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The integration of nitrogen and carbon catabolite repression in *Aspergillus nidulans* requires the GATA factor AreA and an additional positive-acting element, ADA

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The expression of the structural genes of the proline utilization cluster of *Aspergillus nidulans* is repressed efficiently only when both repressing carbon and nitrogen sources are present. Two hypotheses can account for this fact. One is a direct or indirect competition mechanism between the positive-acting AreA GATA factor, mediating nitrogen metabolite repression, and the negative-acting CreA protein, mediating carbon catabolite repression. The second is to propose that CreA prevents the binding or activity of another, as yet unidentified, positive-acting factor, here called ADA. We show the second possibility to be the correct one, and we localize the new positive *cis*-acting element within 290 bp of the *prnD–prnB* divergent promoter.

Keywords: carbon catabolite repression/nitrogen metabolite repression/transcriptional control

Introduction

Proline catabolism is a useful model system for studying the regulation of gene expression in *Aspergillus nidulans*. In this organism, and differently from *Saccharomyces cerevisiae*, proline can serve as both a carbon and a nitrogen source. All the genes required to convert external L-proline to glutamate are clustered in 13 kb on chromosome VII (Arst and MacDonald, 1975; Arst *et al.*, 1981; Jones *et al.*, 1981; Hull *et al.*, 1989; see Figure 1). The cluster comprises the *prnA*, *prnB*, *prnC*, *prnD* and *prnX* genes. *prnA* codes for a transcription factor mediating pathway-specific induction (by proline) which is unconditionally required for the expression of the *prn* structural genes. These structural genes are *prnD*, encoding proline oxidase, *prnB*, encoding the specific proline permease, and *prnC*, encoding L-Δ^1-pyrroline-5-carboxylate dehydrogenase. A fifth gene of unknown function, *prnX*, is also present in the *prn* cluster (Gavrias, 1992).

In *A.nidulans*, the utilization of carbon and nitrogen sources is under the control of, respectively, CreA, a negative-acting zinc finger protein (Bailey and Arst, 1975; Dowzer and Kelly, 1991), and AreA, a positive-acting transcription factor belonging to the GATA family (Arst and Cove, 1973; Kudla *et al.*, 1990). Expression of genes involved in the utilization of less favoured carbon sources is repressed by glucose, irrespective of the nitrogen nutrient present in the medium or of the *areA* allele present in the strain. Good examples of glucose-repressible genes are the genes of the ethanol regulon (Mathieu and Felenbok, 1994). Conversely, expression of genes involved in the utilization of compounds which serve only as nitrogen sources is repressed by ammonium or glutamine irrespective of the carbon source or of the presence of a *creA*^{d} (carbon catabolite-derepressed) mutation (Arst and Cove, 1973; Bailey and Arst, 1975). Examples of such genes are those involved in the utilization of nitrate (Cove, 1966; Arst and Cove, 1973) and of purines (Gorfinkiel *et al.*, 1993; Oestreicher and Scazzocchio, 1993).

The expression of the *prn* cluster is repressible by the simultaneous presence of ammonium and glucose. The prime target of glucose/ammonium repression is the permease gene *prnB*. Although synthesis of the *prnD* and *prnC* products is also repressed, the limiting step for proline utilization under repression conditions is proline uptake (Arst *et al.*, 1980). *prn*^{d} mutations (derepressed, see below), map in the *prnD–prnB* intergenic region and result in derepression of *prnB* and also of *prnD* and *prnC* (Sophianopoulou *et al.*, 1993; see Discussion).

Glucose or ammonium on their own reduce the expression of *prnB*, *prnD* and *prnC*, but full repression requires the simultaneous presence of repressing carbon and nitrogen sources (Gavrias, 1992). Strains carrying *areA* loss-of-function mutations (*areA*$^-$) are unable to utilize proline as a source of nitrogen if a repressing carbon source (e.g. glucose) is present, but grow on proline in the presence of non-repressing carbon sources (Arst and Cove, 1973). This implies that the AreA transcription factor is only required for the expression of the *prn* structural genes in the presence of an active CreA protein. The pattern of transcriptional regulation of the *prn* cluster makes teleonomic sense. It allows the use of proline as a nitrogen source even if a rich carbon source is present and as a carbon source even if a rich nitrogen source is present. The utilization of some other metabolites, which can serve as both carbon an nitrogen sources (e.g. acetamide, γ-aminobutiric acid), is subject to the same pattern of regulation as that of proline (Arst and Bailey, 1977).

