

Regulation of the *glaA* gene of *Aspergillus niger*

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Summary. The glucoamylase gene of *Aspergillus niger*, *glaA*, is expressed at high levels in the presence of starch. We have determined the nucleotide sequence of 1966 bp of the 5' flanking region of the *glaA* gene and have begun to identify sequences important for the control of *glaA* expression by deletion analysis. Constructs containing deletions extending into the *glaA* gene promoter were introduced into an *A. niger* host whose own *glaA* gene had been disrupted by a gene replacement event. Secreted levels of glucoamylase, expressed from each of the recombinant *glaA* genes, were measured by enzyme immunoassay. The effect of each deletion on the expression of *glaA*, when grown on differing carbon sources, was used to determine the limits of sequences upstream of *glaA* responsible for gene regulation. A region between –562 and –318 appears to direct high-level expression, whereas only 214 bp of 5' flanking sequence is required to initiate the start to transcription.

Key words: *Aspergillus* – Glucoamylase – Upstream sequence – Gene regulation

Introduction

The availability in *Aspergillus* sp. of selectable markers and DNA-mediated transformation methods has allowed the analysis of fungal gene regulation at the molecular level. Several workers have recently used deletion analysis of fungal gene promoter sequences followed by re-introduction of the truncated gene back into fungi in an attempt to identify those sequences responsible for regulation at the molecular level (Hamer and Timerlake 1987; Hynes et al. 1988; Goc and Weglenski 1988). We are interested in the *glaA* gene of *A. niger* as a model system for gene expression and regulation. Using similar molecular methods we have begun an analysis into the regulation of the *Aspergillus niger* glucoamylase gene, *glaA*.

The *glaA* genes from *A. awamori* and *A. niger* have been cloned. Molecular analysis has shown that differential splicing of an intron results in two forms of glucoamylase (Boel et al. 1984a, b; Nunberg et al. 1984). The larger of these two glucoamylase enzymes, form I, will saccharify raw starch. The smaller molecular weight glucoamylase, form II, lacks the carboxyl tail responsible for starch particle recognition and is restricted to degradation of maltodextrins (Smiley et al. 1971). Little is known about those sequences necessary for high-level expression and regulation of the *glaA* gene. Experiments in which bovine chymosin cDNA was linked to the glucoamylase promoter indicate that 1966 bp of 5' flanking *glaA* DNA sequences were sufficient to confer *glaA* gene regulation on the heterologous reporter gene (Cullen et al. 1987).

Expression of *glaA* is modulated by growth on different carbon sources (Nunberg et al. 1984; Barton et al. 1972). This regulation was thought to occur at the level of transcription and thus makes the *glaA* gene of *A. niger* an interesting model system for studying gene regulation in this organism. To locate functional regions responsible for regulation and high-level expression within the upstream region of the *glaA* gene we have sequenced 1966 bp of the *glaA* gene promoter and made a series of deletions within this region. Following transformation into a strain deleted for its own glucoamylase, we examined the response of the truncated *glaA* gene to growth in different carbon sources. We provide data that helps functionally define the *glaA* gene promoter and those sequences responsible for high-level expression of the *glaA* gene.

Materials and methods

Strains. *Aspergillus niger* strain ATCC 10864 produces high glucoamylase levels and was used as the parental strain. A uridine requiring auxotroph of *A. niger* ATCC 10864 was constructed by spreading spores on minimal media plates containing uridine and 5-fluoroorotic acid (FOA) (Boeke et al. 1984). FOA will be incorporated into the host's RNA as fluoro-uridine unless a mutation occurs in the uridine biosynthetic pathway [usually at the orotidine monophosphate decarboxylase (*pyrG*) gene]. Resistant colonies were selected

with a frequency of between 10^{-7} to 10^{-8} . One auxotroph (*A. niger* GC44, Ward et al. 1988), called *pyrG44* in the figures, could be complemented by transformation with the cloned *A. niger pyrG* gene (Wilson et al. 1988) on the plasmid pBH2 (Ward et al. 1989, data not shown). The *pyrG* gene was then used as a selectable marker in this host. *Escherichia coli* strains JM101 [*supE*, *thi*, Δ (*lac-proAB*)], (F', *traD36*, *proAB*, *lacIqZ* Δ M5)] and GM48 (*thr*, *leu*, *thi*, *lacY*, *galK*, *galT*, *ara*, *tonA*, *tsx*, *dam*, *dcm*, *supE44*) were used for transformation and production of in-vitro modified DNA.

Plasmid constructions. The plasmid pAGAM NB-pyr (see Fig. 4) was used to replace a portion of the host glucoamylase gene with the *A. nidulans pyrG* gene. To construct this vector a 5.8 kb *XhoI*-*HindIII* fragment from *A. niger*, var. *awamori* (Strain UVK143f, derived from NRRL 3112), containing the glucoamylase gene and flanking regions, was cloned into pUC18. The *glaA* promoter and N terminal coding sequence (contained on a 945 bp *NsiI*/*BglII* fragment) were removed and the resultant ends made flush using Klenow polymerase. Upon religation a *BglII* site was regenerated and a *BamHI* fragment containing a functional *A. nidulans pyrG* gene was cloned into this. pUCpyr-P (see Fig. 3) contains an *A. niger pyrG* gene lacking its promoter and the first 64 codons of the coding sequence and was constructed by cloning the 913 bp *ClaI*-*HindIII* fragment of the *A. niger pyrG* gene into pUC218 (Wilson et al. 1988). The plasmid pUC218 is a pUC18 derivative into which the M13 origin of replication has been cloned to facilitate single stranded sequencing. In addition, a *XhoI*-*BglII*-*ClaI* linker has been inserted between the *BamHI* and *XbaI* sites of the pUC18 polylinker. The *A. niger* ATCC 10864 *glaA* gene had been previously cloned into pBR322 as a 6.5 kb *ClaI* fragment from a Genencor (South San Francisco, USA) production strain. To produce the deletion series diagramed in Fig. 4, *ClaI* linkers were inserted into unique restriction sites 5' to the *glaA* gene. The resultant *ClaI* fragment was then cloned into pUCpyr-P at the unique *ClaI* site and these plasmids were used for the transformation of *A. niger*.

