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The *Aspergillus nidulans npeA* locus consists of three contiguous genes required for penicillin biosynthesis

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Clones of *Aspergillus nidulans* genomic DNA spanning 20 kb have been isolated and shown by a combination of classical and molecular genetic means to represent the *npeA* locus, previously found to be one of four loci (*npeA*, *npeB*, *npeC* and *npeD*) involved in the synthesis of penicillin. As well as containing the gene encoding the second enzyme for penicillin biosynthesis, namely isopenicillin N synthetase (IPNS) (designated *ipnA*), our results show that these clones (pSTA200, pSTA201 and pSTA207) contain two more genes to form a cluster of three contiguous penicillin biosynthetic genes. Our evidence suggests that these genes encode δ(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS) and acyl transferase (ACYT) (designated *acvA* and *acyA* respectively), the first and third enzymes required for penicillin biosynthesis, with the gene order being *acvA*−*ipnA*−*acyA*. Transcripts have been identified for the three genes and their approximate sizes determined—*acvA* 9.5 kb, *ipnA* 1.4 kb and *acyA* 1.6 kb. All three mRNA species are observed in cells grown in fermentation medium but not in cells grown in minimal medium, suggesting that the control of penicillin biosynthesis is, in part, at the level of mRNA accumulation. Finally our results show that *acvA* and *ipnA* genes are divergently transcribed, whilst *acyA* is transcribed in the same orientation as *ipnA*.

Key words: antibiotic genes/filamentous fungi/penicillin biosynthesis/regulation

Introduction

Despite the massive industrial production and world-wide application of β-lactam antibiotics, relatively little is known regarding the molecular biology of their biosynthesis. This is in part due to the sparsity of information concerning the genetics of the filamentous fungi *Penicillium chrysogenum* and *Cephalosporium acremonium*, the two most important commercial sources of β-lactams. Alteration in antibiotic titre by genetic means has been based solely on the laborious empirical method of screening large numbers of mutagenized cells for β-lactam over-producers and non-producers (for reviews see Martin and Demain, 1980; Martin and Liras, 1985; Ingolia and Queener, 1989; Miller and Ingolia, 1989). Recently, molecular techniques have been applied to analyse antibiotic production and its regulation. Such studies have resulted in the identification and isolation of the genes encoding isopenicillin N synthetase (IPNS) from *P.chrysogenum* (Carr *et al.*, 1986), *C.acremonium* (Samson *et al.*, 1985) and *Aspergillus nidulans* (Ramon *et al.*, 1987) and acyl transferase (ACYT) from *P.chrysogenum* (van Solingen *et al.*, 1989; J. Martin, unpublished data), enzymes catalysing the second and third steps of the penicillin biosynthetic pathway respectively (Figure 1).

We have used the filamentous fungus *A.nidulans* as a model organism for the investigation of penicillin biosynthesis. Although *A.nidulans* produces comparatively low levels of penicillin, it has the advantage of being amenable to rigorous formal genetic analysis. Efficient gene-mediated transformation systems for *A.nidulans* have also been generated, including the *argB* system (Johnstone *et al.*, 1985) utilized in this work. Furthermore, a number of

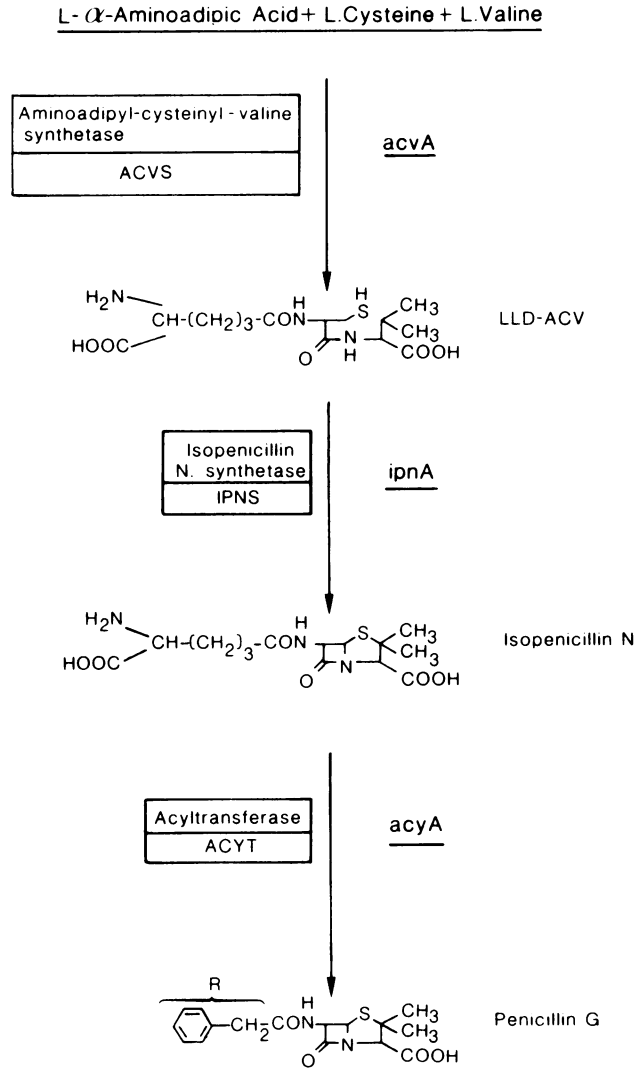

Fig. 1. The biochemical and genetic components of the penicillin biosynthetic pathway in *A.nidulans*.

A.nidulans mutants (designated *npe*) defective in penicillin production have been described.

Previous studies, using chemically or radiologically induced mutants of *A.nidulans*, had distinguished four unlinked complementation groups involved in penicillin biosynthesis—*npeA, npeB, npeC* and *npeD* (Edwards *et al.*, 1974; Holt *et al.*, 1976; Makins *et al.*, 1981, 1983). Of these, the majority of penicillin non-producing mutations fall into the *npeA* category. In addition, there exist naturally occurring penicillin non-producers, which have been shown to correspond to mutational defects mapping at the *npeA* locus (Cole *et al.*, 1976). Biochemical work has implicated this locus in the formation of the tripeptide δ(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV). The observation that mutant strain *npeA*0022 was blocked in this step suggested an association between *npeA* and ACV synthetase (ACVS) (Makins *et al.*, 1981), the first enzyme of the pathway (Figure 1).

We report here the isolation and preliminary analysis of genomic DNA clones that are counterparts of the *npeA* locus. In addition to containing the gene encoding IPNS (designated *ipnA*), we present evidence for the existence of two further penicillin biosynthetic genes on these clones. These genes encode ACYT (designated *acyA*) and ACVS (designated *acvA*) (Figure 1).

Results

Heterologous hybridization of P.chrysogenum IPNS to A.nidulans npeA$^+$ and npeA0049/1 genomic DNA

Figure 2 shows cross-hybridization of the *P.chrysogenum* IPNS gene probe with *A.nidulans* and *C.acremonium* DNA. Differences in hybridization pattern were observed between a naturally occurring *A.nidulans* penicillin non-producing mutant (*npeA*0049/1) and the wild-type strain (*npeA*$^+$). No hybridization was observed in *npeA*0049/1, implying a deletion of sequences homologous to the IPNS gene probe. This suggested that the *npeA* locus in *A.nidulans* may contain the IPNS gene. To investigate this further we isolated several *A.nidulans* IPNS-containing genomic clones.

