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The promoter of the glucoamylase-encoding gene of *Aspergillus niger* functions in *Ustilago maydis*

(Hygromycin phosphotransferase; Basidiomycete; Ascomycete; fungal transformation; starch; recombinant DNA)

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SUMMARY

Promoter sequences from the *Aspergillus niger* glucoamylase-encoding gene *(glaA)* were linked to the bacterial hygromycin (Hy) phosphotransferase-encoding gene *(hph)* and this chimeric marker was used to select Hy-resistant (Hy^R) *Ustilago maydis* transformants. This is an example of an Ascomycete promoter functioning in a Basidiomycete. Hy^R transformants varied with respect to copy number of integrated vector, mitotic stability, and tolerance to Hy. Only 216 bp of *glaA* promoter sequence is required for expression in *U. maydis* but this promoter is not induced by starch as it is in *Aspergillus* spp. The transcriptional start points are the same in *U. maydis* and *A. niger*.

INTRODUCTION

High expression levels and starch induction of the *A. niger* Gla-encoding gene, *glaA*, have made it an attractive source for transcriptional and secretion control elements for expression vectors. Transcript levels are several 100-fold higher when cultures are grown on starch as opposed to xylose; glucose-grown cultures produced less than a third the amount of extracellular Gla as starch-grown cultures (Nunberg et al., 1984). When the *glaA* promoter is fused to the structural genes for bovine chymosin (Cullen et al., 1987a) or a-interferon (Gwynne et al., 1987), regulated expression of these heterologous genes is obtained in *A. nidulans*. In *A. nidulans*, the *A. niger glaA* gene is induced by maltodextrin, the transcript is processed to remove introns, and the Gla protein is synthesized and secreted (Cullen et al., 1987a). In *A. nidulans*, expression of full-length *glaA* or of chymosin fused to the *glaA* promoter requires more than 250 bp of 5'-untranslated DNA (Cullen et al., 1988). The operation of the *glaA* promoter in more distantly related fungi has not been previously reported.

To further investigate the range of organisms that are able to recognize the *glaA* promoter, we have tested the ability of this ascomycete promoter to function in the Basidiomycete *U. maydis*. Previously, we described a vector in which the bacterial gene encoding Hy phosphotransferase, *hph*, was functionally coupled to the *A. nidulans trpC* promoter and terminator (Cullen et al., 1987b). This vector, pDH25, has been used to select Hy^R transformants in

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Abbreviations: *A.*, *Aspergillus;* Δ, deletion; bp, base pair(s); *glaA*, gene (DNA) encoding Gla; Gla, glucoamylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *hph*, gene (DNA) coding for Hy phosphotransferase; Hy, hygromycin B; *hsp*, gene (DNA) encoding heat-shock protein; kb, kilobase(s) or 1000 bp; nt, nucleotide(s); PolIk, Klenow-(large) fragment of *E. coli* DNA polymerase I; ^{R}, resistance/resistant; *tsp*, transcription start point(s); *U.*, *Ustilago;* wt, wild type.

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A. nidulans (Cullen et al., 1987b) and *Fusarium oxysporum* (Kistler and Benny, 1988). However, repeated attempts to transform *U. maydis,* a pathogen of maize, failed. It has been suggested that Ascomycete and Basidiomycete phylogeny are too disparate for their genes to be interchangeable (Mellon et al., 1987).

We report here the successful transformation of *U. maydis* using the pDH25 derivatives pDH33 and pD1, in which the *trpC* promoter has been replaced by portions of the *A. niger gluA* promoter.

EXPERIMENTAL AND DISCUSSION

(a) Strains, plasmids, media and techniques

Escherichia coli strains 294 (ATCC31446) and DH5a (F^-, *endA1*, *hsdR*17(r^{-k}, m^{+k}), *supE*44, *thi*-1, *recA*1, *gyrA*96, *relA* 1, e(*argF-lacZYA*)U169, ϕ80d*lac*Z e M15) were used for routine plasmid propagation. An adenine methyltransferase-deficient strain, GM48 (ATCC39099) was used when required. *U. maydis* wt strain 518 (R. Holliday, MRC, U.K.) was used in all transformation experiments. *U. maydis* strains transformed with pHL1 (Wang et al., 1988) were obtained from J. Wang and D. Holden. pHL1 contains *hph* fused to the *U. maydis hsp* promoter.

The transformation protocol of Wang et al. (1988) was followed except that the selective medium (Holliday, 1974) was supplemented with 5 mg/ml each of maltose and soluble starch to induce expression from the *gluA* promoter. Presumed transformants were transferred to and maintained on Holliday's complete medium (Holliday, 1974) supplemented with 350 µg Hy/ml.