Growth conditions. Approximately 10^7 – 10^8 spores were used to inoculate 50 ml *Aspergillus* minimal medium (Rowlands and Turner 1973) containing 15% (w/v) of each carbon source, and uridine at 2 mg/ml, in a 250 ml shake flask. The mycelia were grown with shaking (150 RPM) at 37°C for 96 h.

Glucoamylase assay. Glucoamylase Enzyme immunoassays (EIA) were performed as described by Cullen et al. (1987). Microtitre plates were pre-adsorbed with rabbit anti-glucoamylase antibodies (6 µg/ml). Horseradish peroxidase (HRP)-conjugated rabbit anti-glucoamylase antibody was at 2.4 µg/ml. The chromogenic HRP substrate was o-phenylenediamine.

***Aspergillus* transformation protocol.** Transformation by electroporation was performed as described by Ward et al. 1989. Briefly, protoplasts were made from a 24 h mycelial culture by incubation in Novozyme 234 (Novo Biologicals Ltd). The washed protoplasts were suspended in electroporation buffer (7 mM sodium phosphate, pH 7.2, 1 mM magnesium sulphate, 1.4 M sorbitol). DNA was pulsed into the cells using Bio-Rad (Richmond, CA) Gene Pulser at 2375v/cm, 25 µFD.

Nucleic acid isolation and hybridization. Total RNA and genomic DNA was prepared essentially as described by Timberlake and Barnard (1981). Slot blot analysis of RNA was performed by transferring the RNA to Nytran membranes using a BRL (Gaithersburg, MD) vacuum slot blot apparatus. Southern blot analysis was as described by Maniatis et al. (1982).

Sequencing. DNA sequencing of phage M13 clones was by the dideoxynucleotide chain termination method (Sanger et al. 1977).

Results

The regulation of glaA gene expression in A. niger GC44

Wild-type *glaA* expression was studied to see how regulation of the gene was affected under different growth conditions. The following experiments compare these wild-type patterns of *glaA* expression with the expression patterns of *glaA* contained in the deletion constructs.

The effect of single, or mixed, carbon sources on the expression of native glucoamylase was determined in two ways. First, by measuring glucoamylase production by enzyme immuno-assay of the supernatants after 96 h growth (Fig. 1 A) and second, by examining the accumulation of *glaA*-specific mRNA after 48 h growth by slot blot analysis (Fig. 1 B) and Northern blotting analysis (data not shown).

Glucoamylase was found to be maximally secreted into the medium if starch or maltose were supplied as the sole carbon source (Fig. 1 A). Lower amounts of glucoamylase were seen when grown on glucose, while undetectable levels were obtained when xylose was used as a sole carbon source. Two explanations are possible for diminished *glaA* expression in glucose medium. Glucose could either be inducing glucoamylase to intermediate levels or acting as a carbon catabolite repressor. Another possibility is that at high levels of glucose, glucoamylase can catalyze the synthesis of maltose or transglucosidase or isomaltose from glucose (Nikolov et al. 1989) which in turn could then act to induce the synthesis of glucoamylase. This latter possibility was ruled out by assaying for the presence of maltose or in isomaltose in the medium of *A. niger* grown in 15% glucose using HPLC. No maltose could be detected down to the lower detection limit of 1 µg/ml maltose (data not shown). Furthermore, cultures of *A. niger* grown in xylose for 2 days were spiked with 1 µg/ml maltose to see if this was sufficient to induce the production of glucoamylase. Using an EIA assay, capable of detecting glucoamylase at levels of 1–5 µg/ml, no glucoamylase could be observed in the culture media (results not shown). Thus it does not appear that levels of maltose of 1 µg/ml in the culture media are sufficient to induce glucoamylase to the levels seen in those cultures grown in glucose alone.

Glucoamylase production during growth on pairwise combinations of the carbon sources gave additional data regarding the regulation of the *glaA* gene. Either maltose or starch in the presence of glucose act to increase levels of glucoamylase synthesis over those seen with glucose alone. This result suggests that, regardless of whether glucose is acting as a repressor or inducer, the effects are overridden upon induction with either starch or maltose. Xylose does not induce glucoamylase synthesis and when xylose is mixed with starch it prevents induction of glucoamylase synthesis. However, in a maltose-xylose mixture induction of the *glaA* gene can occur and maximal levels of glucoamylase synthesis are induced. A glucose/xylose mixture gave levels of glucoamylase synthesis similar to those seen with glucose alone and would suggest that glucose can act as an inducer of *glaA* expression. Thus, starch, maltose and glucose all positively induce glu-