Isolation and characterization of A.nidulans genomic clones

An *A.nidulans* genomic library (Johnstone *et al.*, 1985) was screened at low stringency using the *P.chrysogenum* IPNS gene probe. Of 17 strongly hybridizing clones, two were found to overlap by 3 kb but extend for 10 kb in opposite directions, namely pSTA200 and pSTA201. A map of the genomic *A.nidulans* IPNS region was generated (Figure 3.I) and the IPNS gene located by hybridization to the *P.chrysogenum* IPNS probe. Comparison of this map to that of Ramon *et al.* (1987) confirmed the location and orientation of the *A.nidulans* IPNS gene on pSTA200 and pSTA201.

The *A.nidulans* library was rescreened using a 0.6 kb *Kpn*I−*Sal*I fragment isolated from the end of the pSTA201 genomic insert distal to IPNS (Figure 3.II), with a view to isolating a clone similar to pSTA201 but excluding the IPNS region. Such a clone was isolated and designated pSTA207.

Southern blot analysis of npeA mutants

Figure 4 shows restriction enzyme digests of genomic DNA isolated from various *npeA* mutants and wild-type *A.nidulans* which have been blotted and probed with DNA fragments spanning pSTA200, pSTA201 and pSTA207.

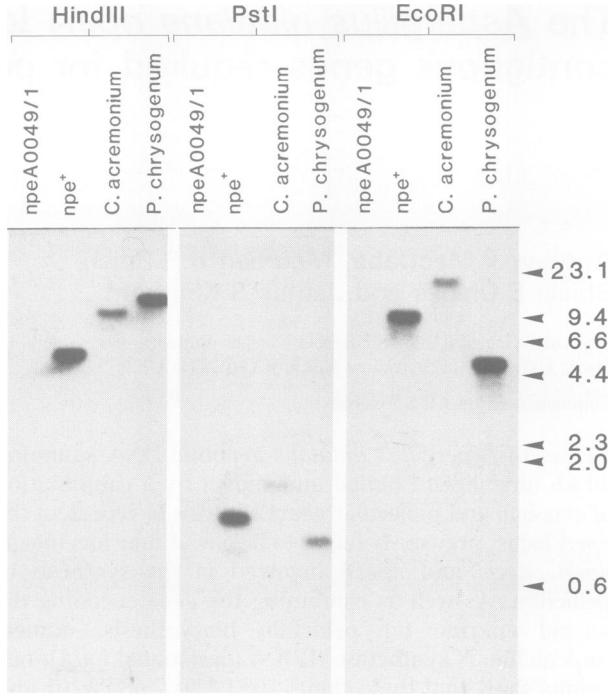

Fig. 2. Hybridization of the *P.chrysogenum* IPNS gene to *A.nidulans* and *C.acremonium* DNA. Genomic DNA digests (2 μg) were electrophoresed on a 1% agarose gel at 70 V for 6 h and transferred onto Amersham Hybond-C. The blot was pre-hybridized at 56°C for 12 h with 4 × SET, 0.1% $Na_4P_2O_7 \cdot 10H_2O$, 0.1% SDS, 100 μg/ml denatured herring sperm DNA, 0.1% polyvinylpyrrolidone, 0.1% Ficoll, 400, 0.1% bovine serum albumin and hybridized overnight to the ^{32}P-labelled *P.chrysogenum* IPNS gene probe in the same buffer at 56°C. Washing was performed at 56°C for 30 min periods with 4 ×, 1 × and 0.5 × SET in 0.1% $Na_4P_2O_7 \cdot 10H_2O$, 0.1% SDS, sequentially. The blot was autoradiographed with Fuji RX film for 12 h. Molecular size markers in kb are shown on the right margin. *npeA*0049/1 and *npe*$^+$ are the relevant markers of the *A.nidulans* strains.

Mutation *npeA*0049/1 appears to be an extensive deletion of genomic DNA extending from the furthest cloned sequences 5′ of IPNS to the region of probe H, 3′ of IPNS. This suggests that the cloned DNA fragments encompassing the IPNS gene correspond to the *npeA* region. Sequences in the vicinity of the right-most extremity of the deletion are probably rearranged as probe J hybridized to fragments of different sizes in *npeA*0049/1 and wild-type. The mutation *npeA*0022 appears to have resulted in a perturbation of genomic sequences homologous to probe C. The data can be interpreted as representing either an insertion of ~14 kb of DNA into the region cross-hybridizing to *Bam*HI fragment C, or a translocation of sequences from C to another genomic location. The mutation *npeA*005 showed no obvious difference in genomic organization within the region probed, in comparison to wild-type. This may reflect a point mutation, or small insertion or deletion of DNA.

Southern blot analysis was also carried out on genomic DNA digests from six independently isolated, naturally occurring *npeA* strains (Birmingham Strain nos 49, 108, 112, 123, 132 and 136) all belonging to heterokaryon-compatibility group F (Cole *et al.*, 1976). The banding patterns observed were identical to those seen for the naturally occurring *npeA*0049/1 mutation, indicating a similar deletion of this 20 kb genomic region in all seven of the wild isolate *npeA* mutants analysed (unpublished data).

