

SHORT COMMUNICATION

A. Aleksenko · I. Nikolaev · Y. Vinetski · A.J. Clutterbuck

Gene expression from replicating plasmids in *Aspergillus nidulans*

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Abstract Plasmids bearing the AMA1 replicator from *Aspergillus nidulans* are capable of extrachromosomal replication in this fungus as well as in other species. Synthetic plasmids bearing the moderately expressed *argB* gene and the highly expressed, inducible β -galactosidase gene (*bgaS*) were introduced into fungal cells. Expression of both genes was monitored by Northern hybridization. It was demonstrated that transcription of *bgaS* is induced and repressed normally, irrespective of whether the gene is integrated into the chromosome or maintained on an extrachromosomal supercoiled plasmid. Transcription of the strongly expressed *bgaS* gene stimulates transcription of the *argB* gene located on the same replicating plasmid irrespective of orientation. This effect also occurs with chromosomally integrated vectors, but to a lesser extent. Episomal vectors are present in 10 copies per nucleus, and the expression level of the *argB* gene is approximately proportional to copy number. However, the amount of mRNA transcribed from the highly expressed *bgaS* gene on the multi-copy replicating plasmid does not exceed that from single-copy integrants. High levels of expression of the plasmid-borne gene do not affect plasmid mitotic stability or copy number.

Key words AMA1 · *Aspergillus nidulans* · fungi · transcription · plasmids

Introduction

Replicating plasmids and artificial minichromosomes are powerful tools of molecular research in both

prokaryotes and lower eukaryotes. Since the AMA1 plasmid replicator was described in *Aspergillus nidulans* (Gems et al. 1991), AMA1-based plasmids have become available for use in a number of *Aspergillus*-related genera of filamentous fungi. One application of replicating fungal plasmids is in gene expression studies, where a synthetic extrachromosomal determinant would allow analysis of gene function in an artificial surrounding, free from effects of different chromosomal integration sites, and eliminating the risk of rearrangement accompanying integration. Such constructions may also be useful in applied studies on standardization of optimal expression for industrial production. Although AMA1 plasmids have already been used for gene expression experiments (Verdoes et al. 1994), it has not been examined whether the extrachromosomal location of a highly expressed gene affects its expression pattern or the behaviour of the extrachromosomal determinant itself.

The chromosomal AMA1 sequence from *A. nidulans* Glasgow strains was shown to confer on plasmids the ability to persist in fungal nuclei as circular supercoiled molecules (Gems et al. 1991; Aleksenko and Clutterbuck 1996). The plasmids are structurally stable and generally do not undergo recombination with the chromosome (Aleksenko and Clutterbuck 1995). Unlike bacteria and yeasts, filamentous fungi in nature have not been reliably reported to contain circular nuclear plasmids (Clutterbuck 1995), and do not necessarily possess enzymatic machinery (topoisomerases, gyrases and other components of replicational and transcriptional complexes) capable of dealing with small circular DNAs. This was hypothesized to account for the difficulty encountered in isolating ARS-like sequences from filamentous fungi (Beguirer et al. 1989), and may have implications for expression of plasmid-borne genes.

In this paper, we report the transcriptional behaviour of genes located on circular extrachromosomal plasmids in *A. nidulans*. The results indicate that gene expression is not generally affected by episomal

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A. Aleksenko · A.J. Clutterbuck
Division of Molecular Genetics, Institute of Biomedical and Life Sciences, University of Glasgow, Church Street, Glasgow G11 6NU, UK

A. Aleksenko (✉) · I. Nikolaev · Y. Vinetski
GNII GENETIKA, 1st Dorozhny 1, Moscow 113545, Russia

location, but some effects (e.g. transcriptional interference between two adjacent genes) are probably enhanced on small circular vectors.

Materials and methods

The *A. nidulans* strain RMS010 (*AargB:trpCABam*; *trpC801*; *methH2*; *biA1*) was kindly provided by Dr. T.H. Adams (Texas A&M University, College Station, Tex.) and used as the recipient for transformation. Standard growth media and conditions were used (Clutterbuck 1974), except for experiments involving induction of β -galactosidase. The standard transformation protocol for poly-ethyleneglycol-stimulated DNA uptake was employed (Johnstone et al. 1985), with modifications for replicative vectors described earlier (Aleksenko and Clutterbuck 1995). Phenotypic stability of transformants was tested as described by Gems et al. (1991) and Aleksenko and Clutterbuck (1995).