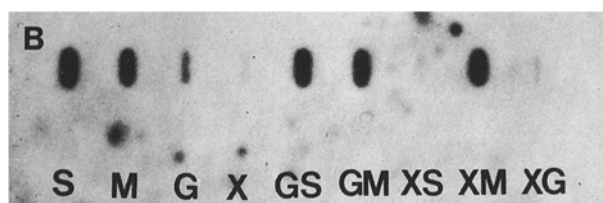
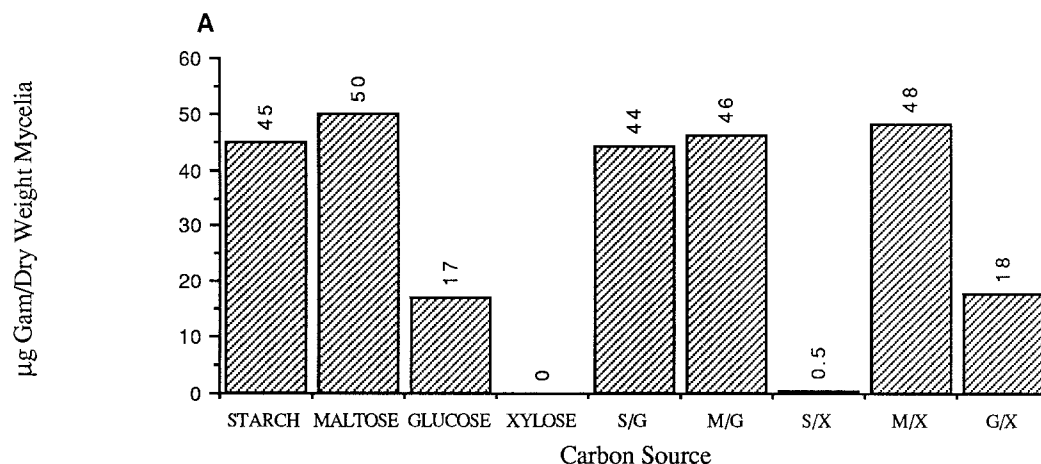


Fig. 1A, B. Regulation of glucoamylase protein levels and *glaA* transcript in differing carbon sources. **A** Bar graph showing glucoamylase production from *pyrG44* after 4 days growth in each carbon source. Glucoamylase production was standardized to the dry weight of mycelia to allow for variation in mycelial growth from flask to flask. Results are from duplicate experiments. **B** Slot blot analysis of *glaA* message production after 2 days mycelial growth. The probe was the 3.4 kb *EcoRI* *glaA* coding region. S, starch; M, maltose; G, glucose; X, xylose

coamylase production. In addition, maltose and glucose can both induce glucoamylase production even in the presence of xylose. Taken together these results indicate a complex regulatory pathway.

Levels of *glaA* mRNA in 2-day old cultures grown in different carbon sources were examined by hybridization analysis (Fig. 1B, Materials and methods). With the exception of the xylose-glucose lane the levels of *glaA* message can be seen to reflect the levels of glucoamylase protein assayed at 4 days (seen in Fig. 1A). It is unknown why so little *glaA* message was seen in the xylose-glucose lane when compared to RNA production in glucose alone. One explanation could be that the presence of xylose, acting as a repressor, may be interfering with *glaA* mRNA production at 2 days. It is possible that higher message levels may be observed at 4 days after xylose has been preferentially utilized, which is when the EIAs were done, but RNA is difficult to extract from mycelia at such a late stage of development. We believe this data shows regulation of the *glaA* gene is exerted primarily at the level of transcription. However, the possibility of additional post-transcriptional control mechanisms has not been ruled out.

Construction and integration of the *glaA* deletion constructs

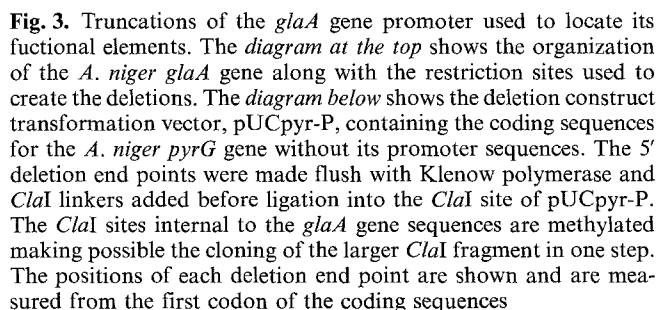
The nucleotide sequence of a 1966 bp region comprising the 5' flanking region of the *glaA* gene is shown in Fig. 2. Using the sequence data, we constructed a series of *glaA* 5'-deletion plasmids using a number of convenient restriction sites in the DNA upstream of the glucoamylase message start site (Fig. 3). Attempts were made to obtain integration events at a single genomic location by selecting

for homologous recombination between the part of *pyrG* contained on the transformation vector and the mutated *pyrG44* locus, to restore the *pyrG* allele (see pUCpyr-P in Fig. 3). Placing all of the constructs at the single *pyrG* locus within the genome would eliminate any variation resulting from differing regulation at other genomic positions, thus enabling more precise quantitation of glucoamylase activity between each construct. However, the low transformation frequency obtained when transforming the *ΔglaA* strains FOA A and FOA 2 to *Pyr*⁺, made the search for homologous integration at the *pyrG* locus unproductive. The deletion constructs were, therefore, co-transformed with the *A. niger pyrG* gene contained on the plasmid pBH2. As a result, the *glaA* plasmid was free to integrate non-homologously elsewhere within the genome, or homologously at the remaining portions of the host *glaA* locus that were unaffected by the *glaA* gene disruption.