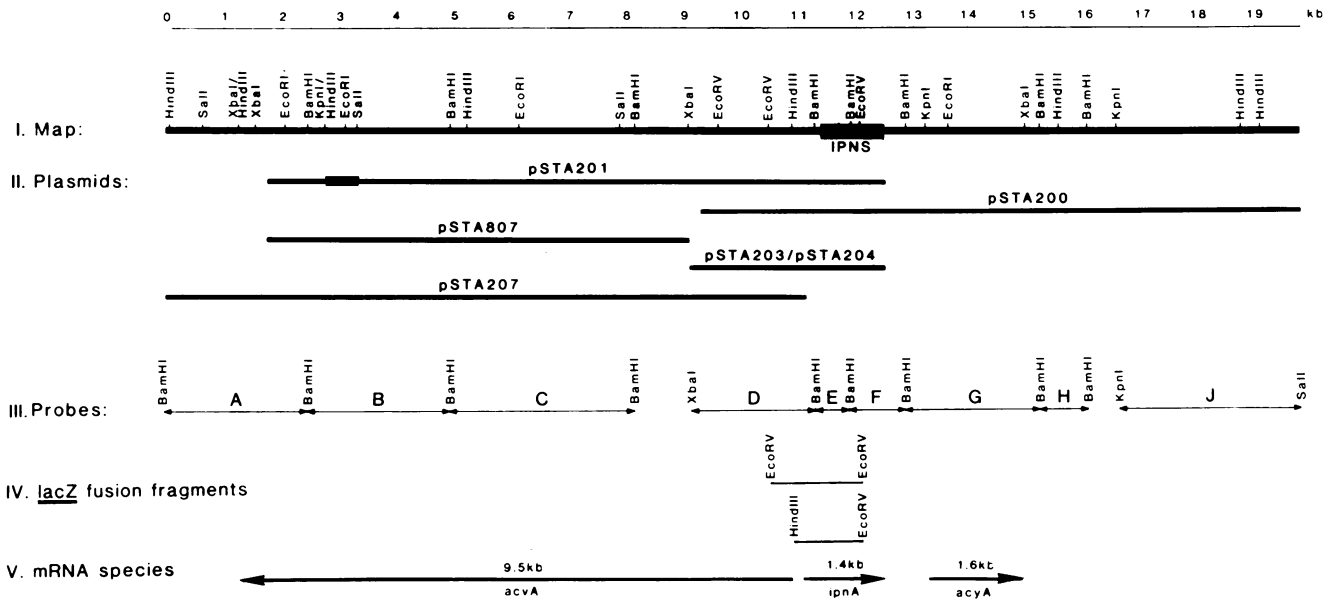

Fig. 3. The *A.nidulans* IPNS region. (**I**) Restriction endonuclease map. The heavy bar represents the gene encoding IPNS (Ramon *et al.*, 1987). Only *Eco*RV sites relevant to the *lacZ* fusions are shown. (**II**) pSTA201, pSTA200 and pSTA207 were isolated from an *A.nidulans* genomic library in pILJ16 (Johnstone *et al.*, 1985). Plasmids pSTA203, pSTA204 and pSTA807 are subclones of pSTA201. (**III**) DNA fragments used to generate ^{32}P-labelled probes for hybridization experiments. Fragment A was isolated from pSTA207, fragments B, C and E from pSTA201 whilst fragments D, F, G, H and J were isolated from pSTA200. The 0.6 kb *Kpn*I−*Sal*I fragment from pSTA201 used as a probe to isolate pSTA207 is represented by a heavy bar on pSTA201. (**IV**) Fragments inserted into *lacZ* fusion vectors in both orientations. (**V**) mRNA species revealed by Northern blotting analyses. The approximate position of the genes was deduced from Northern blotting, complementation and cross-hybridization studies. The direction of transcription is indicated by the arrows (determined by Ramon *et al.*, 1987 for *ipnA*). Not all *Sal*I sites in fragments B and C are located on the map.

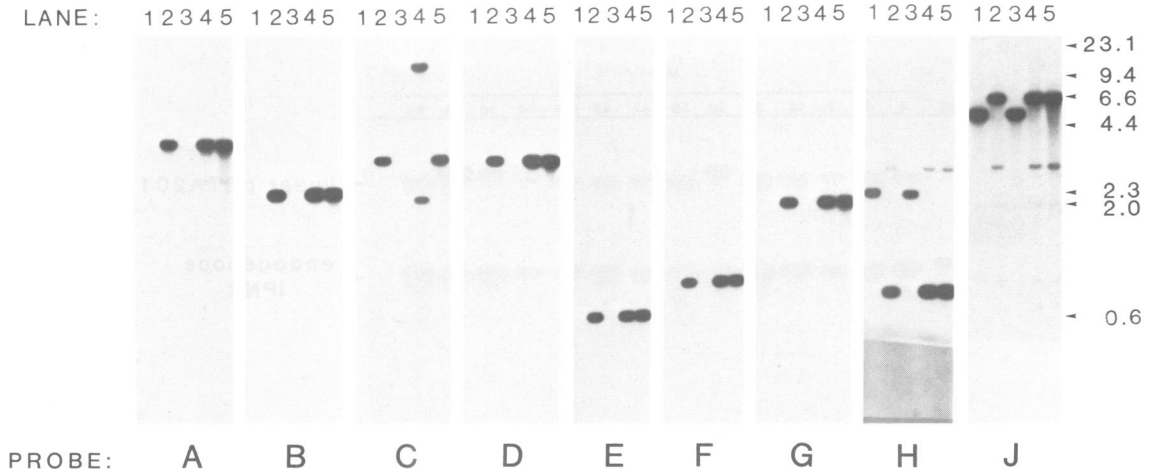

Fig. 4. Southern blot analysis of *A.nidulans npeA* mutants. *Bam*HI genomic DNA digests (5 μg) were electrophoresed on 1% agarose gels at 50 V overnight and blotted onto Amersham Hybond-N. Blots were pre-hybridized at 56°C for 12 h in 5 × SSPE, 6% PEG 6000, 0.5% skimmed milk, 1% SDS, 0.1% $Na_4P_2O_7{\cdot}10H_2O$, 250 μg/ml denatured herring sperm DNA and hybridized overnight in the same buffer at 56°C with the specified ^{32}P-labelled probes (see Figure 3). Washing was performed at 56°C for 30 min periods with 4 ×, 1 × and 0.2 × SSC in 0.1% $Na_4P_2O_7{\cdot}10H_2O$, 0.1% SDS, sequentially. The blots were autoradiographed with Fuji RX film for 12 h. Relevant genotypes of DNA in each track are as follows: **Lane 1**, *npeA*0049/1, *argB*$^+$; **lane 2**; *npeA*$^+$, *argB*$^+$; **lane 3**, *npeA*0049/1, *argB*$^+$; **lane 4**, *npeA*0022, *argB*2; **lane 5**, *npeA*005, *argB*$^+$. Molecular size markers in kb are shown on the right margin.

Since the left-most extremity of the deletion in *npeA*0049/1 and each of these mutants has not been established, they may not be identical.

Complementation of A.nidulans npeA mutants

Double mutant *npeA*, *argB*2 strains were used as recipients for transformation of recombinant plasmids pSTA200, pSTA201 and pSTA207 and their derivatives (Figure 3.II). Transformants were selected for arginine prototrophy and screened for penicillin production (Table I). Of the three genomic clones, only pSTA201 and pSTA207 efficiently complemented the phenotype of mutation *npeA*0022. Subclones pSTA203 and pSTA204, both of which contain the intact IPNS gene, failed to complement this mutation, though pSTA807 yielded a very low frequency of complementation. These results indicate that a penicillin biosynthetic gene lies 5′ to IPNS and may be intact on pSTA201 (but see gene disruption studies) and pSTA207, as judged by the frequency of complementation, but incomplete on pSTA807. Evidence that the fragment responsible for complementation of *npeA*0022 is not IPNS is provided by the fact that pSTA207 (which does not include

IPNS) complemented *npeA*0022 whilst pSTA203 and pSTA204 (which include IPNS) failed to do so.

Only pSTA201 reversed the phenotype of mutant *npeA*005. pSTA200 and pSTA207 complemented at low frequency but only when transformed together into this mutant.

The deletion mutant *npeA*0049/1 was not complemented by pSTA200, pSTA201 or pSTA207 in co-transformation experiments.

Table I. Transformation and complementation of *npeA*, *argB*2 mutants

Allele	Plasmid	No. of *argB*$^+$ transformants analysed	No. of *npeA*$^+$ phenotypes
*npeA*0022	pSTA200	103	0
	pSTA201	259	56
	pSTA207	100	39
	pSTA203	224	0
	pSTA204	194	0
	pSTA807	977	1
*npeA*005	pSTA200	417	0
	pSTA201	99	32
	pSTA207	510	0
	pSTA204	218	0
	pSTA807	322	0
	pSTA200 + pSTA207[a]	203	2
*npeA*0049/1	pSTA200 + pSTA201[a]	399	0
	pSTA200 + pSTA207[a]	418	0

[a]Co-transformation experiments with equimolar plasmid concentrations (5 μg of each).