A model where AreA is strictly necessary, and CreA acts by preventing its binding or action, is incompatible with both the genetic and physiological evidence. If this were the case, either ammonium or glucose alone would repress *prn* expression, and *areA*$^-$ strains would be unable to use proline, irrespective of the carbon source. Two models could account for the pattern of repression of the *prn* genes. The first is that AreA acts by preventing the binding or repressing

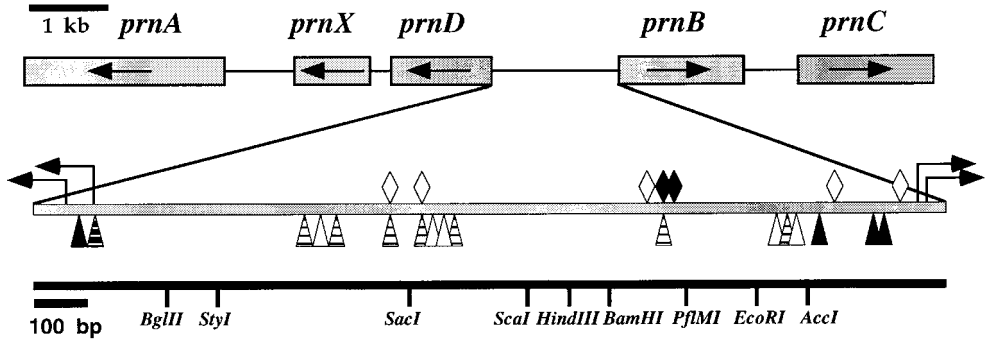

Fig. 1. Diagram of the *prnB–prnD* intergenic region. CreA-binding sites are indicated by lozenges; filled lozenges are the two sites shown to be necessary for repression *in vivo*. GATA sites are indicated by triangles; non-striped triangles for HGATAR sites and striped triangles for other GATA sites. White triangles indicate GATA sites removed by the larger deletions described in this work, black triangles are GATA sites sufficient to explain ammonium repression in these deletions. Arrows at both ends of the intergenic region indicate transcription start points for the *prnB* (Sophianopoulou and Scazzocchio, 1989) and the *prnD* (positions –58 and –100 with respect to the *prnD* start codon, determined by primer extension, data not shown) mRNAs. The restriction sites used in the construction of the deletion strains shown in Figure 4 are indicated.

activity of CreA. We shall call this model the 'AreA/CreA competition model', without pre-judging whether the effect of AreA on CreA binding or activity is direct or indirect. An analogous mechanism, whereby GATA-1 prevents the binding of a negative-acting factor has been demonstrated for the glycophorin B gene (Rahuel *et al.*, 1992). In the second model, the transcription of the *prnB* gene can be elicited by either AreA or, alternatively and only somewhat additively, by an unidentified positive-acting element whose binding or activity is prevented by the CreA protein in conditions of carbon catabolite repression. This model may also be valid for the *prnD* and *prnC* genes (Scazzocchio, 1992; Sophianopoulou *et al.*, 1993). In fact, while in the first model AreA is preventing the action of CreA, in the second model it is by-passing it. In spite of their formal similarity, these two models make strictly different predictions. In the AreA/CreA competition model, the only function of AreA is to prevent, directly or indirectly, the repressing activity of CreA. Thus, a deletion removing the physiologically relevant CreA sites should be derepressed, in every case, except in those in which other essential elements are removed, in which case no transcription will be seen. In the second model, a deletion not removing other essential elements will have two different phenotypes, according to whether the hitherto unidentified positive-acting element is removed or not. If the positive-acting element is not removed, the phenotype will be derepressed, exactly as in strains carrying point mutations of the CreA-binding sites (Sophianopoulou *et al.*, 1993; Cubero and Scazzocchio, 1994). If it is removed, and the physiological AreA-binding sites are not, the promoter will become repressible by ammonium on its own, and strictly dependent on AreA, irrespective of whether the organism is grown under carbon repressing or derepressing conditions or whether the strain carries a *creA*$^{+}$ or a *creA*^{d} allele. The possibility of generating an AreA strictly dependent phenotype is only compatible with the second model.

Here we establish that an AreA/CreA competition model is untenable and that CreA acts by preventing another, hitherto unidentified, positive element from eliciting *prnB* transcription. We call this element ADA (absolute dependence on AreA). This hypothesis, but not the experimental evidence, has been presented previously (Scazzocchio, 1992).

Results

Structure of the prnD–prnB intergenic region

In Figure 1, we show schematically the 1672 bp intergenic region, between the initiation codons of genes *prnB* (Sophianopoulou and Scazzocchio, 1989) and *prnD* (Gavrias, 1992). This sequence has been submitted to the DDBJ/EMBL/GenBank database under accession No. U74465. The direction of transcription of *prnD* has been determined by hybridization of wild-type RNA with single-stranded probes (data not shown). At variance with a previous report (Hull *et al.*, 1989), *prnD* is transcribed from the *prn* intergenic region divergently from *prnB*. The initiation codon shown for *prnD* in Figure 1 is the most 5′ in the transcribed sequences (see below) and also the one which maximizes the protein similarity of *prnD* to PUT1, the isofunctional protein of *S.cerevisiae* (Wang and Brandriss, 1987). Although no sequences corresponding to a TATA box are present in the *prnD* gene promoter, the sequence TCATTAAT at position –75 is similar to the sequence CATTTAAT, which can act as a TATA box for the yeast transcription factor TFIID (Singer *et al.*, 1990). The sequence, transcription initiation point and possible promoter structure of *prnB* have been published (Sophianopoulou and Scazzocchio, 1989). The region contains seven actual CreA-binding sites (shown in Figure 1), of which two have been shown to be necessary for repression of the gene cluster (see figure legend) (Sophianopoulou *et al.*, 1993; Cubero and Scazzocchio, 1994), and 17 GATA sites of which 14 follow the HGATAR consensus established for the AreA protein (Ravagnani *et al.*, 1997). There are also 21 GATT and 16 GATG sites which may bind AreA, mainly if another, canonical, strongly binding site is adjacent (D.Gomez, C.Scazzocchio and B.Cubero, unpublished). Only true GATA sites are shown in Figure 1. The binding ability and role of the different putative AreA-binding sites will be published elsewhere.