Deletion of the *A. niger* 10864 *glaA* gene

To facilitate the investigation into the effect of each deletion on the expression of *glaA* in the construct transformants, a part of the host *glaA* gene was deleted. The plasmid pGAM NB-pyr (Fig. 4) was linearized with *XhoI* and *HindIII*, releasing a fragment containing the *A. niger pyrG* gene flanked by DNA from the 5' and 3' regions of the *glaA* locus. This DNA was used to transform the strain to uridine prototrophy with a frequency of approximately one transformant per 1 µg of DNA. Figure 4 also illustrates how a double homologous recombination event can excise the host *glaA* and replace it with the *A. nidulans pyrG* gene. Screening of *pyr*⁺ transformations for the lack of glucoamylase expression (by EIA) identified

Fig. 2. Sequence of the 5' upstream region of the *A. niger* ATCC10864 *glaA* gene. DNA for sequence analysis was obtained from the clone described in the Materials and methods. Numbering is from the first codon of the glucoamylase signal sequence. Restriction sites shown in *bold case* were used in the construction of the deletion series



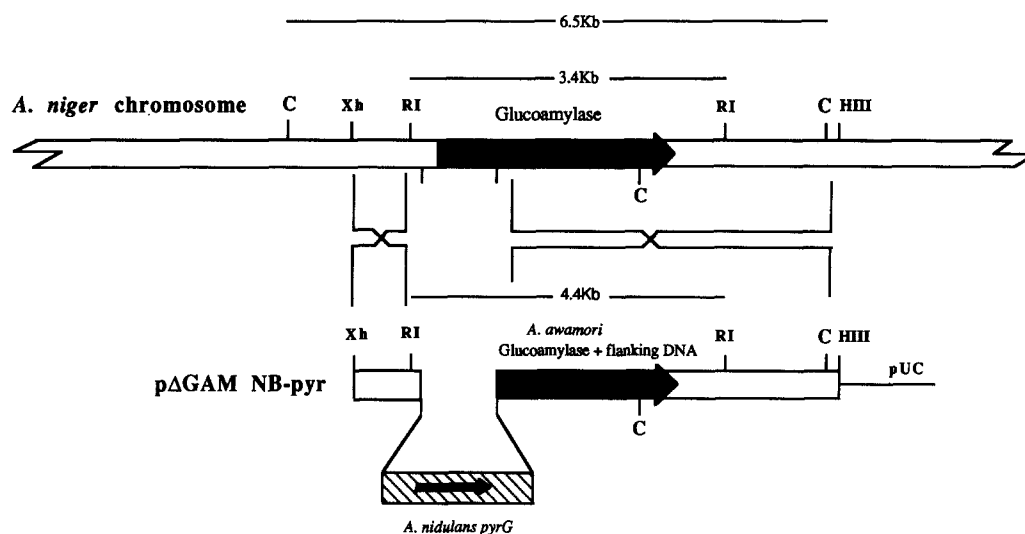


Fig. 4. The disruption of the native glucoamylase gene by the targeted integration of pΔGAM NB-pyr. The *bottom diagram* shows pΔGAM NB-pyr in which the *A. nidulans pyrG* gene has been cloned into the promoter region of the *A. awamori glaA* gene, thus inactivating it. Homologous recombination events between the *glaA*

flanking regions result in replacement of the host *glaA* gene, shown in the *top diagram*, by the inactive form. The increased size of the replacement *pyrG* gene fragment into the promoter of *glaA* results in a diagnostic increase in size of the *EcoRI* fragment from 3.4 kb to 4.4 kb (see Fig. 5). *Xh*, *XhoI*; *C*, *ClaI*; *RI*, *EcoRI*; *HIII*, *HindIII*

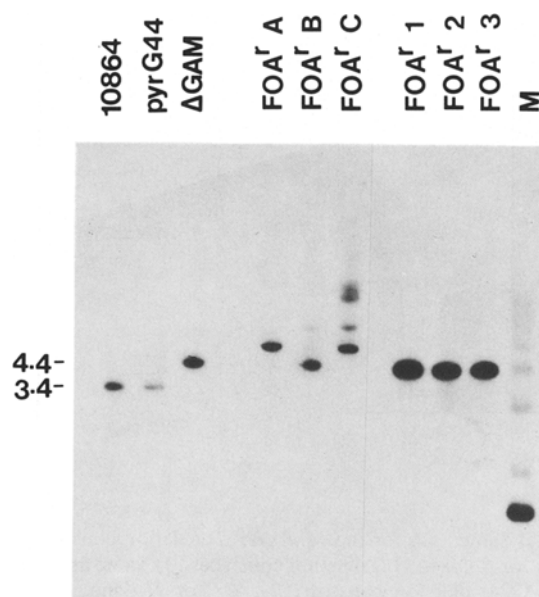


Fig. 5. Southern analysis of the glucoamylase-deletion hosts. Genomic DNA from *A. niger* strains were digested with *EcoRI* and probed with the isolated 3.4 kb *EcoRI* fragment containing the *glaA* coding region (see Fig. 4). Lanes are marked as follows: 10864, *A. niger* ATCC 10864; *pyrG44*, a uridine auxotroph of ATCC 10864 selected for by growth on FOA; ΔGAM, *pyrG44* into which pΔGAM NB-pyr has been integrated; FOA' A, B, C, 1, 2, and 3 are six separate isolates of ΔGAM selected on FOA

putative *glaA*-deficient hosts. Confirmation that a gene disruption event had occurred was provided by Southern blotting of genomic DNA that had been digested with *EcoRI*. The predicted increase in size of the *EcoRI* fragment from 3.4 kb to 4.4 kb was observed (Fig. 5). This host strain (*A. niger*, Δ*glaA*) produced no detectable levels of glucoamylase RNA or protein.