Southern analyses of npeA0022 complementing transformants

A random sample of 27 arginine prototrophs (designated MacT) arising from pSTA201 complementation of the *npeA*0022, *argB*2 double mutant, which were found concomitantly to produce penicillin (i.e. *npeA*$^+$), were examined by Southern blotting. *Xba*I digests of genomic DNA were blotted and hybridized with probe E (Figure 3.III), containing a fragment of the *A.nidulans* IPNS gene, the results of which are shown in Figure 5. Whilst wild-type and all transformants analysed exhibited the endogenous IPNS fragment, an additional larger band was present in all transformants. For the majority (>70%) this signal corresponded to the size of linearized pSTA201 (e.g. MacT5, MacT48, MacT69). This observation is consistent with homologous integration of the transforming plasmid at the genomic IPNS site.

Of the remaining transformants, only one (MacT83) was shown, by reprobing with *argB*, to have integrated at the *argB* locus (data not shown). The sites of integration in MacT28, MacT56, MacT65, MacT82, MacT86 and MacT91 are unknown.

Classical genetic analysis of transformants

Five transformants analysed by Southern blotting were also subjected to classical genetic analysis to determine the genomic location of the integrated sequences by crossing to

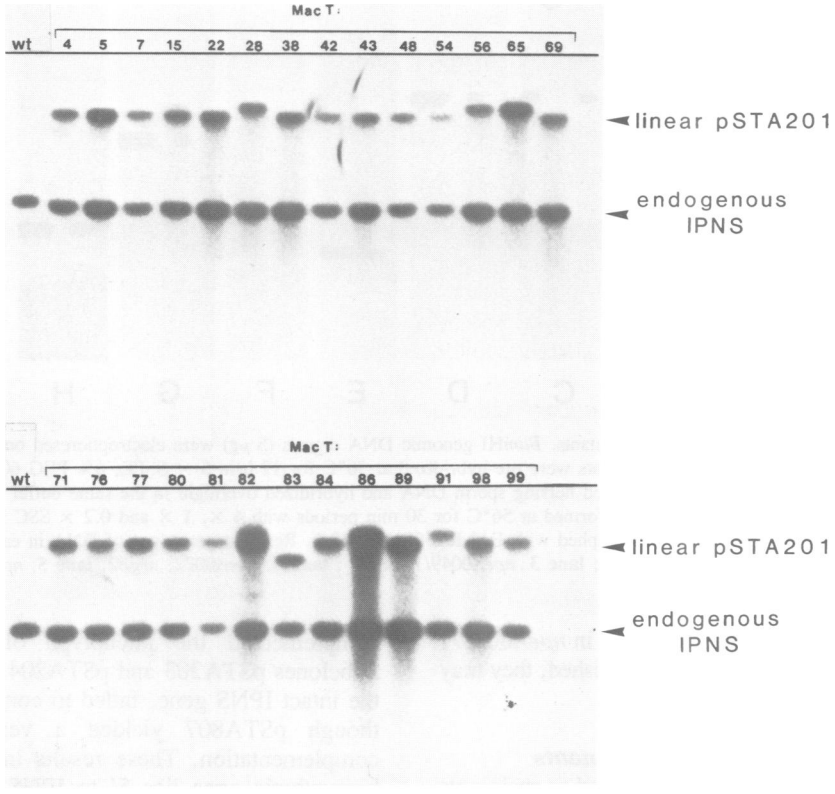

Fig. 5. Southern blot analysis of transformants. DNA mini-preps were made from 10 ml cultures of MacT transformants and digested with *Xba*I. Samples were electrophoresed for 12 h at 50 V on a 1% agarose gel and transferred to Amersham Hybond-N. The blot was pre-hybridized and hybridized at 68°C as described in the legend to Figure 4 using probe E, and washed down to 0.2 × SSC at 68°C prior to autoradiography with Fuji RX film for 20 h. The blot was stripped by three successive 30 min washes in distilled water at 90°C. Pre-hybridization and hybridization were performed as before using the *argB* probe (data not shown).

an *npeA*$^+$, *argB*$^+$ strain (Table II). Integration of pSTA201 at the *npeA* or *argB* loci would result in the absence of *npeA*$^-$ or *argB*$^-$ progeny respectively. The genetic data show that MacT5, MacT48 and MacT69 carry integrates at or near the *npeA* locus (no *npeA*$^-$ phenotypic progeny were recovered from such crosses). The integration of pSTA201 at the IPNS site in transformants MacT5, MacT48 and MacT69 as judged by Southern analyses combined with formal genetic mapping of such integration events to the *npeA* locus support the suggestion that the IPNS region and the *npeA* locus are counterparts. Furthermore, classical genetic analyses confirm that pSTA201 has integrated in MacT83 and MacT91 at sites other than *npeA*; in MacT83, pSTA201 mapped at the *argB* locus, whilst in MacT91 integration occurred at an ectopic site.

A summary of the combined physical and genetic transformation data is given in Table III.

Hybridization studies of sequences 5′ of IPNS

Complementation studies of the *npeA*0022 mutation indicate the existence of a gene 5′ of IPNS which has a role in the biosynthetic pathway of β-lactams. We have carried out a heterologous hybridization using probe C (Figure 3III) to *Eco*RI digests of genomic DNA isolated from different fungal species. Specific cross-hybridization did occur to DNA from *P.chrysogenum, C.acremonium* and *A.oryzae*, all of which produce β-lactams. Significantly, a specific signal was not observed for *Neurospora crassa*, an organism from which we have been unable to detect antibiotic activity on bioassay plates (data not shown).

Identification of bidirectional promoter activities 5′ of IPNS

In order to investigate the control of expression of the *A.nidulans* IPNS gene, fragments of DNA in the region 5′ of IPNS were cloned into *lacZ* fusion vectors (van Gorcom *et al.*, 1986). This system was designed to report the presence of promoter sequences on cloned stretches of DNA by producing a hybrid protein product comprising the N-terminal region of the gene under analysis and the β-galactosidase activity of a partially deleted *Escherichia coli lacZ* gene. Two DNA fragments in the IPNS region—a 1.6 kb *Eco*RV−*Eco*RV fragment and a 1.2 kb *Hin*dIII−*Eco*RV fragment (Figure 3.IV)—were cloned into these vectors in all possible reading frames and orientations with respect to the *lacZ* reporter gene. For preliminary analysis the 12 different recombinants so generated were transformed into the *npeA*0022, *argB*2 double mutant and grown on medium in which the endogenous *A.nidulans* β-galactosidase gene is repressed. Transformants containing the 1.2 kb *Hin*dIII−*Eco*RV fragment showed the presence of promoter activity reading only in the direction of transcription of the IPNS gene. Transformants containing the 1.6 kb *Eco*RV−*Eco*RV fragment, however, showed two promoter activities— one apparently identical to that in the *Hin*dIII-*Eco*RV fragment (i.e. same direction and same reading frame) and a second in the opposite orientation. This suggests that the transcription start site of a gene 5′ to, and reading away from the IPNS gene, is within the 0.4 kb *Eco*RV−*Hin*dIII stretch.