Deletions prn301 and prn304 and construction of prn^{+} strains carrying a deleted prnD–prnB intergenic region

Two previously characterized deletions have proven useful to generate strains suitable for distinguishing the two

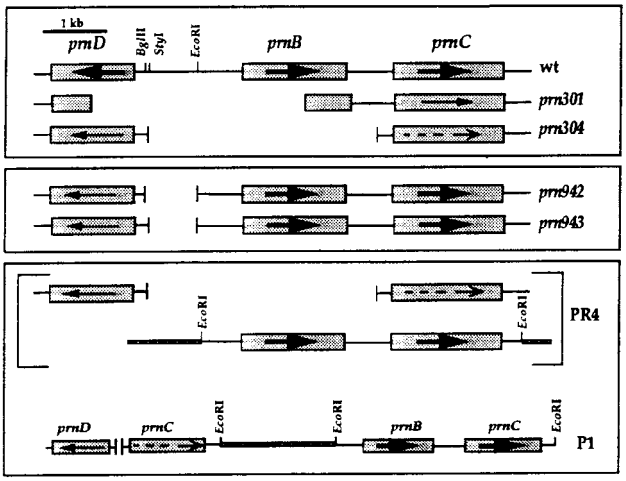

Fig. 2. Diagram of the *prn301* and *prn304* deletions and of four transformants lacking the ADA element. The restriction sites used *in vitro* to generate the deletions are shown. Arrows indicate the direction of transcription of the *prn* genes. Thick arrows indicate the wild-type level of expression in induced derepressed conditions; thin arrows, indicate weak but detectable levels, and broken arrows, almost undetectable levels of transcription. The wide striped line indicates the plasmid sequences.

possible models discussed in the Introduction. Deletion *prn304* is a *prnD–prnB–prnC* deletion by complementation and recombination criteria (Arst *et al.*, 1981; Hull *et al.*, 1989). The relevant portion of the *prn* cluster of a strain carrying this mutation was sequenced. It is a 3479 bp deletion, from position –284 of *prnD* to 373 bp downstream of the last codon of *prnB*, 310 bp before the ATG of *prnC* (Figure 2). Northern blots show that deletion *prn304* results in almost non-detectable transcription of *prnC* and reduced, but clearly inducible and ammonium-repressible, transcription of *prnD* (not shown, see below). This deletion was used to generate two types of strains altered in the *prnB* promoter: (i) strains carrying the *prnB* and *prnC* genes in an ectopic position; and (ii) similar strains generated by homologous recombination at the *prnC* gene. A *prn304pabaA1riboB2* strain was co-transformed with plasmids pAN225 and pPL5 (see Materials and methods for the structure of these plasmids). Transformants were selected for complementation of the *riboB2* mutation and tested for growth on proline as sole nitrogen source. A number of transformants were able to utilize proline, albeit at a reduced rate when compared with a wild-type strain. The fact that such transformants were obtained at all indicates that the low level of *prnD* transcript is sufficient to allow some proline utilization (see below). Several transformants were characterized by Southern blot analyses, and two of them, P1 and PR4, which contain single-copy integrated plasmid pAN225, were selected for transcript analysis (see below). Transformant P1 contains the *prnB–prnC Eco*RI fragment inserted homologously in the *prn* gene cluster, while transformant PR4 contains the same construction inserted at an unknown chromosomal location (Figure 2). The *riboB*$^+$-containing plasmid pPL5 has integrated independently from the pAN225 plasmid in both P1 and PR4. Both transformants have an intact *prnB–prnC* intergenic region and have the same sequences upstream of *prnD* as the original deletion strain. The physiological CreA-binding sites are not present. These transformants carry two copies of the *prnC* gene, the copy present in the recipient strain, which should be virtually non-transcribed, and a second one under the control of the intact *prnC* promoter contained in the transforming plasmid.

Deletion *prn301* has been shown by both genetic and physical mapping to terminate within the *prnD* and *prnB* open reading frames (ORFs) (Arst *et al.*, 1981; Hull *et al.*, 1989). Sequencing has shown that it is a 3058 bp deletion with boundaries at positions +459 of *prnD* and +946 of *prnB* (from the ATGs of each gene, Figure 2). This deletion is a null mutation for both *prnB* and *prnD*, and thus results in an inability to use proline as nitrogen and/or carbon source under any conditions. This deletion has been used to generate strains with altered *prnD–prnB* intergenic regions. A strain carrying this deletion has been transformed with DNA fragments containing *prnD* and *prnB* genes truncated at their 3′ ends, so that transformants selected for growth on proline can only be generated by gene conversion (or double cross-over). This is a way to introduce any modification in the intergenic region, at the original chromosomal location, with the only proviso that *prnD–prnB* intergenic regions not restoring the growth on proline under at least some conditions cannot be obtained by this method. Strains carrying deletions *prn942* and *prn943* have been constructed using this system. The missing sequences in the *prnD–prnB* intergenic region in these two strains are almost identical to those in transformants P1 and PR4. The left boundaries of these two deletions were chosen so as to bracket that of deletion *prn304* and, consequently, that of transformants P1 and PR4 (Figure 2). The second non-transcribed copy of *prnC* and the heterologous plasmid sequences are absent in strains carrying deletions *prn942* and *prn943*. No expression of the *prnD*, *prnB* and *prnC* genes is seen in any of these four transformant strains in the absence of proline induction, exactly as for the wild-type strain (results not shown).