(b) Transformation

Using the transformation protocol of Wang et al. (1988), vectors pDH33 and pD1 (Fig. 1) yielded 20–30Hy^R *U. maydis* colonies per µg DNA after three to five days incubation. Growth rates of these presumed transformants varied. All colonies continued to grow when transferred to fresh media containing 350 µg Hy/ml suggesting that few, if any, were 'abortive transformants'. Repeated attempts to transform with pDH25 failed.

Plasmids pDH33 and pD 1 transformation frequencies were dramatically increased by their linearization before transformation. For example, in one experiment *Xba*I-linearized pDH33 yielded 20 transformants per µg DNA, while the uncut circular vector yielded only 0.5 transformants per µg. An increase in transformation frequency for linearized vector has also been observed for pHL1 (Wang et al., 1988). Frequencies for pDH33 are generally lower than for pHL1.

The stability of integrated sequences is dependent upon

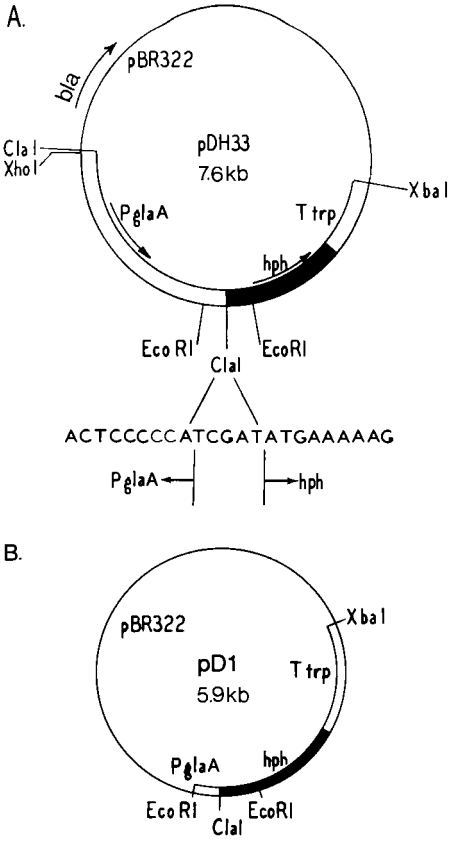

Fig. 1. Plasmid constructions. (**A**) Plasmid pDH33 was constructed in several steps, the components of which originated as follows: *Cla*I-*Hin*dIII fragment (thin line) is from pBR322 (Bolivar et al., 1977). The *Eco*RI site was removed by digesting with *Eco*RI, blunt-ending with PolIk and ligating. The *Eco*RV-*Sal*I region was removed by digesting with the two enzymes, blunt-ending, and ligation. The *gluA* promoter *(PglaA,* open box) was isolated in two fragments. The first, a 1768-bp *Cla*I-*Eco*RI fragment, was isolated from pGRG1 (Cullen et al., 1987a). The second, a 220-bp *Eco*RI-*Cla*I fragment, was derived from plasmid pARS163 which had a *Cla*I site introduced immediately preceding the start codon of the glucoamylase coding sequence (Arjun Sing, pers. commun.). The sequence surrounding the introduced *Cla*I site is shown. The *hph* gene (blackened box) and the *A.* nidulans *trpC* terminator *(Ttrp,* open box) sequences were isolated on an 1800-bp *Xba*I-*Cla*I fragment from pDH25 (Cullen et al., 1987b). (**B**) Plasmid pD1 was constructed by deleting the 1670-bp *Cla*I-*Eco*RI *glaA* promoter region from pDH33.

continued selective pressure (Fig. 2). Transformants designated D1A (lanes 9, 10), D1B (lanes 11, 12), DH33a (lanes 15, 16), DH33B (lanes 17, 18) and HLlB (lanes 7, 8) have significantly reduced signals in the lanes representing nonselective media. In contrast, transformants HLlA (lanes 5, 6) and DH33C (lanes 19, 20) show no apparent differences in copy number when grown on selective vs. nonselective media. Upon prolonged exposure, light bands of equal intensity appeared at 5.4 kb in lanes 13 and 14 suggesting that D1C was also stable (data not shown).