Table II. Genetic linkage observed in transformant strains

Transformant[a]	No. of randomly isolated progeny screened	Phenotype of progeny			
		argB$^+$	*argB*$^-$	*npeA*$^+$	*npeA*$^-$
MacT5	60	56	4	60	0
MacT48	60	54	6	60	0
MacT69	120	99	21	120	0
MacT83	60	60	0	36	24
MacT91	60	54	6	30	30

[a]Transformants of the putative genotype *argB*2, (*argB*$^+$), *npeA*0022, (*npeA*$^+$) where the genes in brackets denote incoming DNA, were crossed to strain B418 of genotype *argB*$^+$, *npeA*$^+$.

Gene disruption studies using a fragment 5′ of IPNS

pSTA807 is a subclone of pSTA201 that contains a 7.5 kb region 5′ of IPNS (Figure 3.II). This stretch probably does not contain an intact penicillin biosynthetic gene since pSTA807 complements *npeA*0022 only at a very low frequency (Table I). pSTA807 was introduced by transformation into an *argB*2, *npeA*$^+$ strain. It was observed that 59% of arginine prototrophic transformants showed penicillin non-producing phenotypes. The high frequency of penicillin non-producers is indicative of homologous integration of an

Table III. Summary of plasmid integration from DNA hybridization and classical genetic analysis

Transformant	DNA hybridization	Classical genetics
MacT5	IPNS	*npeA*
MacT48	IPNS	*npeA*
MacT69	IPNS	*npeA*
MacT83	*argB*	*argB*
MacT91	elsewhere	elsewhere

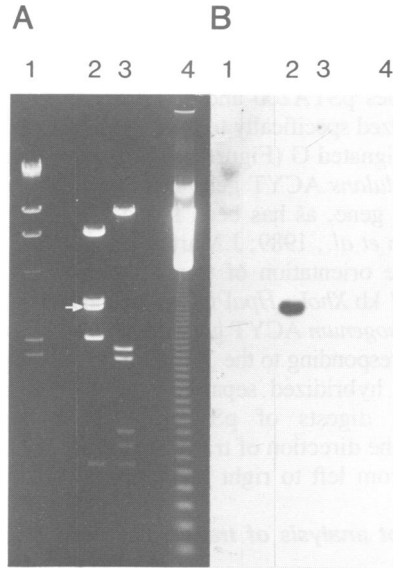

Fig. 6. Identification of the ACVS coding region. **Panel A** shows the *Bam*HI digestion pattern of plasmids pSTA200 (**lane 2**) and pSTA201 (**lane 3**) after electrophoresis on a 1% agarose gel. **Lanes 1** and **4** contain λ/*Hin*dIII and BRL 123 bp markers respectively. DNA was transferred onto Amersham Hybond N and the blot pre-hybridized at 30°C overnight with 5 × SSPE, 6% PEG 6000, 0.5% skimmed milk, 1% SDS, 0.1% $Na_4P_2O_7 \cdot 10H_2O$, 250 g/ml denatured herring sperm DNA and hybridized overnight in the same buffer at 30°C with 100 ng end-labelled mixed 23mer probe. Washing was performed in 5 × SSC, 0.1% SDS, 0.1% $Na_4P_2O_7 \cdot 10H_2O$ at 30°C for 40 min, and repeated in the same buffer at 40°C. **Panel B** shows the result of autoradiography with Fuji RX film for 6 h.

incomplete gene and, by extension, the existence of a penicillin biosynthetic gene 5′ of IPNS.

Finally, that gene disruption by pSTA807 occurs, suggests that this construct lacks both 5′ and 3′ termini. pSTA201 therefore must also lack a terminus (see Figure 3II), probably the 3′ in view of the *lacZ* fusion data detailed earlier. Consequently, complementation of *npeA*0022 by pSTA807 must be a rare transformation event, involving a double crossover at the *npeA* site as well as a second integration at *argB*. It is possible to speculate on the discrepancy between complementation of *npeA*0022 by pSTA201 following integration at non-homologous sites and the apparent lack of the 3′ terminus. The molecular basis of this contradiction is, however, as yet unclear.

Identification of the ACVS coding region

ACVS protein was purified and used to generate partial amino acid sequence data, from which a set of mixed 23mer oligonucleotides was synthesized and subsequently used to probe Southern filters of *Bam*HI-digested pSTA200 and pSTA201 (Figure 6). Only a 3.0 kb *Bam*HI fragment of pSTA201 (Figure 3.III) was detected using this probe. That no hybridization was observed to any fragment of pSTA200 suggested that the 1 kb *Bam*HI−*Xba*I nucleotide stretch between fragments C and D (see Figure 3III) contained the region homologous to the probe. Preliminary DNA sequence data (to be published elsewhere) of this region has confirmed this conclusion and has shown that the ACVS gene is transcribed in the opposite orientation relative to IPNS (Figure 3V).

Hybridization studies of sequences 3′ of IPNS

A 1.9 kb *Hin*dIII−*Eco*RI *P.chrysogenum* probe isolated from pSTA18, which contains part of the gene coding for ACYT (van Solingen *et al.*, 1989; J. Martin, unpublished data), was hybridized to *Bam*HI digests of the *A.nidulans* genomic clones pSTA200 and pSTA201 (Figure 7). This probe hybridized specifically to the 2.1 kb pSTA200 *Bam*HI fragment designated G (Figure 3.III). It appears therefore, that the *A.nidulans* ACYT gene is located close to, and 3′ of, the IPNS gene, as has been found for *P.chrysogenum* (van Solingen *et al.*, 1989; J.Martin, unpublished data). To determine the orientation of transcription of ACYT, two probes—a 0.7 kb *Xho*I−*Hpa*I fragment containing the 5′ end of the *P.chrysogenum* ACYT gene and a 0.9 kb *Eco*RI−*Sal*I fragment corresponding to the 3′ region (van Solingen *et al.*, 1989)—were hybridized separately to various restriction endonuclease digests of pSTA200. Such experiments suggest that the direction of transcription is the same as for IPNS, i.e. from left to right in Figure 3V.

Northern blot analysis of transcripts from the npeA region

Three major transcripts were detected by Northern blot analysis (Figure 8A−C) of total RNA prepared from wild-type mycelium grown in fermentation medium (derepressed conditions). No transcripts were detected in RNA isolated from mycelium grown in minimal medium (repressed conditions). The presence of transcripts followed a pattern similar to the production of penicillin as judged by bioassay, i.e. antibacterial halos were observed around wild-type cells grown on fermentation medium but not those grown on minimal medium (Figure 8E).