Plasmids were constructed using fragments of the ARp1 plasmid (Gems et al. 1991) and the gene for a secreted β -galactosidase from *Penicillium canescens* (Nikolaev et al. 1992a). β -Galactosidase activity in growth media was determined as described by Nikolaev et al. (1989). For experiments on induction and repression of the β -galactosidase gene, transformants of *A. nidulans* were grown for 24 h in liquid minimal medium (MM) supplemented with methionine and biotin, with 1% glucose as carbon source and 5 mM glutamine as nitrogen source. Mycelium was collected by filtration, washed with water and transferred to fresh medium (5 g of wet mycelium per 400 ml of medium). The fresh medium was supplemented MM with either 10 mM arabinose (inducing medium) or 50 mM glucose (repressing medium) as carbon source. After 4 h of growth a portion of each culture was left to grow to saturation (48 h), and the remaining mycelium was collected by filtration and frozen in liquid nitrogen. At this time β -galactosidase activity in the growth medium of induced cultures was 5–7% of the level of saturation, which, as was shown, corresponded to the point of maximum yield of the specific mRNA. RNA was isolated from the frozen mycelia as described by Chirgwin et al. (1979). Equal amounts of RNA (10 μ g) were loaded on an agarose gel containing formamide, electrophoresed and transferred to Hybond N membranes (Amersham); Northern blot hybridization with radioactively labeled restriction fragments (see Fig. 1) was performed according to the membrane supplier's protocol. Auxiliary manipulations of DNA and RNA were as described by Sambrook et al. (1989). *E. coli* strain DH5 α (relevant markers *recA1*, *endA1*, *hsdR17* (rk⁻, mk⁺), *gyrA1*, *relA1*) was used for plasmid propagation and reisolation from fungal mycelia (Aleksenko and Clutterbuck 1995) throughout this study.

Results and discussion

Expression constructions used in this study utilized the gene for a secreted β -galactosidase (*bgaS*) from *P. canescens*, which was shown to exhibit a high level of expression in response to arabinose in the growth medium, and is almost completely repressible by glucose (Nikolaev and Vinetski 1994). Since laboratory strains of *A. nidulans* do not secrete β -galactosidases into the growth media, and *A. nidulans* genomic sequences do not cross-hybridize with the *bgaS* gene, this is a convenient model for monitoring both enzyme level and mRNA synthesis by an introduced gene. In addition, the recipient strain carried a total deletion of the *argB* gene, which made it possible precisely to monitor

expression of the introduced copy. The *argB* gene is expressed at an effectively constant rate independently of the growth conditions (Yelton et al. 1983), and much less efficiently than the *bgaS* gene. Therefore, the system enabled us to monitor plasmid replication (via stability tests) and expression of a plasmid-borne constitutive gene in response to induction of a second highly expressed gene.

Plasmids used in this study (Fig. 1A) contain the *bgaS* and *argB* genes in both relative orientations. Plasmids pRHG2 and pRHG4 also bear the AMA1 replicator and are capable of extrachromosomal maintenance, whereas plasmids pRHG1 and pRHG3 do not contain the replicator and are designed for chromosomal integration. The plasmids were introduced into the recipient strain by transformation. Replicative plasmids pRHG2 and pRHG4 transformed at a similar high frequency of 50 000 colonies per μ g DNA; integrative plasmids pRHG1 and pRHG3 exhibited characteristic low transformation frequencies of 30 and 75 colonies per μ g DNA, respectively. On induction with arabinose, all transformants with the replicative plasmids produced similar levels of β -galactosidase in the growth medium (0.48 to 0.53 units per ml after 48 h). In contrast, enzyme levels for integrative transformants varied over a wider range (0.3 to 0.7 U/ml). These data, however, may not necessarily exactly measure differences in gene expression, since growth rates vary slightly between integrative transformants, and are noticeably lower for replicative transformants.

