YEAST
Yeast **15**, 1269–1274 (1999)

Yeast Functional Analysis Reports

A Strong Carbon Source Effect Is Mediated by pUC Plasmid Sequences in a Series of Classical Yeast Vectors Designed for Promoter Characterization

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While using YIp356 and YEp356R *lacZ* reporter plasmids, we found *lacZ* expression driven by the *ARG2* promoter to be much higher in cells grown on a non-glucose carbon source than in glucose-grown cells (5–10-fold higher on galactose and up to 40-fold higher on ethanol). Furthermore, expression increased 30-fold upon shifting from a high-glucose to a low-glucose medium. This carbon source regulation requires Snf1p and possibly Ssn6p. It appears, however, to be artefactually mediated by plasmid sequences located upstream from the multicloning site. This emerged from the following observations: (a) the derepressive effect disappears if any extra piece of DNA is inserted upstream from the *ARG2* promoter; and (b) similar derepression on low glucose is observed with another yeast promoter (*ARG11*), provided that the flanking 5′ region is short. We determined that the *cis*-elements responsible for this physiologically irrelevant glucose regulation are located between positions 636 and 879 of the pUC18 DNA sequence. Copyright © 1999 John Wiley & Sons, Ltd.

KEY WORDS — YEp356; *lacZ* fusion reporter plasmid; glucose repression

INTRODUCTION

To analyse the functional organization and regulation of promoters, especially those of genes whose products are difficult to assay, a common strategy is to construct translational reporter gene fusion plasmids. Myers *et al.* (1986), notably, have developed a series of yeast/*Escherichia coli* shuttle vectors suitable for fusing yeast promoter and coding sequences with the *E. coli lacZ* gene: the self-replicating YEp plasmids 353–368, the integrative YIp plasmids 353–368, and the equivalent R vectors with reversed orientation of the multicloning site.

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Contract/grant sponsor: IWT Specialisation Fellowship, Belgium.

We have used some of these vectors to study the *ARG2* promoter and to resolve contradictory data concerning the existence of catabolite repression on this gene's expression: Wipf and Leisinger (1979) reported strong derepression of acetylglutamate synthetase in the post-diauxic phase, but Jauniaux (1987) found no *ARG2* derepression in glycerol-grown cells. Difficulties inherent in the enzymatic assay of the synthetase (low specific activity, protein instability, difficult elimination of strongly bound feedback-inhibiting arginine) or strain differences appeared as possible causes of this discrepancy.

In this short communication we report that the strong carbon-source regulation of the *ARG2* promoter observed in our constructs is, in fact, a physiologically irrelevant plasmid effect mediated by DNA sequences from the pUC vector.

Since the incriminated vectors are widely used, even in recent carbon-source regulation studies, it

CCC 0749–503X/99/121269–06$17.50

Received 23 February 1999
Accepted 26 April 1999

seemed important to issue this warning to experimenters in the field.

MATERIALS AND METHODS

Saccharomyces cerevisiae *strains and media*

MG471 (Mat α, *ura3*$^-$) is a derivative of our Σ1278b wild-type strain. Strain PI3 (Mat α, *ura3*$^-$, *snf1:: gen*^{R}) is a derivative of MG471 (see below). MCY1974 (Mat α, *ssn6-Δ9, ade2*$^-$, *his3*$^-$, *trp1*$^-$, *ura3*$^-$) is a gift from Marian Carlson. The strains were grown at 30°C on a minimal medium (M.am) containing 0·02 M $(NH_4)_2SO_4$, 3% glucose, vitamins and trace minerals (Messenguy, 1976). Where required, L-amino acids or nucleotides were added to a concentration of 25 μg/ml. Where indicated, glucose was replaced with 3% galactose or 2% ethanol. For a shift from high to low glucose concentration, cells growing exponentially on M.am were centrifuged, washed twice with water, and resuspended in M.am medium containing only 0·05% glucose.