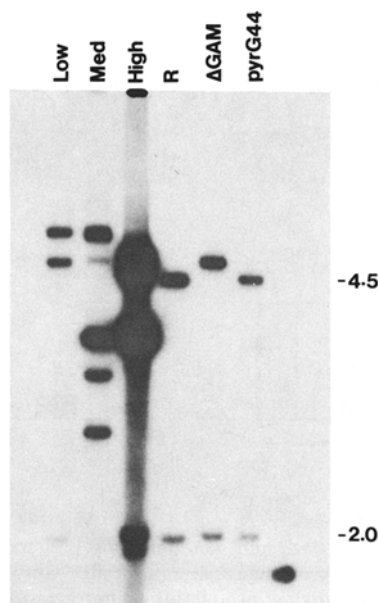


Fig. 6. A Southern blot showing representative examples of low (Δ318 #1), medium (Δ318 #4), and high (Δ318 #8) copy numbers of the integrated Δ318 (*SmaI*) deletion construct in genomic DNA digested with *ClaI*. A homologous integration event is shown in the lane marked R (Δ318 #9) which demonstrates the same banding pattern as the host strain *pyrG44*

In order that *pyrG* could be used again in Δ*glaA* as a selectable marker, spontaneous *pyr*⁻ auxotrophs of the Δ*glaA* strain were isolated by selection on FOA. Six *Pyr*⁻ colonies were picked and the nature of the mutation analyzed by Southern analysis. As shown in Fig. 5, two *Pyr*⁻ isolates, FOA A and FOA C, show rearrangements at the *glaA* locus. Only two of the six *Pyr*⁻ mutants selected

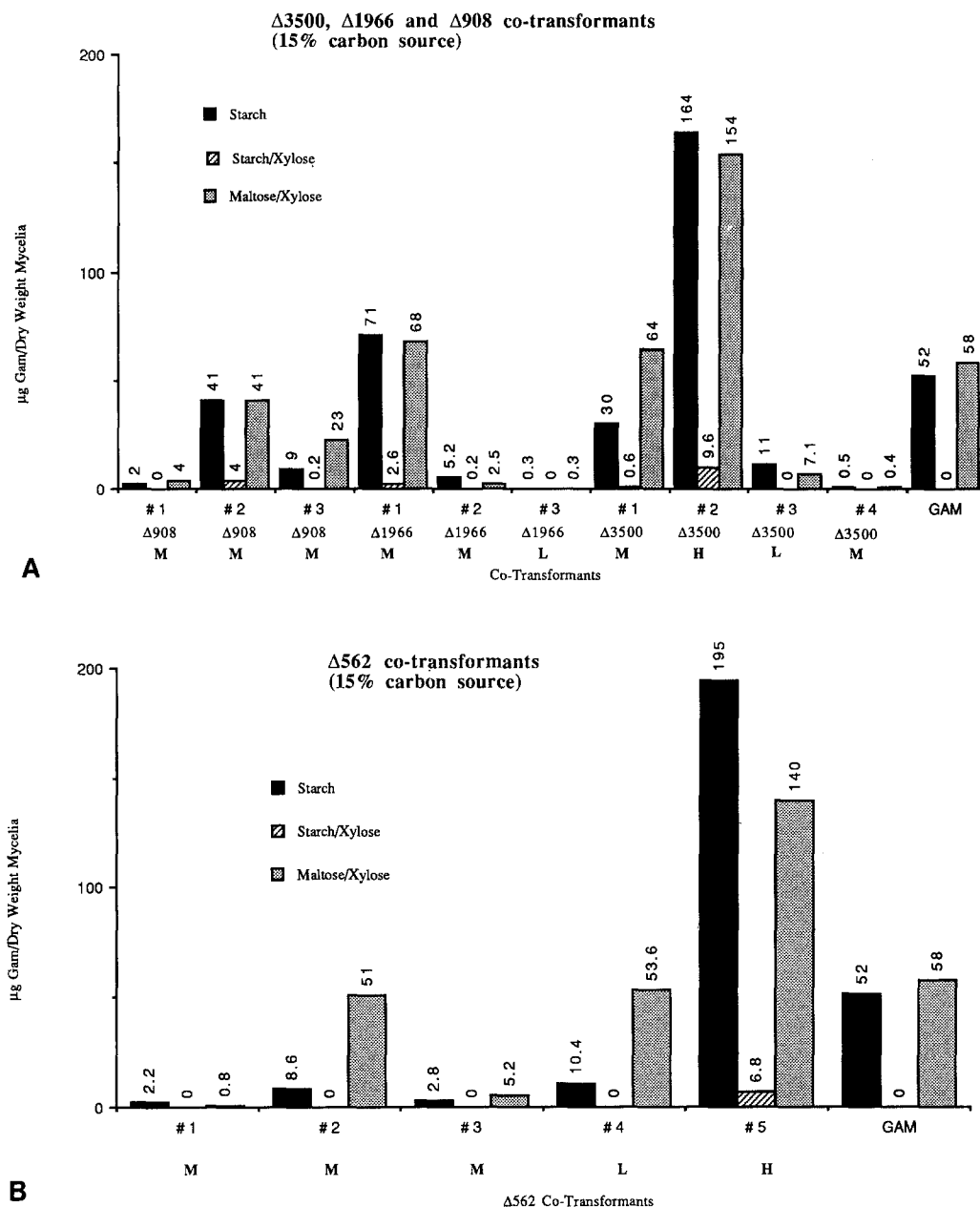


Fig. 7. Effect of promoter-truncation, and genomic copy number and location, of each transformant on the expression of glucoamylase. **A** Bar graph showing glucoamylase production from transformants of constructs with deletions extending from the *Cla*I (Δ3500), *Xho*I (Δ1966) and *Nco*I (Δ906) restriction sites upstream of *glaA*, grown in starch, starch+xylose, and maltose+xylose. **GAM**, *A.*