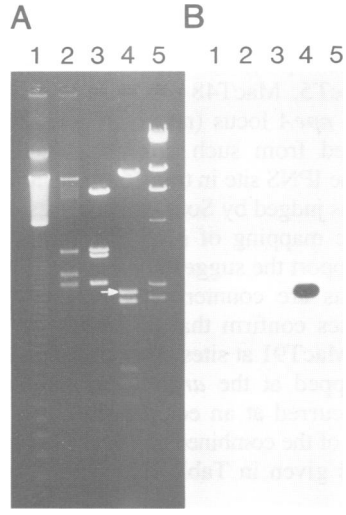

Fig. 7. Identification of the *A.nidulans* acyl transferase gene. **Panel A** shows the *Bam*HI digestion pattern obtained from plasmids pSTA207 (**lane 2**), pSTA201 (**lane 3**) and pSTA200 (**lane 4**). **Lanes 1** and **5** contain the BRL 123 bp ladder and λ/*Hin*dIII markers respectively. **Panel B** shows the result of hybridization of the *P.chrysogenum* ACYT probe to the plasmid digests under the same conditions as described in the legend to Figure 4, after washing down to 1 × SSC at 56°C.

A probe specific for the IPNS gene (Figure 3.III, fragment E) hybridized to an RNA species of 1.4 kb. This species was of the size expected from the inferred IPNS protein sequence (Ramon *et al.*, 1987). A probe specific for the ACVS gene (Figure 3.III, fragment C) hybridized to a species of 9.5 kb. We suggest that this is the ACVS message although this mRNA size is rather larger than that predicted from the protein size of 230 kd (van Liempt *et al.*, 1989). This discrepancy may be due to post-translational proteolytic processing or to specific cleavage of the protein by a protease during purification. Alternatively, such variation could be as a result of the protein size being underestimated, a reasonable possibility considering its large size. Total RNA probed with a nucleotide stretch 3′ of IPNS (Figure 3.III, fragment G) revealed a transcript of 1.6 kb. This mRNA size is in reasonable agreement with the inferred polypeptide length of 358 amino acids reported for the *P.chrysogenum* ACYT gene (van Solingen *et al.*, 1989). We suggest that the 1.6 kb transcript is the ACYT message.

The appearance of all three transcripts is in accordance with the regulation of the three genes being, at least in part, at the level of mRNA accumulation.

Discussion

The data presented identify the *A.nidulans npeA* locus as the genomic site for at least one gene, designated *ipnA*, coding for the second enzyme (IPNS) involved in penicillin biosynthesis. DNA clones harbouring the *A.nidulans* IPNS gene were found to integrate at the resident IPNS genomic site in the majority of transformants analysed by Southern hybridization. Significantly, such integrations were found by formal genetic analysis to locate at the *npeA* locus. Furthermore, that the *npeA*0049/1 mutant strain is deleted in the IPNS region supports the notion that the IPNS region and the *npeA* locus are counterparts. Additionally, our results (i) confirm the restriction endonuclease map of the *ipnA*

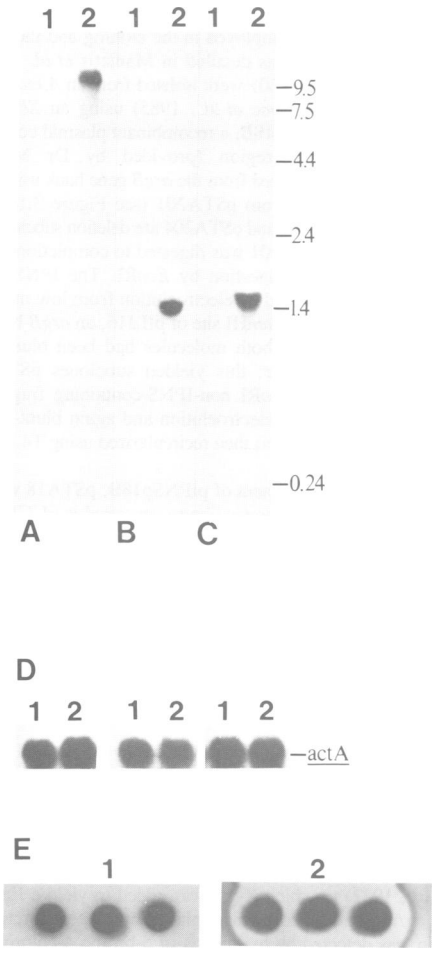

Fig. 8. Northern blots of transcripts. Total RNA was prepared, from *A.nidulans* wild-type mycelium (**1**) grown under repressed conditions in minimal medium and (**2**) grown under derepressed conditions in fermentation medium. Following electrophoresis of 10 μg total RNA in 1% agarose containing 0.66 M formaldehyde, gels were washed twice in 10 × SSC for 20 min before blotting onto Amersham Hybond-N. Blots were pre-hybridized for 5 h at 42°C in 5 × SSPE, 40% deionized formamide, 250 μg/ml denatured herring sperm DNA and hybridized overnight in the same buffer at 42°C with the ^{32}P-labelled probes. Filters were washed sequentially in 5 ×, 3 × and 1 × SSC, containing 0.1% $NA_4P_2O_7{\cdot}10H_2O$, 0.1% SDS before autoradiography with Fuji RX film. (**A**) The putative ACVS fragment C (Figure 3.III) was used as probe. (**B**) The IPNS specific fragment E was used as probe. (**C**) The putative ACYT fragment G was used as probe. The molecular sizes (kb) of the BRL RNA ladder are indicated. (**D**) In order to verify uniformity of RNA transfer, blots were stripped by boiling twice in distilled water containing 1% SDS and reprobed with a fragment specific for *A.nidulans actA* (Fidel *et al.*, 1988). (**E**) Bioassay of wild-type mycelium grown on (**1**) minimal medium; (**2**) fermentation medium. Areas of clearing around plugs represent antibacterial activity (indicating the presence of β-lactam antibiotics).

region reported by Ramon *et al.* (1987) and indeed significantly expand the region; (ii) indicate that the *ipnA* mRNA is 1.4 kb, which is in accord with the expected size of the transcript from the protein data of Ramon *et al.* (1987); and (iii) suggest that the regulation of *ipnA* is at the level of mRNA accumulation.

An additional gene required for penicillin biosynthesis is located 5′ of IPNS. Alteration in DNA sequences in mutant *npeA*0022, which is defective in ACVS activity (Makins *et al.*, 1981), is found between 3 and 6 kb upstream of IPNS coding sequences. The fact that pSTA207 does not include IPNS coding sequences but complements *npeA*0022 at a relatively high frequency (39%) suggests that it harbours an intact penicillin biosynthetic gene. Furthermore, sequences leading into the putative gene (namely 1.6 kb *Eco*RV−*Eco*RV fragment but not the 1.2 kb *Hin*dIII−*Eco*RV fragment) give promoter activity. Such results suggest that the transcriptional start of this second antibiotic biosynthetic gene lies in the 0.4 kb *Eco*RV−*Hin*dIII stretch. Transformation of a penicillin-producing strain by a plasmid carrying an incomplete gene lacking both termini would lead to the loss of penicillin production due to disruption of the functional gene as a consequence of homologous integration. This is indeed observed following transformation of the wild-type with pSTA807. Sequence conservation between β-lactam producing fungi detected in the region designated C may also be indicative of another gene. Sequences 5′ to IPNS hybridize to an mRNA species of 9.5 kb isolated from cells grown under the conditions which lead to the derepression of penicillin biosynthesis. That a transcript is observed of sufficient length to encode a protein of the size obtained from mol. wt estimates (van Liempt *et al.*, 1989) supports the conclusion that this DNA region encodes ACVS (designated *acvA*). Finally, hybridization of a 23 base oligomer probe, derived from ACVS amino acid sequence data, to a fragment on pSTA201 is definitive proof of identification, location and orientation of *acvA*.