For further studies, one transformant was selected from each group. Southern blotting of DNA from the transformants indicated that replicative plasmids were present in mycelia as uniform pools of unarranged molecules (Fig. 1B); this conclusion was confirmed by restriction mapping of recovered plasmids. It was previously shown by a variety of methods that average plasmid copy number is 10 per nucleus (Gems et al. 1991; A. Aleksenko, unpublished). In integrative transformants integration occurred at different loci for pRHG1 and pRHG3 transformants, T1 and T3. T3 is a single-copy transformant, whereas T1 is unlikely to contain more than three (probably tandemly) integrated plasmid copies, and in both cases ectopic integration has probably occurred through pUC8 sequences. The results also indicate that in both cases integration of the entire plasmid has taken place, i.e. the two genes remain closely linked in the integrants.

In all transformants tested β -galactosidase was produced at a maximum level in medium with arabinose, no activity was detected on glucose, and only low levels of activity on some other carbon sources (data not shown; a complete account of β -galactosidase induction and repression in *A. nidulans* is to be published elsewhere); this is similar to the regulation pattern in the natural host *P. canescens* (Nikolaev et al. 1992b; Nikolaev and Vinetski 1994). This indicates that the heterologous promoter retained its pattern of induction

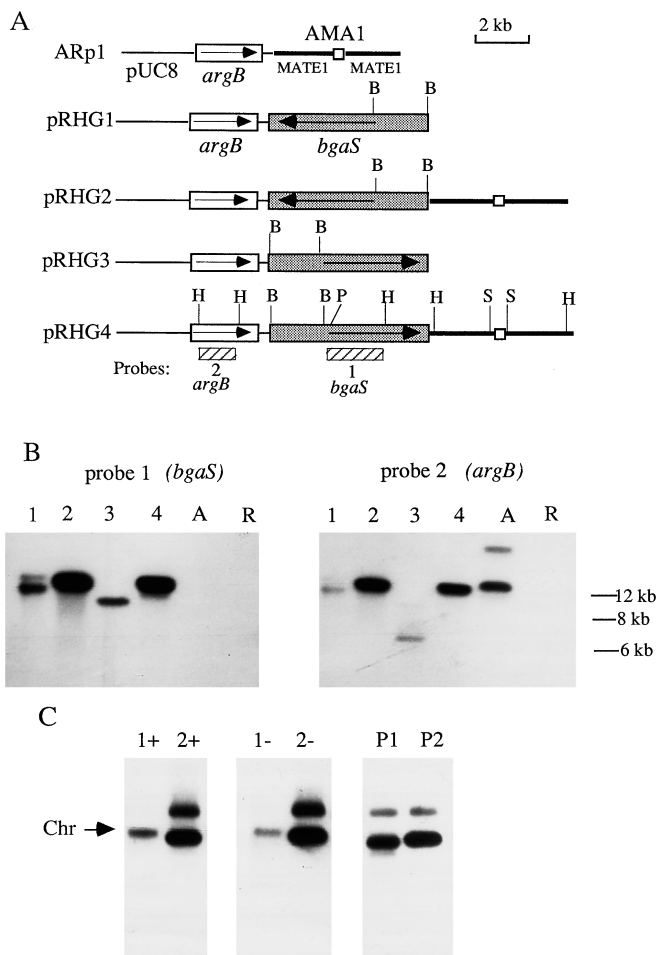


Fig. 1A–C Linearized maps of vectors (**A**) and Southern blot analysis of transformants (**B**, **C**). Thin line, pUC8 bacterial plasmid; open box, the *argB* gene from *Aspergillus nidulans*; shaded box, the *bgaS* gene from *Penicillium canescens*; thick line and open square, the AMA1 sequences, i.e. two inverted copies of MATE1 flanking a unique central spacer. Arrows indicate direction of transcription. Restriction sites: B, *Bam*HI; H, *Hind*III; P, *Pst*I; S, *Sal*I (only relevant sites are shown). Restriction fragments used as probes (dashed boxes) are shown below the plasmid pRHG4. **B** Southern blot hybridization of DNA from transformants; probes indicated in **A**. Lanes 1 to 4, transformants with pRHG1, pRHG2, pRHG3 and pRHG4, respectively; lane A, a transformant with ARp1; lane R, recipient strain. Positions of molecular weight markers are indicated on the right. Two microgram DNA samples were digested to completion with *Bam*HI and electrophoresed on a 0.6% agarose gel at 4 V/cm for 3 h. ARp1 does not contain *Bam*HI sites. **C** Southern blot hybridization of uncut DNA from transformants with a linearized pUC8 probe. Lanes 1+ and 1–, the pRHG1 transformant grown on arabinose and glucose, respectively; 2+ and 2–, the pRHG2 transformant grown on arabinose and glucose, respectively; P1 and P2, 10 ng of pure plasmids pRHG1 and pRHG2 (extracted from *E. coli*). Some 3 µg of fungal DNA was loaded per lane. Position of the ethidium-stained band of chromosomal DNA is indicated on the left (Chr)