The prnB 5′ truncated promoters extant in transformants P1, PR4, prn942 and prn943 lack ADA: genetic evidence

A diagnostic test for the presence of the ADA element is as follows. A strain carrying a *prnB* wild-type promoter (ADA$^+$) and an *areA*$^-$ null allele will use proline as sole carbon and nitrogen sources or proline as nitrogen source in the presence of non-repressing carbon sources (such as ethanol). However, such a strain will be unable to use proline as nitrogen source when a repressing carbon source (glucose) is present. A strain carrying the wild-type promoter will use proline under all conditions if, together with an *areA* null mutation, a *creA*^{d} mutation is also present in the strain. In the presence of an *areA* null allele, an ADA$^-$ strain will be unable to use proline under any conditions, regardless of whether a *creA*^{d} mutation is also present in the strain. We have constructed *P1areA600*, *PR4areA600*, *prn942areA600* and *prn943areA600* double mutants. In each case, the introduction of an *areA* null mutation resulted in inability to use proline under any conditions. The results are illustrated in Figure 3 and summarized in Figure 4. We then crossed each of the *areA600*-containing strains to a *creA*^{d}*1* strain. While the strain containing the wild-type promoter in an *areA600*-

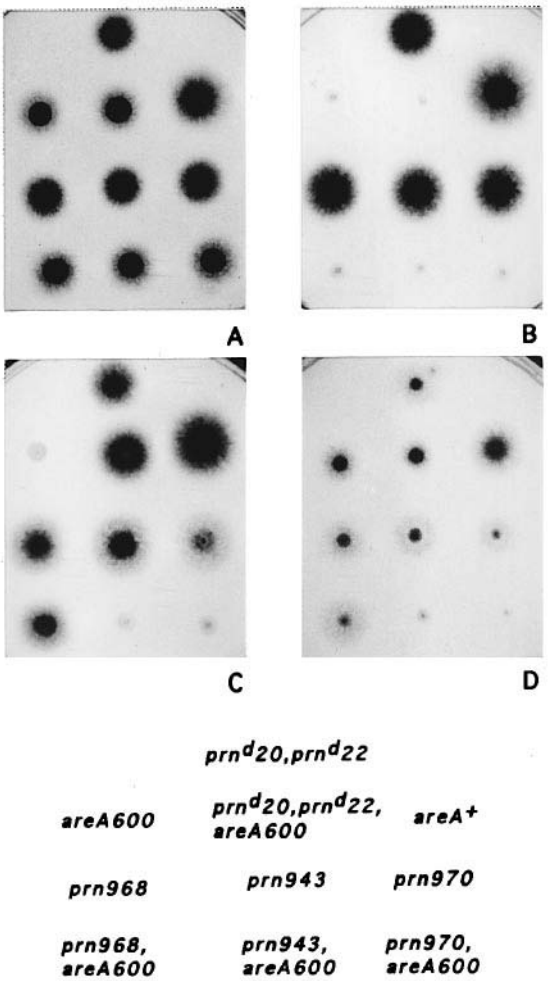

Fig. 3. Growth of strains carrying three different deletions of the *prnD–prnB* intergenic region, as well as the *prn^d20prn^d22* double mutation, in two different *areA* backgrounds, at 37°C, in the presence of different carbon and nitrogen sources: (**A**) glucose 1%, ammonium tartrate 5 mM; (**B**) glucose 1%, sodium nitrate 10 mM; (**C**) glucose 1%, L-proline 5 mM; (**D**) L-proline 5 mM. The position and relevant genotype of each strain are shown at the bottom. Ammonium is utilized by *areA*⁻ mutants while nitrate is never utilized by *areA*⁻ mutants, regardless of the carbon source.

creA^d1 context is able, as expected, to use proline as nitrogen source in the presence of glucose, no such ability is present in the deleted strains (data not shown). We can deduce that all four transformant strains have lost ADA.