(*FOA A* and *FOA 2*, in Fig. 5) were complemented by transformation with a *pyrG*-containing vector at a frequency of approximately one transformant per 10 μg DNA (i.e., a 10-fold reduction in transformation frequency compared to *pyrG44*). No differences in transformation frequency or expression levels of the same reintroduced *glaA* gene were observed between the two mutants, both of which were used in further experiments.

Truncated *glaA* gene expression

The previous experiments describe how *glaA* is maximally induced in starch or in maltose-xylose mixtures and

niger 10864. **B** Same as **A** for the *Mlu*I (Δ562) deletion constructs. **C** Same as **A** for the *Sma*I (Δ318) deletion constructs. **D** Same as **A** for the *Eco*RI (Δ224) deletion constructs. *L*, *M*, or *H* denotes low, medium, or high copy number integration of the construct. Integration events that reconstitute the *glaA* gene at the disrupted *glaA* locus are indicated by an asterisk

unmixed in starch/xylose mixtures. To determine the regions of the *glaA* promoter important for regulation of the *glaA* gene, each deletion construct was transformed into the *ΔglaA A. niger* host. A number of transformants of each construct were then assayed for glucoamylase production by growing on either starch, starch-xylose or maltose-xylose and measuring the expression levels of the truncated *glaA* gene compared to expression levels of wild-type *glaA*.

Transformants were grown for 4 days, the levels of secreted glucoamylase were determined by EIA and integration of the corresponding construct viewed by Southern blotting analysis. Genomic DNAs from all of the