and repression in both extrachromosomal and integrated states. Northern blot hybridization with a *bgaS*-specific probe (Fig. 2A) revealed the same major bands as in the natural host *P. canescens*. No *bgaS*-specific

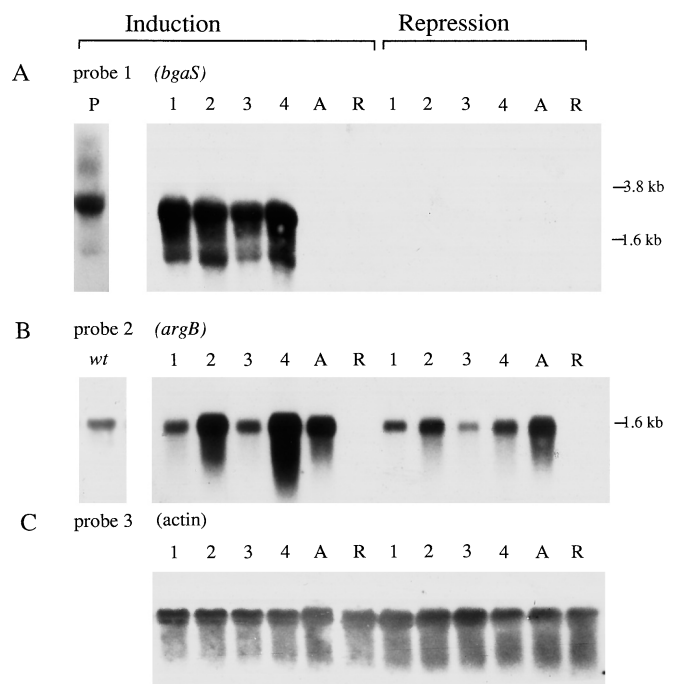


Fig. 2A–C Northern blot analysis of transformants under conditions of induction and repression of the *bgaS* gene (as indicated). **A**, **B** and **C**. Hybridization with probes 1, 2 and the actin gene from *A. nidulans* (probe 3; kindly provided by Dr. B. Felenbok, Université Paris-Sud, France) as indicated; see also Fig. 1A. Positions of RNA molecular weight markers are indicated on the right. Lanes 1–4, A and R: total RNA from transformants with pRHG1 to pRHG4, ARp1, and the recipient strain, respectively, as in Fig. 1. Lane P, RNA from *P. canescens*; wt, RNA from the wild-type *A. nidulans*. Some 10 µg of RNA per lane was electrophoresed in a 1% agarose MOPS-formamide gel (Sambrook et al. 1989) for 3 h at 4 V/cm. Probes of similar size (see Fig. 1) were labeled with 32 P by random priming (Boehringer Mannheim kit and protocol) to similar specific activities; exposure times were 12 h (**A**), 40 h (**B**) and 18 h (**C**)

mRNA was detected in repressed cultures. In spite of copy number differences, levels of mRNA in induced cultures were very similar in all transformants (slot-blot data indicated variation no higher than 1.5 fold, data not shown). The absence of a universal dependence of transcription level on gene copy number has been described and exhaustively discussed earlier for integrative transformants in yeasts and fungi (see, for example, Cregg and Madden 1988; Sreekrishna 1993; van Gorkom et al. 1994). It is usually attributed to the effect of different integration sites. It is possible that transcription from circular plasmids is limited by conformational problems, e.g. superhelicity. However, the most natural interpretation is that a single copy of the *bgaS* gene is sufficient for a saturating level of expression, while further copies result in titration of transcriptional or RNA-stabilizing factors, so that the total amount of mRNA in the cell is not increased.