Construction of plasmids

All plasmids were derived from YIp356R (integrative vector) or YEp356R (2 μ-based vector) by ligating appropriately restricted promoter fragments (obtained by PCR amplification on Σ1278b genomic DNA) into the correspondingly restricted vector DNA. Plasmids pKAT11 (YIp-derived) and pKAT1 (YEp-derived) bear a *Pst*I–*Xba*I fragment obtained with primers PK3/PK1 and contain an *ARG2* promoter region extending from position −1233 to position +15, the first five codons of *ARG2* being fused with *lacZ*. Plasmids pKAT13 (YIp-derived) and pKAT3 (YEp-derived) likewise bear a *Pst*I–*Xba*I fragment obtained with primers PK4/PK1 and contain an *ARG2* promoter region extending from position −270 to position +15. Plasmid pKAT22 is derived from pKAT13 and was obtained by inserting a 1453 bp *Sph*I–*Pst*I fragment bearing the ARG3 ORF (amplified by PCR with primers PK12/PK11 on plasmid pMC200; Crabeel *et al.*, 1983) so that the ARG3 ORF occupies the same position and displays the same orientation as the YJLO72C ORF in pKAT11. To make pAA23 and pAA23-w, we amplified by PCR the YEp356 sequence from position 7709 to the *Pst*I site of the multicloning site, using primers AA63/AA64, and inserted the 268 bp fragment into the *Pst*I site of pKAT22, with the original (pAA23) or inverted (pAA23-w) polarity. Plasmids pNAN12, pNAN14 and pNAN15 are YIp plasmids bearing *Sal*I–*Eco*RI fragments comprising different portions of the ARG11 promoter region, including the ARG11 initiation codon (A = +1); these fragments were amplified by PCR with VN1/VN3 (from position −682), VN1/VN5 (from position −360) and VN1/VN6 (from position −203), respectively.

Oligonucleotides

PK1 (GGCCTCTAGATATTCTCCTCCACAT GATAAAAGTAACCGAC)
PK3 (GGCCCTGCAGCCATAACGACAAAC AAGAATCAATTCACGAAAC)
PK4 (GGCCCTGCAGGCGTCTATGAAACT AGTCTTATTTCAGATC)
PK11 (GGCCCTGCAGACCATGTCAACCAC AGCATCCACGCCTTCA)
PK12 (GGCCGCATGCCTATTGCTATTGTA GTGGAACTGTCTAAAG)
AA63 (GGCCCTGCAGTCAGGGGGGCGGA GCCTATG)
AA64 (GTCGACCTGCAGGCATCGAAGC)
VN1 (GGCCGAATTCAGACATTATACGTT GACCTTCCAGAATTTGAG)
VN3 (GGCCGTCGACTACACGTTTTAACG TGAATGTACTAAACTGC)
VN5 (GGCCGTCGACAAGCACAACAGCAG CCTACCCTTTTTGTGG)
VN6 (GGCCGTCGACCATATACGTTTTCCG CTGTGGCCGCTGTGG)

Construction of strain PI3

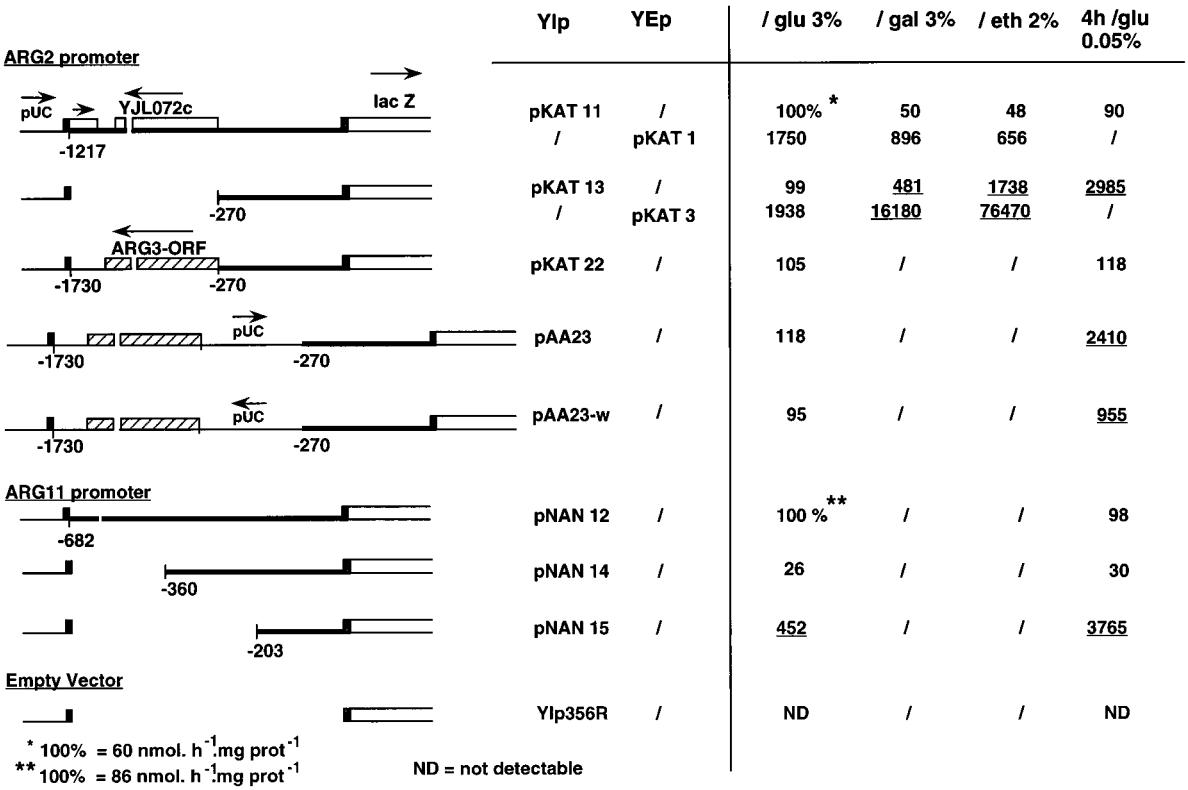
In strain P13, derived from MG471, the whole *SNF1* ORF has been replaced with a geneticin-resistance cassette introduced by 'the long flanking homology region' procedure developed by Wach (1996). Proper substitution in PI3 of the Kan^R cassette for *SNF1* was assessed by colony-PCR using suitable primers.

