding. CreA5 and CreA6 are located contiguously on the same DNA strand and have the consensus sequence GCCGGG. EMSA experiments revealed the formation of a protein-DNA complex (AG1) with probe A and protein extracts from both glucose and lactose-grown cells (Fig. 3A). It is likely that the AG1 complex is modified (perhaps by phosphorylation) in lactose-grown cells. When the box A DNA fragment was divided into two smaller probes (A1 and A2), EMSA experiments indicated that the binding activity was retained only by probe A2 (Fig. 6), excluding the possibility that sites CreA5 and CreA6 participated directly in the formation of the complex.

The results of the uracil interference assay showed that the DNA sequence participating in the AG1 complex formation was TTAGTAA (centered at position −679). There are no other sequences in the pcbAB-pcbC intergenic region matching exactly this heptanucleotide. The TTAGTAA sequence resembles target sequences for other transcriptional regulators (33), particularly the core of the target sequence of BAS2 (PHO2) (TTAGTAA), a factor with two zinc fingers required for basal and induced transcription of different genes in Saccharomyces cerevisiae (34).

Deletion of box A causes a decrease of 41% in the transcriptional activity of the promoter in lactose-grown mycelium and approximately the same (40%) in glucose-grown cells; therefore the protein that binds to TTAGTAA is expected to be a transcriptional activator, and for this reason it was named PTA1. To confirm this hypothesis, the target sequence of PTA1 was deleted from the promoter. In vivo studies comparing the resulting β-galactosidase activity obtained with the mutated or deleted heptanucleotide sequence (mutants MUT1, MUT2, and MUT5) with the activity of the entire unmodified promoter (transformant DEL0) confirmed that PTA1 is a transcriptional activator. Mutations in the first thymine (MUT2) or simulta-

neous substitution of both Ts at positions 1 and 5 altered the PTA1-box A interaction and reduced the in vivo expression of the reporter gene, whereas the activity found in the MUT1 transformants (with the deleted heptamer) dropped to the level of endogenous activity. This interesting result may be due to existence of complex protein-protein interactions among the multiple transcriptional factors that presumably regulate pcbAB-pcbC expression.

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