Fine mapping of the ADA element

Using deletion *prn301* and the methodology described above, we have constructed strains carrying a series of deletions in the *prnD–prnB* intergenic region. Each of the deletion strains was crossed with *areA600*. The results of the whole deletion analysis are shown in Figure 4. We include results with strains where the two relevant CreA-binding sites were eliminated by point mutation (*prn^d20prn^d22*), with a small deletion encompassing both physiologically important CreA-binding sites (*prn927*) and with the large deletions discussed above. Figure 3 illustrates the phenotypes associated with three significant deletions. *prn943* has the characteristics expected for an ADA⁻ mutation as described above. *prn968* is the largest of a series of deletions which remove the CreA-binding

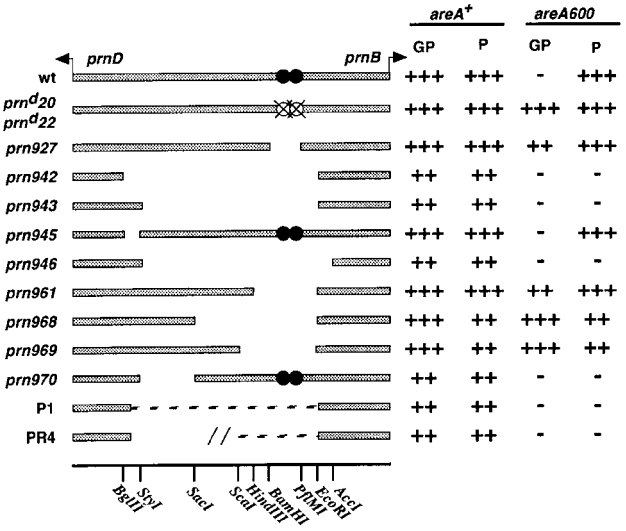

Fig. 4. Deletion analysis of the *prnB–prnD* intergenic region. Filled circles represent the two CreA-binding sites shown to be functional *in vivo*. Open, crossed circles represent mutated CreA-binding sites unable to bind the CreA protein. Plus and minus symbols indicate the different growth levels of strains carrying these deletions, in two different *areA* backgrounds, and in two different culture conditions: GP, for glucose and proline as carbon and nitrogen sources; and P, for proline as the only carbon and nitrogen source. The restriction sites used in the construction of the deletion strains are indicated. Transformants P1 and PR4 are also shown for comparison. Refer to Figure 2 for a detailed diagram of these two constructions.

sites, and whose phenotype is similar to that of point mutations in the CreA sites (*prn^d20prn^d22*). ADA activity is not affected in these strains. We can conclude that ADA maps upstream of the *prnD* proximal border of *prn968*. *prn945* (not shown in Figure 3) is a small deletion, adjacent to *prn943* (Figure 4), which has no effect on proline utilization. It brackets the left boundary of the ADA element. Deletion *prn970* has the expected ADA⁻ phenotype, locating the ADA element within a 290 bp DNA fragment. Thus the ADA element is located between nucleotides 369 and 657 in the sequence GenBank U74465.

Deletion of the ADA element: transcriptional evidence

Figure 5 confirms, using mRNA steady-state levels, earlier data obtained measuring proline uptake and proline oxidase activity (Arst and MacDonald, 1975; Arst *et al.*, 1980). These data indicate that, in the wild-type strain, full repression necessitates the simultaneous presence of repressing carbon and nitrogen sources. A further prediction of the 'ADA' model is that *prnB* promoters lacking ADA will be repressed by ammonium, irrespective of the carbon source. This prediction is borne out for a number of the deletion strains described above (Figure 5). We have tested transformants P1 and PR4 and strains carrying deletions *prn943*, *prn942* and *prn970*. In these transformant strains, *prnB* and *prnD* transcripts are inducible. As in the wild-type strain, no transcript is detectable in the absence of proline induction (results not shown). For all transformants, the steady-state levels of *prnB* and *prnD* were determined under non-repressing conditions, under carbon-repressing conditions, under nitrogen-repressing conditions and under carbon- and nitrogen-repressing conditions. Figure 5 shows the results for PR4 (lower

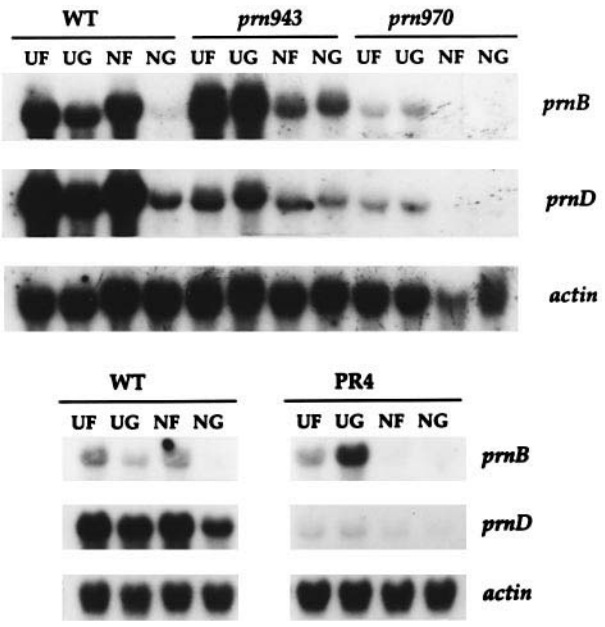

Fig. 5. *prnB* and *prnD* transcript levels of strains lacking the ADA element under different conditions of repression. Upper panel, strains carrying deletions *prn943* and *prn970*; lower panel, transformant PR4. WT stands for a strain carrying no mutation in the *prn* cluster and no additional sequences introduced by transformation. Mycelia were grown for 8 h at 37°C in 0.1% fructose, 5 mM urea, and then induced simultaneously with 20 mM L-proline and either non-repressed, 0.1% fructose, 5 mM urea (UF); carbon repressed, 1% glucose, 5 mM urea (UG); nitrogen repressed, 0.1% fructose, 20 mM ammonium tartrate (NF); or carbon and nitrogen repressed, 1% glucose, 20 mM ammonium tartrate (NG), for 2.5 h at 37°C.