Yeast transformation

Before yeast cell transformation, plasmids of the integrative type were linearized by *Apa*I restriction within *URA3* so as to target their integration to that locus. Correct targeting was verified by colony-PCR using appropriate primers.

Enzyme assays

β-Galactosidase was assayed according to Miller (1972) on yeast extracts obtained with a French



YIp	YEp	/ glu 3%	/ gal 3%	/ eth 2%	4h /glu 0.05%
pKAT 11	/	100% *	50	48	90
/	pKAT 1	1750	896	656	/
pKAT 13	/	99	481	1738	2985
/	pKAT 3	1938	16180	76470	/
pKAT 22	/	105	/	/	118
pAA23	/	118	/	/	2410
pAA23-w	/	95	/	/	955
pNAN 12	/	100 % **	/	/	98
pNAN 14	/	26	/	/	30
pNAN 15	/	452	/	/	3765
YIp356R	/	ND	/	/	ND

Figure 1. Relative β-galactosidase activity in yeast cells transformed with YIp356R and YEp356R plasmid derivatives bearing various promoter fragments.

pressure cell. The protein content was measured by the Folin procedure. The values provided in Figure 1 are based on means of at least three independent assays (maximal standard deviation 25%).

RESULTS AND DISCUSSION

Apparent glucose regulation of the ARG2 *promoter*

In recent efforts to characterize open reading frame YJL071w of the yeast genome, encoding a protein similar to the *N. crassa* acetylglutamate synthetase, we identified this ORF as *ARG2*, the orthologous *S. cerevisiae* gene (unpublished data). Between the *ARG2* ORF and the divergently polarized upstream YJL072c ORF lies a 270 bp promoter region with a consensus TATA sequence 219 bp upstream from the *ARG2* initiator codon. This region, including the first five codons of Arg2p, was cloned in fusion with *lacZ* in the integrative plasmid pKAT13 and the 2 μ-based pKAT3. Similarly, we generated pKAT11 and pKAT1, in which the inserted upstream region of *ARG2* extends to position − 1233, thus comprising the YJL072c ORF. In MG471 yeast transformants containing an integrated pKAT13 plasmid, β-galactosidase activity (see Figure 1) was much higher after growth on non-glucose carbon sources than after growth on glucose. This suggested relief from glucose repression on the former carbon sources. Typically, the effect was moderate on a poor but fermentable carbon source (five-fold increase on galactose) and more pronounced on a non-fermentable carbon source (close to 20-fold increase on ethanol). Likewise, 4 h after a shift from a high- to a low-glucose medium, a 30-fold increase in β-galactosidase activity was observed. Expression of another arginine gene, *ARG3,* remained constant under all growth conditions tested (not shown). Since both *ARG3* and *ARG2* are subject to the general control of amino acid biosynthesis, the derepression occurring in the absence of glucose cannot be an indirect effect of

Gcn4p-dependent transactivation. The results were similar for yeast cells transformed with the multicopy pKAT3 plasmid, but the amplitude of the effect was greater (40-fold increase on ethanol) and, as expected, the basal β-galactosidase activity was 18-fold higher.