The hybridization patterns or relative copy number cannot be directly correlated to stability (Fig. 2). Hybridization patterns suggest multiple tandem integrations for all trans-

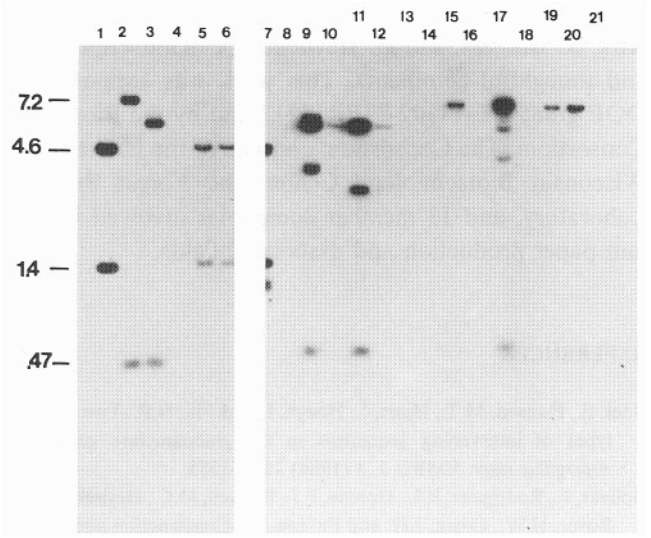

Fig. 2. Stability of the selectable marker in presence or absence of selective pressure, as analyzed by Southern hybridization. Transformants were grown approx. 100 generations in Holliday's (1974) complete medium with and without 20 µg Hy/ml. The 100-ml cultures were harvested by centrifugation, frozen in liquid N_2, lyophilized, and the DNA was extracted by the 'gentle-extraction method' (Specht et al., 1982). Transformant DNA (approx. 5 µg) was digested with *Eco*RI, electrophoresed in 0.6% agarose (SeaKem GTG, FMC, Rockland, ME) and blotted to Nytran (Schleicher & Schuell, Keene, NH). A 1-kb *Cla*I-*Bam*HI fragment of pDH25 (Cullen et al., 1987), which contains most of the *hph* coding region, was isolated, nick-translated and 5×10^5 cpm/ml hybridization buffer was used as the probe under moderate stringency (42°C; 50% formamide/0.25 M Na^+/7% SDS). Blots were exposed to Kodak XAR-5 film overnight (lanes 1–6) or for five days (lanes 7–21). Lanes 1–3, *Eco*RI-digested plasmids pHL1, pDH33, and pD1, respectively. The length (in kb) of the *Eco*RI fragments of pHL1 and pDH33 is indicated on the left margin. Lanes 5–20 are arranged in pairs containing DNA extracted from the same transformant grown with (odd-numbered lanes) and without (even-numbered lanes) Hy: transformants HL1A (lanes 5, 6); HL1B (lanes 7, 8); D1A (lanes 9, 10); D1B (lanes 11, 12); D1C (lanes 13, 14); DH33A (lanes 15, 16), DH33B (lanes 17, 18); DH33C (lanes 19, 20). Lane 21 contains DNA from untransformed *U. maydis* and lane 4 is blank.

formants except D1C (lanes 13, 14), in which only a single 5.4-kb band is observed. In transformant HL1A, bands at 1.4, 4.6 and, after prolonged exposure, 7.4 kb indicate homologous recombination at the *Asp70* locus (Wang et al., 1988). The lack of 7.2-or 7.4-bands in HL1B indicates nonhomologous recombination. In contrast to pHL1, pDH33 and its derivative pD1 contain no homology to the *U. maydis* genome as determined by Southern hybridizations under low stringency (not shown), so they must integrate by nonhomologous recombination. The banding patterns of D1A, D1B, DH33A, and DH33C are consistent with tandem duplications at different single loci for each. The 465-bp fragment corresponds to the *Eco*RI fragment that spans the *hph-glaA* fusion whereas the 5.4- and 7.2-kb fragments correspond to the remainder of pD1 and pD33. Transformant DH33B may also involve multiple tandem