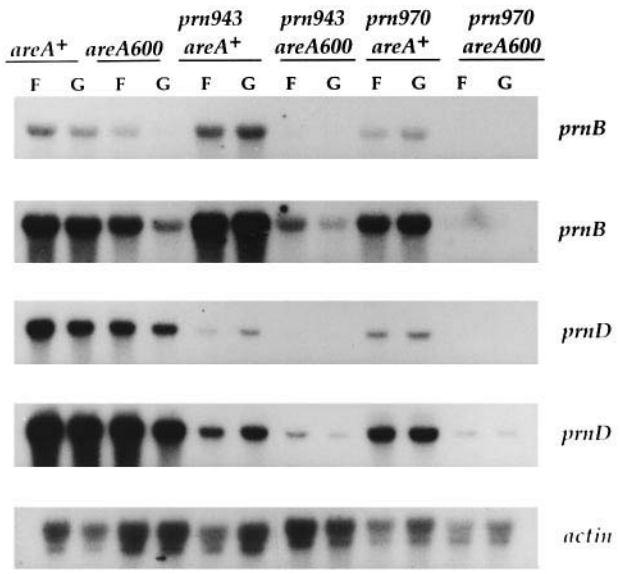

Fig. 6. *prnB* and *prnD* transcript levels of strains lacking the ADA element in *areA*$^+$ and *areA*$^-$ backgrounds. The genotype of each strain is indicated in italics. Mycelia were grown for 9 h at 37°C in 0.1% fructose, 5 mM acetamide, 0.625 mM ammonium tartrate (carbon non-repressing, nitrogen non-repressing conditions allowing the growth of *areA600* mutant strains), and then induced with 20 mM L-proline. At the same time, either 0.1% fructose (non-repressed conditions, F), or 1% glucose (repressed, G) were added, and mycelia grown for an additional hour. Two exposures (top, short; bottom, long) of the blots probed for the *prnB* and *prnD* mRNAs are included in order to show clearly the steady-state levels of the strains lacking the ADA element.

panel) and for transformants *prn943* and *prn970* (upper panel). Results for P1 and *prn942* (data not shown) are equivalent to those for PR4 and *prn943* respectively.

The transcription of *prnD* is, under inducing, non-repressing conditions, as low as it is in the original *prn304* deletion in all the deletion strains tested in Northern blots (see below for discussion of *prn970*). Surprisingly, an increase of transcription of *prnB* in the presence of glucose is seen for transformants P1 and PR4. No such increase is seen in strains carrying deletions *prn942* and *prn943*. In every one of the deleted strains, including *prn970* carrying the 290 bp deletion, ammonium is by itself a repressor of *prnB* and *prnD* independently of the carbon source, as predicted for mutants lacking the ADA element.

We then compared repression by glucose in the absence of a repressing nitrogen source in strains which lacked the ADA element in an *areA*$^+$ and an *areA600* background. We compared the *prnB* and *prnD* mRNA levels in strains carrying deletions *prn970* and *prn943* with those in strains carrying an intact *prnD–prnB* intergenic region. This experiment is the 'mRNA equivalent' of the growth tests shown in Figure 3 and summarized in Figure 4. The results (Figure 6) are strikingly in accord with the predictions. An *areA*$^+$ strain shows marginal repression by glucose (as shown before, Figure 5) and an *areA600* strain shows repression by glucose; in both deletion strains, transcript levels are not repressed by glucose. In an *areA600* background, both deletion strains show, irrespective of whether a repressing or non-repressing carbon source is included in the culture medium, levels of transcription which are equivalent or lower than those observed for an *areA600* single mutant in the presence of glucose. This is what was predicted; repression by glucose in an *areA600* background is equivalent to repression by ammonium and glucose in an *areA*$^+$ background; the ADA element is dispensable in the presence of *areA*$^+$ in the absence of a repressing nitrogen source, but indispensable in an *areA600* background. We note, however, that while the mRNA steady-state levels of *prnB* and *prnD* are clearly dependent on the presence of an active *areA* gene product for both deletions tested, the absolute levels are higher for the longer deletion. This was already observed in Figure 5 (see Discussion).

It was to be expected that the induced, non-repressed level of expression of *prnD* in transformants P1, PR4 and in those carrying deletions *prn942* and *prn943* would be as low as those found in the original *prn304* deletion, as the same (for P1 and PR4) or almost the same sequences are missing from the promoter. Deletion *prn970* shows that within the 290 bp which define the ADA element, there are sequences which are important to set the maximal level of *prnD*. What is quite surprising is that the induced non-repressed level of expression of *prnB* in the small deletion *prn970* is significantly lower than in the wild-type and the large deletions *prn943* and *prn942* (the latter not shown).