We also have evidence that a third gene required for antibiotic biosynthesis locates at the *npeA* locus. The *P.chrysogenum* gene for ACYT strongly cross-hybridizes to a single band located 3′ to *A.nidulans ipnA*, which clearly suggests that the ACYT gene (designated *acyA*) is likewise found near this region in *A.nidulans*. A 1.6 kb mRNA is observed in derepressed cells but not repressed cells and it is likely that this transcript represents the *acyA* mRNA.

In summary, the gene encoding IPNS (*ipnA*) has been identified as corresponding to the *npeA* locus. From the evidence presented here, two other antibiotic biosynthetic genes, which encode ACVS and ACYT, also reside within the *npeA* locus, in the genetic order *acvA*−*ipnA*−*acyA*. The biochemical and genetical relationships of the pathway are presented in Figure 1. Therefore, this region represents a complex gene cluster—a situation that is rather unusual but not unique for lower eukaryotes (for reviews see Arst, 1984; Ballance, 1986; Gurr *et al.*, 1986; Rambosek and Leach, 1987).

The *acvA* and *ipnA* genes appear to be divergently transcribed. The direction of *ipnA* transcription has been previously determined (Ramon *et al.*, 1987) whilst evidence that *acvA* is transcribed in the opposite orientation comes from *lacZ* fusion and preliminary DNA sequence data. The direction of *acyA* transcription appears to be in the same orientation relative to *ipnA*. Identification and characterization of specific mRNAs involved indicates that only monocistronic transcripts are produced, a common feature of fungal gene clusters (Gurr *et al.*, 1986). The results also suggest that the expression of *acvA*, *ipnA* and *acyA* genes is mediated by the mRNA accumulated in response to the growth conditions. This could be due either to increased transcription or to slower turnover of these mRNAs in cells grown in fermentation medium.

Amino acid and DNA sequence similarity between IPNS encoding genes from the prokaryotes *Streptomyces clavuligerus* (Leskiw *et al.*, 1989), *S.lipmannii* (Wiegel

et al., 1988) and those from the fungi *C.acremonium* (Samson *et al.*, 1985), *P.chrysogenum* (Carr *et al.*, 1986) and *A.nidulans* (Ramon *et al.*, 1989; Wiegel *et al.*, 1988) suggest an evolutionary kinship. It has been proposed from DNA sequence comparisons that this gene was originally present in the bacteria and horizontally transferred to filamentous fungi ~370 million years ago (Carr *et al.*, 1986; Ramon *et al.*, 1987). Our results showing that *acvA* and *acyA* are tightly linked with *ipnA* might suggest the genes for the whole pathway were transferred as a single unit. DNA and predicted amino acid sequence data of *acvA* and *acyA* should give us more information on the possibility that the cluster was transferred to filamentous fungi from *Streptomyces*.

Finally, regarding the mutation events, pSTA201 alone is able to achieve complementation of mutation *npeA*005. Co-transformation of *npeA*005 by pSTA200 and pSTA207 also gives complementation, albeit at a reduced frequency. No obvious changes have taken place in *npeA*005 at the DNA level according to our Southern analyses. We are unable, therefore, to detect the precise location of mutation *npeA*005 and its primary physiological defect. The deletion mutation *npeA*0049/1 has not been complemented by transformation or co-transformation with any of the plasmids described. This may be due to several factors, not the least of which is the absence of a homologous *npeA* genomic location, which has been demonstrated to be the preferred site of integration of pSTA201. Alternatively, there may be a further genetic determinant required for penicillin biosynthesis such as a regulatory gene that is deleted in *npeA*0049/1 and is not represented on our recombinant clones.

Materials and methods

Media and strains

Escherichia coli strain DH5 (F^-, *recA*1, *endA*1, *hsdR*17 (r_k^-, m_k^+) *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1) was used for the propagation of all plasmids and the preparation of competent cells as detailed in Maniatis *et al.* (1982). Plasmid preparation was carried out by alkaline lysis and CsCl purification (Maniatis *et al.*, 1982).

Complete medium for *A.nidulans* was as described in Cove (1966). Fermentation medium was prepared according to the following recipe: 3.5% lactose, 0.45% $MgSO_4 \cdot 7H_2O$, 1% $CaCO_3$, 0.21% nitrogen as corn steep liquor, and made to pH 5.2 with 5 M KOH; fermentation plugs were prepared by addition of agar to a final concentration of 4%. Minimal medium was that described by Cove (1966) with glucose and ammonium as the carbon and nitrogen sources respectively.

Bioassay medium was prepared thus: 0.5% peptone, 0.3% Lab-Lemco, 0.1% sodium citrate, 1.2% agar. Once the autoclaved medium cooled to 55°C, 1.5 ml of a 5 day culture of *Bacillus subtilis* C107 and 0.85 ml of 0.5% 2,3,5-triphenyl tetrazolium chloride were added to 170 ml of medium. Petri dishes (22 × 22 cm) were used for penicillin biosassays.

Natural penicillin non-producing isolate, *npeA*0049/1 (strain G0049/1) was obtained from Dr A.J.Clutterbuck (University of Glasgow) whilst other natural isolates (Birmingham Strain nos 49, 108, 112, 123, 132 and 136) were from Dr J.Croft (University of Birmingham). These strains belong to heterokaryon-compatibility group F (Cole *et al.*, 1976; J.Croft, unpublished work). Other natural isolates given to us, Birmingham Strain nos 51 and 143 belonging to heterokaryon-compatibility group G, were found to be penicillin producers and consequently their study was not pursued further. Induced penicillin non-producing mutants *npeA*0022 (strain GH79) and *npeA*005 (strain GH44) were obtained from Professor G.Holt (Polytechnic of Central London). Various *A.nidulans argB*2 mutant strains were crossed with *npeA*0049/1, *npeA*0022 or *npeA*005 alleles to construct the double mutants *npeA*0049/1, *argB*2 (strain SAA246) and *npeA*0022, *argB*2 (strain SAA101) and *npeA*005, *argB*2 (strain SAA243) for use in complementation experiments. *npeA*1 (strain G69), an *A.nidulans* penicillin over-producer, was used for the isolation of the ACVS protein. *Penicillium chrysogenum* and *C.acremonium* strains used in this study were V992 and M8650 respectively.

Plasmids

All standard DNA techniques employed in the cloning and the generation of recombinant plasmids were as detailed in Maniatis *et al.* (1982).

Plasmids pSTA200 and pSTA201 were isolated from an *A.nidulans argB*-based genomic library (Johnstone *et al.*, 1985) using an *Xba*I−*Bam*HI fragment prepared from pIPNSp18B, a recombinant plasmid containing the *P.chrysogenum* IPNS-coding region (provided by Dr M.Ramsden, Glaxochem). pSTA207 was isolated from the *argB* gene bank using a 600 bp *Kpn*I−*Sal*I fragment isolated from pSTA201 (see Figure 3.II).