Under similar conditions, however, no derepression was observed with plasmid pKAT11 or pKAT1 containing the longer 5′ region. Although unexpected, such behaviour might occur if glucose induction mediated by the distal region were compensating for glucose repression mediated by the proximal region. If so, the induction effect might be absent in Leisinger's strains (Wipf and Leisinger, 1979), leaving the repressive effect to show itself. In our strains (isogenic with those of Jauniaux, 1987), on the other hand, the two effects would counteract each other and no carbon source regulation of the chromosomal *ARG2* gene would be detected.

To identify the signal transduction pathway responsible for the apparent glucose repression affecting the 270 bp *ARG2* promoter, we transformed strain P13, a *snf1* deletion mutant isogenic with MG471, and a non-isogenic *ssn6* mutant with the pKAT13 plasmid, and monitored β-galactosidase activity in these transformants before and after a shift from high- to low-glucose medium: derepression was no longer observed in either of these genetic backgrounds (data shown in Figure 2 for the *ssn6* mutant).

Yet an alternative explanation remained possible, such as an effect mediated by plasmid sequences upstream from the multicloning site that would fortuitously contain yeast regulatory targets mediating a carbon source control. Should this be the case, insertion of any DNA sequence upstream from the 270 bp ARG2 promoter would be expected to have the same effect as the presence of YJL072C sequences. Therefore, we inserted the ARG3 ORF into pKAT13, creating pKAT22. No derepression was observed during a shift to low-glucose medium in yeast cells transformed with this construct. This supports the hypothesis that fortuitous plasmid-controlled glucose regulation occurs in cells harbouring pKAT13 and pKAT3.

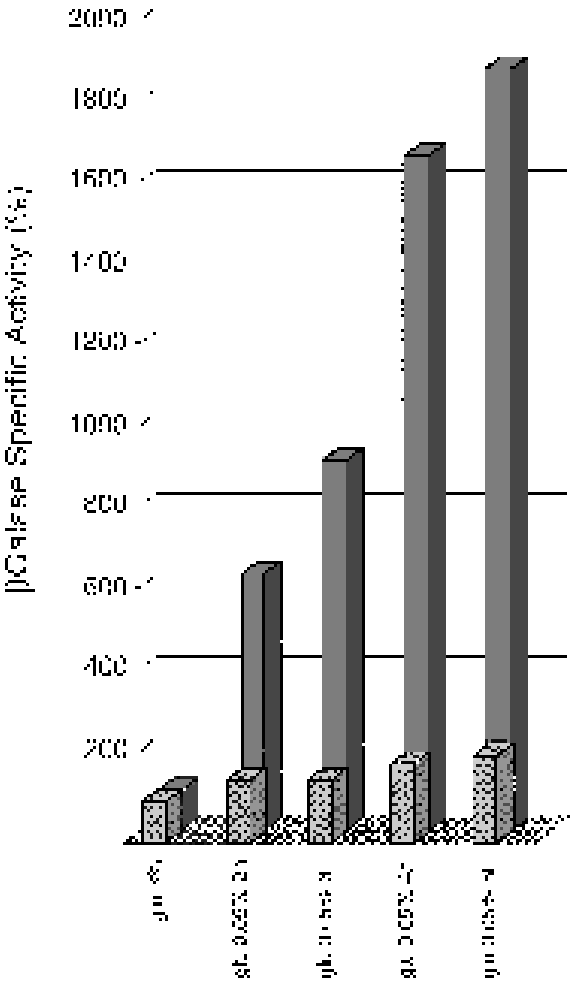


Figure 2. Time course of β-galactosidase derepression after a shift from high- to low-glucose growth medium: rear stack is MG471::pKAT13 (100%=60 nmol/h/mg prot), front stack is the ssn6-Δ9 mutant strain MCY1974::pKAT13 (100%=51 nmol/h/mg prot).