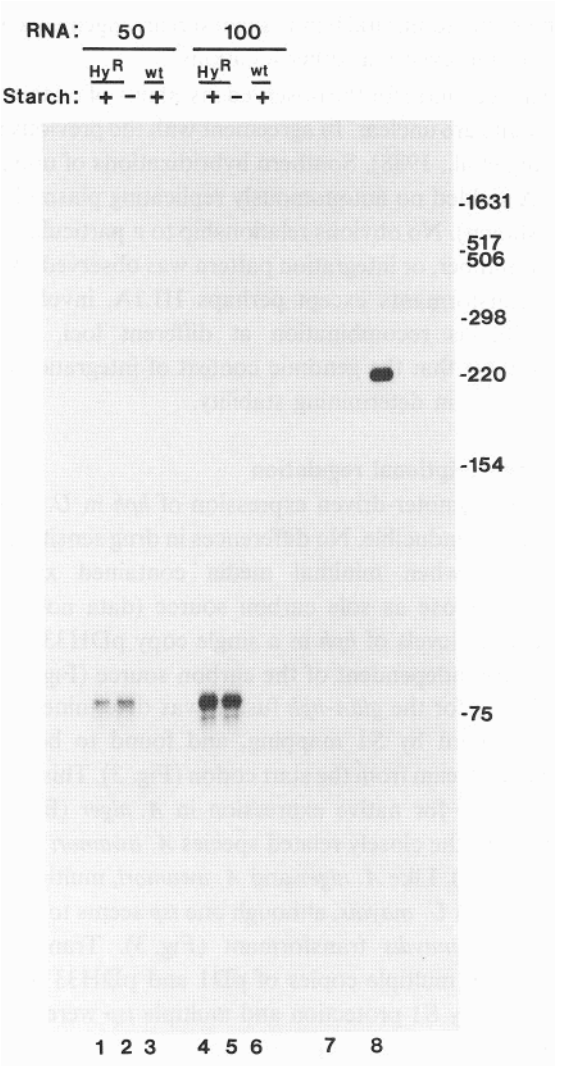

Fig. 3. S1 nuclease protection analysis of transcript levels. A single copy pDH33 transformant was grown overnight on minimal medium containing xylose (5 mg/ml, practical grade) or maltose/starch as sole carbon source. Total RNA was extracted as described by Timberlake and Barnard (1981). S1 protection experiments were carried out essentially as described by Favaloro et al. (1980). An *Eco*RI-*Cla*I fragment of pDH33 spanning the *tsp* was radiolabeled at the *Cla*I site and hybridized overnight at 50°C to 50 or 100 µg total RNA. Following hybridization, 300 units of S1 nuclease (Amersham Inc., Arlington Heights, IL) was added to each sample and incubated 30 min at 37°C. Samples were then extracted with phenol-chloroform and the nucleic acids ethanol precipitated. The pellets were washed with 70% ethanol, dried, and redissolved in 10 µl gel loading buffer (90% formamide/10 mM EDTA/10 mM NaOH/0.2% bromophenol blue/0.2% xylene cyanol). After heating to 80°C for 5 min, samples were loaded onto a 40-cm denaturing gel (5% polyacrylamide/8 M urea), and electrophoresed at 30 W. The gel was exposed to Kodak XAR 5 film with an amplifying screen for 2 days at −70°C. Lanes: 1,4, RNA from maltose/starch-grown cells; 2,4, RNA from xylose grown cells; 3, 6, 50 and 100 µg RNA, respectively, from untransformed wt *U. maydis* cells grown on maltose/starch; 7, 100 µg yeast tRNA; 8, undigested probe. Molecular size markers (bp) shown on the right margin were obtained from ^{32}P-labeled pBR322 digested with *Hin*f1. No bands are visible in lanes 3, 6 and 7.

copies, but additional bands suggest rearrangements and/or integration events at other locations.

The reason(s) for the observed instability of certain transformants are unclear. In agreement with the previous report (Wang et al., 1988), Southern hybridizations of undigested DNA yielded no autonomously replicating plasmids (data not shown). No obvious relationship to a particular vector, copy number, or integration pattern was observed. As all of the transformants except perhaps HLlA, involved non-homologous recombination at different loci, it seems reasonable that the genomic context of integrations might play a role in determining stability.

(c) Transcriptional regulation

glaA promoter-driven expression of *hph* in *U. maydis* is not starch-inducible. No differences in drug sensitivity were observed when minimal media contained xylose vs. starch/maltose as sole carbon source (data not shown). Transcript levels of *hph* in a single copy pDH33 transformant are independent of the carbon source (Fig. 3).

The *tsp* for the *glaA-hph* fusion was determined for this transformant by S1 mapping, and found to be approx. 80 bp upstream from the start codon (Fig. 3). This is similar to *glaA tsp* for native expression in *A. niger* (Boel et al., 1984) and the closely related species *A. awamori* (Nunberg et al., 1984). Like *A. niger* and *A. awamori,* multiple *tsp* are observed in *U. maydis,* although one *tsp* seems to dominate in the *U. maydis* transformant (Fig. 3). Transformants containing multiple copies of pD1 and pDH33 were also analyzed by S1 protection and multiple *tsp* were observed again at approx. –80bp (not shown).

In contrast to *glaA* promoter function in *A. nidulans,* substantial 5'-untranslated sequences are not required for expression in *U. maydis.* Plasmid pD1 (Fig. 1), which contains only 216 bp of upstream untranslated sequence and does not function in *A. nidulans,* transforms *U. maydis* strain 518 with approximately the same frequency as pDH33.

(a) Conclusions

We have demonstrated that the *A. niger glaA* promoter functions in *U. maydis,* even though these fungi represent disparate taxonomic groups. In view of the present results it seems likely that pDH33 and pD1 will function in other fungi. However, the full taxonomic range of usefulness remains to be investigated.

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