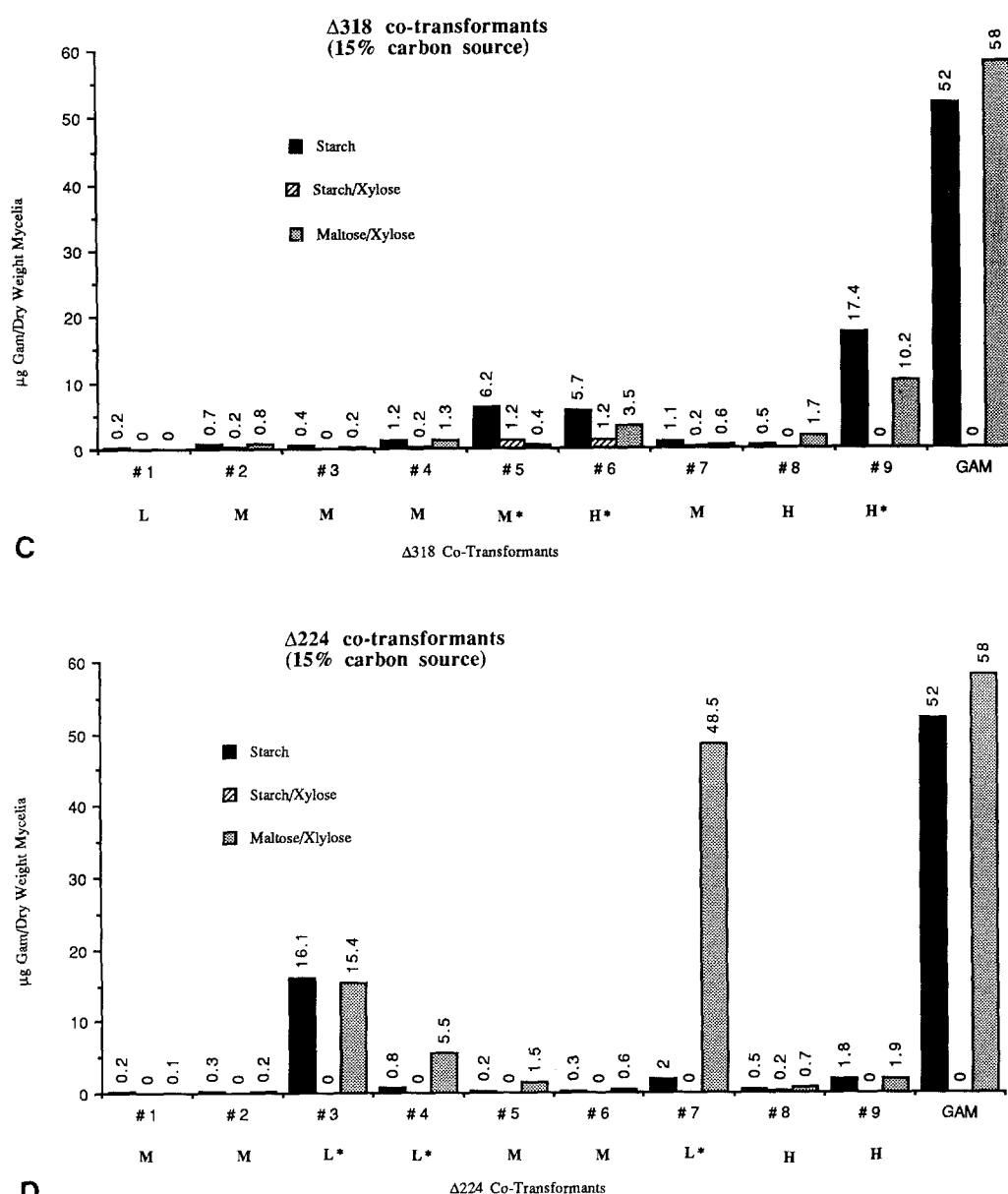


Fig. 7. C, D

transformants were digested with *Cla*I and subjected to Southern analysis using the internal 3.4 kb *Eco*RI *glaA* fragment as a probe (see Fig. 4). The native *glaA* gene is identified as 4.5 and 2.0 kb fragments (increasing to 5.5 and 2.0 kb following *glaA* inactivation by insertion of the *A. nidulans pyrG* gene into the *glaA* promoter, see Fig. 6).

Southern analysis showed that the deletion plasmids could integrate at various sites within the genome. Integration was often associated with tandem duplication of the deletion plasmid giving a variety of copy numbers. While this result complicates the interpretation of the effect of the deletions on glucoamylase levels, obvious trends in the results could be discerned by studying sufficient numbers of transformants.

Glucoamylase levels from the truncated *glaA* gene transformants are shown as bar graphs in Fig. 7. Each transformant line was also investigated by Southern blot analysis and the copy number of the integrated deletion construct estimated relative to the single copy of the *glaA*

gene in the parental strain. A low (1–10 copies per cell), medium (10–50 copies per cell) or high (> 50 copies per cell) copy number is denoted under each transformant in the bar graphs. Figure 6 shows representative examples of low, medium and high copy number integration events for three Δ318 deletion constructs. Occasionally the deletion plasmid integrated homologously at the native *glaA* locus (inactivated by the *A. nidulans pyrG* gene). This homologous integration led to the loss of the *A. nidulans pyrG* gene and reconstitution of an intact *glaA* gene with a functional promoter. An example of such an integration event is shown in Fig. 6, lane R. The deletion construct Δ318 #5 has integrated back at the *glaA* locus regenerating an intact *glaA* gene. Homologous integration of Δ318 #5 at the host *glaA* locus was further confirmed by re-probing the blot replace with- and showing the loss of the *A. nidulans pyrG* gene segment unique to the position of the deleted promoter sequences at the *glaA* locus (data not shown). Figure 6, lane R, shows a banding pattern, at

the *glaA* locus, identical to that of the parental strain suggesting that a functional *glaA* gene has been reconstituted. However, in all examples where this event was seen (all similar homologous integration events, confirmed by Southern blot analysis, are marked on the bar graphs in Fig. 7 with an asterisk) the levels of *glaA* expression were lower than wild-type levels. One explanation for lowered expression levels could be an inexact recombination event creating small deletions or rearrangements in the *glaA* promoter. However, subsequent rescue and sequence analysis of the promoter region was not performed.

Figure 7A shows glucoamylase production from a selection of co-transformants with plasmids containing the full length *Clal* fragment ($\Delta 3500$) or deletions which remove all sequences 5' to the *XhoI* ($\Delta 1966$) or *NcoI* ($\Delta 908$). Co-transformants $\Delta 3500$ #1 and #2, $\Delta 1966$ #1 and $\Delta 908$ #1 are transformants of deletion constructs which appear to maintain high-level expression and correct regulation of the *glaA* gene. Furthermore, these integration events have not reconstituted a functional *glaA* gene at the disrupted *glaA* locus that could be responsible for the observed high level of *glaA* gene expression (data not shown).

Correctly regulated, high-level expression was also seen in co-transformants with the $\Delta 562$ deletion construct (truncated at the *MluI* site) as illustrated by co-transformant #5 shown in Fig. 7B. Co-transformants $\Delta 562$ #2 and #4 gave wild-type levels of expression when grown in maltose. However lower than expected levels of expression are seen when the same co-transformants are grown in glucose. Transformant $\Delta 562$ #1 gave low levels of expression (presumably due to the site of plasmid integration) and Southern blot analysis showed $\Delta 562$ #3 to have integrated at the inactivated *glaA* locus and so reconstituted the *glaA* gene (data not shown).

In contrast to the $\Delta 3500$, $\Delta 1966$, $\Delta 908$ and $\Delta 561$ constructions, high-level *glaA* expression was never observed in six $\Delta 318$ and six $\Delta 214$ vector co-transformants (Fig. 7C and D). Three $\Delta 318$ and three $\Delta 214$ construct co-transformations integrated back at the *glaA* locus to give a wild-type full length *glaA* gene and were, therefore, discounted in this analysis. Thus, between position 561 and 318 bp upstream of the *glaA* gene are sequences responsible for high-level gene expression.

All of the transformants show variation in copy number and integration sites. However, there does not appear to be a correlation between the levels of *glaA* expression and copy number. Surprisingly copy number did not have a major effect on the expression levels of the truncated *glaA* gene. Indeed transformants $\Delta 562$ #4 (Fig. 7B) $\Delta 908$ #2 and $\Delta 1966$ #1 each have 2–10 integrated copies (data not shown) which all give wild-type levels of glucoamylase, whereas $\Delta 562$ #1, $\Delta 908$ #1 and $\Delta 3500$ #3 have between 10 and 50 copies are genome (data not shown) and give relatively low expression levels.