Plasmids pSTA807, pSTA203 and pSTA204 are deletion subclones derived from plasmid pSTA201. pSTA201 was digested to completion with *Xba*I, and then subjected to partial digestion by *Eco*RI. The IPNS-containing *Xba*I−*Eco*RI region was isolated by electroelution from low melting point agarose and subcloned into the *Bam*HI site of pILJ16, an *argB*-based vector (Johnstone *et al.*, 1985), after both molecules had been blunt-ended by treatment with Klenow enzyme; this yielded subclones pSTA203 and pSTA204. The large *Xba*I−*Eco*RI non-IPNS-containing fragment from pSTA201 was also isolated by electroelution and again blunt-ended with Klenow enzyme; this molecule was then recircularized using T4 DNA ligase and designated pSTA807.

Using the *Xba*I−*Bam*HI fragment of pIPNSp18B, pSTA18 was isolated from a *Eco*RI *P.chryosgenum* genomic library generated in pUC13. pSTA18 contains the *P.chrysogenum* gene for IPNS as well as sequences downstream that have been shown to encode the gene for ACYT (van Solingen *et al.*, 1989; J.Martin, unpublished work).

The *lacZ* fusion series of vectors, designated pAN923-41B, -42B, -43B, was given to us by Dr C.A.M.J.J.van den Hondel (TNO, Netherlands). The strategy used for subcloning the relevant fragments is described by van Gorcom *et al.* (1985).

Probes

All probes were radiolabelled with [α-^{32}P]dCTP by the method of Fienberg and Vogelstein (1983), with the exception of the 23 base oligomer which was end-labelled according to Maniatis *et al.* (1982).

β-Galactosidase activity

Qualitative assay of *lacZ* fusion product activity was made on M9 medium (Maniatis *et al.*, 1982) to which X-gal was added to yield a final concentration of 40 μg/ml, and glucose was replaced by maltose. Activity of the hybrid protein was detected by hydrolysis of 5-bromo-4-chloro-8-indolyl-β-D-galactopyranoside (X-gal) to yield a blue mycelium.

Isolation of A.nidulans genomic DNA

Aspergillus nidulans strains were grown for 20 h at 37°C at 200 r.p.m. in appropriately supplemented minimal medium. Two methods of DNA isolation were employed. All large-scale preparation of DNA was as described by Tilburn *et al.* (1983). Small-scale preparation of DNA from transformants for rapid analysis used the technique of Leach *et al.* (1986).

Protoplast preparation and transformation

Protoplasts were prepared by the method of Tilburn *et al.* (1983) with a number of modifications. *Asperigillus nidulans* conidia were seeded into 300 ml minimal medium and grown at 37°C for 14 h at 200 r.p.m. The mycelium was filtered through sterile muslin, rinsed with 0.8 M $MgSO_4$, 10 mM sodium phosphate, pH 5.8, and resuspended in the same buffer. Novozym 234 (0.1 g) was added to the suspension and the whole incubated at 30°C for 2 h with gentle shaking. The mixture was decanted into sterile 20 ml plastic containers and centrifuged at room temperature in an MSE 'Minor' bench-top centrifuge at full speed for 20 min. The protoplast pellicle was removed and washed four times in 1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris, pH 7.5, at room temperature by centrifugation. The final pellet was resuspended in 1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris, pH 7.5, to yield 3×10^8 cells/ml.

For transformation, 200 μl of protoplast suspension was incubated with 5 μg of plasmid at room temperature for 20 min. Ten volumes (2 ml) of 50% PEG 4000, 10 mM $CaCl_2$, 10 mM Tris, pH 7.5, were added and the mixture left at room temperature for 10 min. The preparation was diluted with 5 vol (10 ml) of 1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris, pH 7.5, and aded to 300 ml of molten minimal agar solution containing 1.2 M sorbitol and supplements, maintained at 50°C. The mixture was plated out and incubated at 37°C for up to 4 days.

Penicillin bioassay

Fermentation plugs were inoculated with conidia and incubated in a humid environment at 25°C for 5 days. Plugs were then placed on thin bioassay medium seeded with *B.subtilis* spores and incubated at 37°C overnight.

Antibiotic production was detected as clear halos due to inhibition of the indicator bacterium *B.subtilis*.

Isolation of mRNA

Wild-type *A.nidulans* was grown for 26 h at 25°C, 200 r.p.m. in minimal medium (repressed conditions) and fermentation medium (derepressed conditions). Mycelium was harvested by filtration, washed with sterile distilled water and 4 g pressed wet weight ground in liquid nitrogen. Total RNA was isolated by the method of Cathala *et al.* (1983). Electrophoresis and Northern blotting of RNA were carried out as described by Davis *et al.* (1986).

Protein preparation and sequencing

ACVS protein was prepared from *A.nidulans* (strain G69) by the method of van Liempt *et al.* (1989) from 6 × 10 fermentation cultures grown for 32 h at 28°C—conditions that result in penicillin production. Aliquots (1 mg) of crude ACVS material were diluted with buffer B to reduce sample conductivity of <7 milli-Siemens, and bound to a mono-Q FPLC matrix (Pharmacia) equilibrated at room temperature with buffer B (van Liempt *et al.*, 1989). Protein was eluted with a NaCl gradient made in buffer B. A total of ~3.5 mg of ACVS material was isolated by this method, the active fractions eluting at between 170 and 190 mM NaCl. A final purification of ACVS was made by FPLC gel filtration of protein which had been concentrated by elution from DEAE fastflow (Pharmacia) with a small volume of 1 M NaCl in buffer B. Batchwise application and elution of the recovered protein on a Suparose 12 column (Pharmacia) yielded homogeneously pure ACVS as analysed by SDS−PAGE and Coomassie blue staining. Purified ACVS was extensively dialysed against 0.5% ammonium bicarbonate at 4°C, and lyophilized. The dried material was resuspended (it did not redissolve) in 100% formic acid and aliquoted into ~500 pmol quantities. Vapour-phase pyridylethylation and CnBr cleavage of several aliquots of protein were performed as detailed by Amons (1987) and the Applied Biosystem Technical Support group (personal communication) respectively. Dried material was resolubilized in SDS gel buffer, electrophoresed and blotted onto Immobilon-P (Millipore) as detailed by Matsudaira (1987). Amino acid sequencing was performed directly from immobilon fragments using a gas-phase Applied Biosystems sequencer at the SERC protein sequencing facility (Department of Biochemistry, University of Aberdeen).

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Note added in proof

Recombination analyses were carried out between *npeA*0049/1 and *npeA*0022. No *npeA*$^+$ progeny were observed in 100 progeny analysed from a *npeA*0049/1 × *npeA*0022 cross, indicating that the mutations are closely linked. A recent report by Diez *et al.* (1989) *Mol. Gen. Genet.*, **218**, 572−576 provides physical evidence that the genes for IPNS and ACYT are linked in *P.chrysogenum*. Both transcript sizes have been found to be 1.15 kb. The sizes reported in the present communication are 1.4 kb (IPNS) and 1.6 kb (ACYT). We have no explanation for this discrepancy except that it may be species related.