Further evidence in favour of this hypothesis is provided by the fact that, unlike the highly expressed *bgaS* gene, the *argB* gene exhibits clear dependence of level of

expression upon copy number, in both integrative and replicative transformants (Fig. 2B). This difference in behaviour between the two genes is not due to position effects, since in all cases they are closely linked to one another on plasmids or integrated into the same locus. The level of *argB* mRNA in an Arp1 plasmid transformant is approximately 4–8 times higher than in the single-copy integrant, which roughly correlates with the copy number.

Surprisingly, the level of expression of the *argB* gene is dependent on induction of the *bgaS* gene when both are localized on the same extrachromosomal plasmid. This dependence is also noticeable in the integrative transformants with pRHG1 and pRHG3, although it is less pronounced (two-fold stimulation under β -galactosidase induction conditions for integrative transformants against 6- to 10-fold stimulation in replicative transformants; Fig. 2B and slot blot data, not shown). This effect was slightly stronger where the genes were arranged head-to-tail rather than head-to-head. However, in both orientations transcription of the *bgaS* gene stimulated, rather than suppressed, *argB* transcription. The growth medium itself did not affect *argB* transcription, as is seen in Fig. 2B in the case of the Arp1 transformant. The basal level of *argB* transcription from the repressed single-copy integrant (with pRHG3) was comparable to the normal expression of the gene in its wild-type location.

One possible explanation is that it is not transcription itself but an activated state of the *bgaS* promoter which exerts an influence on gene expression over a distance of several kilobases. This influence may be mediated by DNA supercoiling. It was recently demonstrated that localized DNA bending is able to alter dramatically the tertiary structure of a covalently closed plasmid molecule (Yang et al. 1995), and it is well known that some regulatory proteins function by introducing (or eliminating) localized bends into (from) gene upstream regions. Modulation of some promoters by template supercoiling is also a well-known effect (see, for example, Bowater et al. 1994). This effect, if it occurs, would not necessarily depend on gene orientation, in accordance with our data. The hypothesis also accounts for the fact that the influence is stronger on small circular molecules, although also noticeable for chromosomally integrated genes.

It might have been expected that superexpression in replicating plasmids would affect origin function. Efficiency of plasmid replication is partially reflected in phenotypic stability of transformants and absence of rearranged plasmid molecules (Gems et al. 1991; Aleksenko et al. 1996). The pRHG2 and pRHG4 transformants were subcultured on selective medium containing arabinose or glucose as a carbon source, and 5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside (X-gal). Transformant colonies growing on arabinose, unlike those growing on glucose and non-transformed colonies on any media, had a brightly stained halo

indicative of *bgaS* expression, but stability of both pRHG2 and pRHG4 transformants under induction (61% and 63%, respectively) did not differ from that under repression (65% and 60%). No evidence of plasmid rearrangement has been detected by Southern blotting, or by recovering and mapping plasmids from mycelia grown in liquid minimal medium with arabinose. Plasmid copy number estimated by Southern blot hybridization did not differ in glucose and arabinose cultures (Fig. 1).

The experiments reported here suggest that AMA1-bearing plasmids are suitable for gene expression studies provided that their limitations are understood. They have the advantage over integrative systems that transformation rates are high, the context of the expressed gene is predetermined, and copy number is dependent only on the selective system. Moreover, there is no evidence that expression of plasmid-borne genes affects vector replication or stability. On the other hand, replicating vectors, even if present at high copy number, are no better than multicopy integrative transformants in guaranteeing increased expression levels if these are dependent on other factors. Verdoes et al. (1994) used expression of the *E. coli uidA* gene to estimate copy number in *A. niger* transformants with an AMA1-bearing plasmid. Our data predict that this might be an accurate method for a relatively poorly expressed gene such as *argB*, but is not of universal application.

Episomal expression systems are by definition free from unpredictable effects of chromosomal surroundings. However, transcriptional interference between adjacent genes may well be related to position effects reported for integrated genes. The data reported indicate that a single strong promoter adjacent to a gene under investigation (rather than extensive regions of "inactive chromatin" usually invoked for interpretation of position effects) can significantly disturb expression pattern. Replicative vectors provide a tool for investigating this type of transcriptional interference in detail.

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