Discussion

We have described that sequence of 1966 bp of DNA upstream of the *A. niger glaA* coding region and shown

how this gene responds to growth on differing carbon source. We also present an initial characterization of sequences required for expression of the *glaA* gene. Our data reveal that a minimum promoter, which directs low-level expression of the *glaA* gene, lies within the first 214 bp upstream of the translation initiation site. Sequences required for high-level expression appear to lie further upstream of the promoter, between position –318 and –562.

Regulation of the undisrupted *glaA* gene of *A. niger* on a variety of carbon sources was studied at both the RNA and protein level. Glucoamylase is secreted at high levels when the fungi are grown on starch; intermediate levels are produced on glucose and very low levels are produced on xylose (Nunberg et al. 1984). We have extended this data to include maltose, which also gives high levels of glucoamylase secretion (Barton et al. 1972), and mixtures of the above carbon sources. Hybridization analysis of total mRNA from cells grown under the same carbon sources revealed that the level of *glaA* mRNA appeared to correlate well with glucoamylase protein levels detected by EIA. The correlation of *glaA* message with glucoamylase protein suggests that regulation of glucoamylase is controlled primarily at the level of transcription.

In our experiments, xylose acts as a carbon catabolite repressor, either directly or indirectly, to limit production of *glaA* mRNA. Interestingly, this response is overcome by the addition of maltose or glucose to levels seen when maltose or glucose are used as the sole carbon source. However, starch will not overcome the repression effect of xylose, indicating that starch itself is not the signal for *glaA* synthesis. One possible explanation is that starch could be broken down by a low level of some other starch hydrolyzing enzyme, the product(s) of which may act to induce high-level *glaA* synthesis (Barton et al. 1972). Xylose may then act to fully repress the production of this other enzyme. This situation would be analogous to the situation seen in the breakdown of cellulose in *Trichoderma reesei*. It was recently demonstrated that cellulose is not the inducer for the production of cellobiohydrolase, but rather a low constitutive level of other cellulolytic enzymes first acts to break cellulose down into smaller subunits which are then taken up by the cell and induce transcription of the CBHI gene (El-Gogary et al. 1989).

Sequences in the 5' flanking region of many eukaryotic genes are known to contain not only promoter sequences important for transcription initiation, but also sequences responsible for both elevated levels of gene expression and correct regulation of the gene in response to various effector molecules. We have observed: (1) *glaA* to be maximally expressed in the presence of starch, (2) lack of *glaA* expression in the presence of xylose even when mixed with starch and (3) induction of *glaA* expression when cells are grown in xylose mixed with maltose. An attempt to identify sequences important in mediating *glaA* regulating was initiated by assaying for the production of glucoamylase in a *glaA*-deleted host containing truncated versions of the *glaA* gene with deletions extending into the 5' flanking sequences. Figure 8 presents a summary of the 5' flanking sequences important in the regulation of *glaA*. Deletion of all sequences upstream of

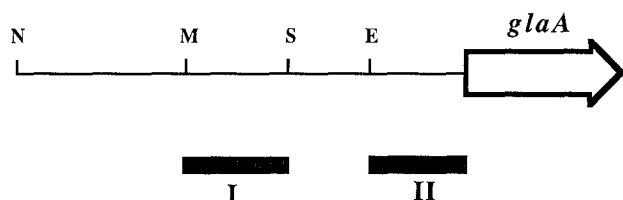


Fig. 8. Functional elements within the upstream region of the *glaA* gene. *I*, region required for high-level expression. *II*, region required for transcription initiation resulting in low-level expression. *N*, *NcoI*; *M*, *MluI*; *S*, *SmaI*; *E*, *EcoRI*

the *MluI* site at -562 bp (represented by the constructs $\Delta 3500$, $\Delta 1966$, $\Delta 908$ and $\Delta 562$) appeared to have no effect on glucoamylase synthesis. That is, for each of the above classes of deletion constructs transformants were observed which had normal regulation and expression patterns. However, when a region between the *MluI* site and the *SmaI* site ($\Delta 318$) was also removed, only low-level expression of glucoamylase was ever observed indicating that this region (called element I) could be responsible for high-level expression. As low-level expression of *glaA* is seen even in the largest deletion ($\Delta 224$), we argue that the basic elements of the promoter must lie within the first 224 bp 5' of the translation start point (called element II). Because of the low levels of expression seen in the $\Delta 318$ and $\Delta 224$ transformants we cannot say with certainty whether the *glaA* promoter was repressed and induced normally.

Upstream activating sequences (UASs) have been previously described in fungi, particularly yeast. These UASs occur in approximately the same position as we see for element I of the glucoamylase gene in *A. niger*. For example, the genes required for galactose metabolism, *GAL 1* and *GAL 10*, amino acid biosynthesis, *HIS 3*, and the cytochrome C genes, *CYC1* and *CYC7*, each have upstream activating sequences that occur 80–600 bp upstream of the major transcription start site and which recognize and bind activator proteins resulting in an enhancement of transcription (for a review see Struhl 1987). In addition, at least three UAS elements are present upstream of the yeast *Saccharomyces diastaticus* glucoamylase gene *STAI* (Shima et al. 1989). We have compared our sequence against those given for *STAI*, *GAL 1* and *GAL 10*, *HIS 3* and *CYC 1* and *CYC 7* upstream sequences and have found no striking similarities or homologies to the yeast consensus UAS.

These results will be used as the basis for additional studies of the *glaA* gene control regions. A more detailed series of deletions within element I and site-directed mutagenesis of elements I and II should provide comprehensive data on the exact role of these sequences.

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