Discussion

We have proposed two different models to explain the repression pattern of the *prn* cluster in *A.nidulans*. These models differ in the role of the AreA protein. Strains carrying our larger deletions are sensitive to ammonium repression and necessitate an *areA*$^+$ allele for *prnB* (and

prnD) expression. This implies that the few AreA potential binding sites left in deletions *prn304* (and its P1 and PR4 derivatives), *prn943* and *prn942* are sufficient for the promoter(s) to respond to AreA transcriptional activation. Note that deletion *prn946*, which results in an *in vivo* phenotype identical to the above-mentioned deletions, leaves only five canonical AreA-binding sites in the *prnD–prnB* intergenic region (see Figure 1). Work which will be published elsewhere shows that these sites bind both to a GST–AreA fusion protein and, for those which have been tested, to native *A.nidulans* AreA protein (D.Gomez, C.Scazzocchio and B.Cubero, unpublished).

The deletion analysis carried out in this study shows conclusively that besides AreA, a second positive-acting element is operative in the *prnD–prnB* intergenic region, and that CreA works by preventing its action. Every single prediction of the ADA model has been confirmed by this deletion analysis. Deletion of ADA results, as predicted, in (i) unconditional dependence on AreA and (ii) repression by ammonium whether the carbon source be repressing or derepressing. The promoter of *prnB* (and *prnD*, but see below) behaves, when ADA is deleted, as do the promoters of, for example, nitrate and nitrite reductase or urate permease. These are only repressible by ammonium and are indifferent to carbon source (Cove, 1966; Arst and Cove, 1973; Gorfinkiel *et al.*, 1993; Oestreicher and Scazzocchio, 1993).

We have used a dual approach to define ADA. We have analysed the same ADA$^-$ deletion in three different genomic locations and carried out nested deletions in an otherwise unmodified *prn* cluster. The agreement of both approaches is satisfying and precludes artefacts due to chromosomal position effects. The comparison of the strains carrying deletions *prn942*, *prn943* and transformants P1 and PR4 is illustrative. All these constructions contain a *prnB* promoter truncated at the *Eco*RI site (Figure 2). *prn942* and *prn943* strains have a deletion and no other modification of the *prn* gene cluster, except that the distances between other remaining *cis*-acting elements (e.g. AreA- and PrnA-binding sites) are altered. P1 contains the same *prnB* truncated promoter in the same chromosomal location, but separated from the truncated *prnD* promoter by ~8 kb, including a non-transcribed *prnC* gene and the plasmidic sequences. PR4 has a *prnB* gene with a truncated promoter at an unknown location in the genome. Besides the unexplained glucose dependence found in P1 and PR4, which may be due to plasmidic sequences absent in *prn942* and *prn943*, the response of the three topographically different constructions is the same: the three behave as predicted by a deletion of ADA. The nested deletion approach has finally allowed mapping of the ADA element within the limits of 290 bp of the *prnD–prnB* intergenic region. Finally, we have shown the equivalence of the genetic and transcriptional analyses by testing the mRNA levels of critical deletions defining the ADA element, *prn970* and the large deletion *prn943* in an *areA600* background. As expected, these double mutants, which cannot utilize proline under any conditions, also have very low levels of *prnB* and *prnD* mRNA irrespective of the carbon source.

Within the 290 bp deletion *prn970*, there is an element or elements that set the maximum induced level of both *prnB* and *prnD*. This is not a PrnA-binding site for *prnB*, as all larger overlapping deletions tested are normally inducible for this gene (see below). It cannot contain an essential PrnA-binding element for *prnD*, as this and larger deletions are still inducible, albeit at a lower level than in the wild-type. This element is conceptually different from ADA, but may be overlapping with it. It is surprising that *prn970* has an effect on the induced, non-repressed level of *prnB* and that this effect is not seen in larger deletions, completely overlapping with *prn970*. This may indicate the presence of still other, unknown negative-acting factors in this complex promoter, which must be counteracted by sequences in the 290 bp fragment defined by *prn970*. What is relevant here is that the pattern of repression is identical in all these deletions and this fact is not altered by this apparent anomaly.

The pattern of repression of *prnD* is identical to the pattern of repression of *prnB*. Two mechanisms may account for this fact. The whole intergenic region may be acting bi-directionally on both genes. Alternatively, the repression of *prnD* may be due to inducer exclusion mediated through the repression of *prnB*. The latter mechanism is also sufficient to account for the repression pattern of *prnC*. These two mechanisms are not exclusive. It is relevant here that *cis–trans* tests using *prn^d* mutations have shown that the expression of *prnB* is the limiting factor affecting growth on proline under conditions of repression (Arst *et al.*, 1980).

The model proposed here might be quite general; other metabolites (acetamide, γ-aminobutiric acid) are utilized as the nitrogen source in an *areA*$^-$ background if a non-repressing carbon source is present (Arst and Cove, 1973; Arst and Bailey, 1977). Recent work has identified *areA* and *creA* homologues in a number of filamentous fungi (Fu and Marzluf, 1990; Drysdale *et al.*, 1993; Haas *et al.*, 1995; Strauss *et al.*, 1995), and it is thus likely that the integration mechanism proposed here is not limited to *A.nidulans*. An apparently similar pattern of repression has been described for the gene encoding the NAD-dependent glutamate dehydrogenase of *S.cerevisiae* (Coschigano *et al.*, 1991). However, the mechanism proposed to account for this pattern is completely different from the one proposed here (Miller and Magasanik, 1991).