Apparent glucose regulation of the ARG11 *promoter*

If glucose regulation of *ARG2* expression from pKAT13 is indeed mediated by plasmid sequences, it should appear when other promoters are inserted into the multicloning site of the YIp356R and YEp356R vectors. In a parallel study we happened to be analysing, using the same vectors, the promoter region of *ARG11* encoding a mitochondrial ornithine carrier (Crabeel *et al.*, 1996; Palmieri *et al.*, 1997). The *ARG11* ORF is preceded by a long (1151 bp) intergenic region, and the promoter region has an atypical functional organization which is not yet understood. We thus used the pNAN12, pNAN14, and pNAN15 plasmids bearing different portions of the *ARG11* 5′-upstream region to test them for the presence of glucose regulation. As seen in Figure 1, a 203 bp

fragment proximal to the *ARG11* ORF conferred derepression of reporter gene expression on low glucose medium. This derepressibility disappeared when the length of the inserted promoter region was increased to 360 bp. Using an empty integrated YIp356R plasmid, we checked that no β-galactosidase activity was detectable on either high- or low-glucose, confirming Myers *et al.*'s (1986) assertion that the plasmid sequences alone cannot function as *bona fide* core promoters.

From these various results we conclude that plasmid sequences upstream from the multicloning site of the YEp plasmids 353–368 and the YIp plasmids 353–368 mediate artefactual carbon-source regulation of core promoters, and that Snf1p and possibly Ssn6p (the experiment concerning the latter was conducted only with a non-isogenic mutant) are necessary elements in the signalling pathway for this regulation. We may speculate that the regulation involves transactivation during growth on low-glucose medium or non-glucose carbon sources, rather than down-regulation due to the presence of glucose, since the basal β-galactosidase activity measured on glucose medium was the same in cells harbouring the regulated pKAT13 plasmid as in ones harbouring the non-regulated pKAT11 and pKAT22 plasmids. In the case of the peculiar *ARG11* promoter, however, the plasmid sequences appear to partially stimulate expression, even on high-glucose medium.

A pUC vector fragment mediates glucose derepression

The plasmid sequence preceding the multicloning site (MCS) of YIp and YEp vectors 353–368 comes from the pUC18 vector: the 1.8 kb DNA sequence, extending clockwise from position 5729 to the start of the MCS at position 7964, corresponds to the pUC sequence extending from position 183, counter-clockwise through position 2687, to position 636. Thus, the sequence adjacent to the MCS comprises 44 bp of *lacI* (from M13 mp), followed by pBR322 sequences containing the plasmid ORI. Within the first 255 bp, there are two STRE consensus sequences (TCAGGGG) and three potential Abf1 binding sites (WDCNYNN NNNACGAD sequences containing 1 mismatch), a combination that might mediate transactivation by Msn2p/Msn4p (Schmitt & McEntee, 1996). The sequence CCGAACGACCG, starting 82 bp upstream from the MCS, might also possibly function as a carbon source responsive element (CSRE, consensus CCRTYSRNCCG). No ANWATSYG GRGR consensus binding site for Mig1p (Lundin *et al.*, 1994) was found in the pUC sequences. Whatever the case may be, we showed that the 255 bp fragment is sufficient to mediate the observed carbon-source regulation. We amplified by PCR the sequence from position 7709 to 13 of the YEP356 vector and inserted it into the non-regulated pKAT 22 plasmid between the ARG3 ORF and position −270 of the *ARG2* promoter, either in the original polarity, creating plasmid pAA23, or in the opposite orientation, creating pAA23-w. As seen in Figure 1, the fragment restored the artefactual regulatory effect, practically fully in pAA23 and partially in pAA23-w.

The YEp and YIp plasmids 353–368 are still often used, notably in studies concerning carbon-source-responsive promoters (e.g. Brown and Trumpower, 1995; Caspary *et al.*, 1997; Randez-Gil *et al.*, 1997). The incriminated pUC sequences might also be present at an inopportune position in other plasmid vectors. Hence, when using such vectors to characterize the regulatory features of promoter regions, it is important to be aware that those sequences can artefactually stimulate gene expression from core promoters on non-glucose medium. On the other hand, the same sequences could be used deliberately to achieve increased gene expression in artificially engineered promoters.

ACKNOWLEDGEMENTS

We are indebted to Juana Gancedo for suggesting construction of a pKAT22-like control plasmid, and to N. Glansdorff for reading the manuscript. K. Pauwels is the recipient of an IWT Specialization Fellowship.

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