We purposely have left the nature of ADA undefined. The most obvious possibility is that the 290 bp region contains the binding site for an unidentified transcriptional activator, and indeed preliminary experiments show that *A.nidulans* nuclear extracts contain proteins which bind the 290 bp fragment. Binding of CreA to both sites shown in Figure 1 (see legend) is necessary for repression (Scazzocchio, 1992; Sophianopoulou *et al.*, 1993; and this study, Figures 3 and 4). Thus, CreA must prevent the binding or activity of an 'ADA factor' at a distance of at least 500 bp. However, a less obvious possibility should be considered. This is that there is no *trans*-acting factor, but rather that ADA is a sequence which maintains the promoter in a transcriptionally competent 'open' configuration, which is 'closed' at a distance by CreA, and that binding of AreA to sites near the transcription start points of *prnB* and *prnD* can by-pass this effect. Further work, including the analysis of chromatin structure in the *prnD–prnB* intergenic region, may distinguish these formally equivalent but mechanistically different possibilities.

Materials and methods

Strains, plasmids and genetic methods

biA1pantoB100 and *pabaA1* strains were used as wild-types. *prn304* is a *prnD–prnB–prnC* deletion (Arst *et al.*, 1981). *prn301* is a deletion removing all the *prn cis*-acting region and most of the coding regions of *prnD* and *prnB* (Arst *et al.*, 1981). *creA*^{d}*1* is a derepressed, partial loss-of-function mutation (null mutations in *creA* are lethal) and *areA600* is an early chain termination, null mutation (Bailey and Arst, 1975; Kudla *et al.*, 1990). *biA1*, *pabaA1*, *riboB2* and *pantoB100* are standard auxotrophic markers (Clutterbuck, 1993). pPL5 is a pUC derivative containing a *riboB*$^{+}$ gene (Oakley *et al.*, 1987). pAN225 contains a 6.6 kb *Eco*RI–*Eco*RI fragment, comprising genes *prnB* and *prnC*, in vector pBR322 (Hull *et al.*, 1989). bAN926 contains a 3 kb *Xho*I–*Asp*718 fragment, comprising a 907 bp fragment of the *prnD* ORF, the *prnD–prnB* intergenic region and 1512 bp of the *prnB* ORF, in vector Bluescript KS+. Other plasmids were constructed by site-directed mutagenesis (*prn*^{d}*20prn*^{d}*22*), using standard methods (Kunkel *et al.*, 1991), or by restriction and ligation of bAN926, in order to obtain the corresponding deletions of the intergenic region (see Results). Nucleic acid manipulations followed standard procedures (Sambrook *et al.*, 1989). Genetic methods for *A.nidulans* were as described by Pontecorvo *et al.* (1953).

Media and growth conditions

Media and supplements were described by Cove (1966). Inducing and repressing conditions are described in the figure legends.

Sequencing

The sequence of the *prnB*-proximal half of the *prnD–prnB* intergenic region has been published (Sophianopoulou and Scazzocchio, 1989). The *prnD*-proximal half was sequenced from subclones of plasmid pAN224 (Hull *et al.*, 1989). Deletions *prn301* and *prn304* were sequenced from PCR fragments amplified from DNA of the corresponding mutant strains. All DNA fragments were sequenced by the method of Sanger *et al.* (1977).

A.nidulans transformation

This followed Tilburn *et al.* (1990). A strain *prn304pabaA1riboB2* was co-transformed with plasmid pAN225 and plasmid pPL5. *riboB*$^{+}$ transformants were selected and tested for co-transformation in medium containing 5 mM proline as the sole nitrogen source. To construct the strains used in the mapping of the ADA element, *prn301pabaA*1 was transformed with the insert of the corresponding bAN926 derivative plasmids. Transformants were selected on medium containing 20 mM proline as the sole source of nitrogen. Transformants were checked by Southern blots, using the 6.3 kb *Eco*RI–*Eco*RI fragment of plasmid pAN225 and a 2.2 kb *Pst*I–*Pst*I fragment of plasmid pPL5 (for *prn304* transformants) or a 2.2 kb *Xho*I–*Sma*I fragment of plasmid bAN942 (for *prn301* transformants) as probes.

Northern blots

RNA was isolated from *A.nidulans* mycelia as described (Chomczynski and Sacchi, 1987; Lockington *et al.*, 1987), glyoxal treated and separated on a 1% agarose gel (Sambrook *et al.*, 1989). Northern blots were carried out by standard techniques, using a PCR-amplified whole *prnD* gene, a 1.8 kb *Pst*I–*Pst*I fragment of plasmid pAN225, containing most of the ORF of *prnB*, or a 2 kb *Kpn*I–*Sma*I fragment of plasmid pSF5, containing the *A.nidulans* actin gene (Fidel *et al.*, 1988), as a probe.

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