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Enhanced expression of heterologous proteins by the use of a superinducible vector in budding yeast

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Summary. We report the effects of a strong overexpression of the GAL4 activator protein on the expression of UAS_{GAL} regulated genes, obtained by cloning the *GAL4* gene and the GAL1–10 upstream activating sequence (UAS_{GAL})-lacZ fusion in the same high copy number plasmid. Comparable amounts of active enzyme were obtained by host strains usually producing different levels of cloned proteins due to their different genetic background. The transformed cells constitutively produced low levels of β-galactosidase (1–2% of total proteins) both in glucose and in raffinose minimal media. Nevertheless, expression was still inducible and a tenfold induction could be rapidly obtained by the addition of 0.5% (w/v) galactose to the culture, even when glucose was still present in the medium.

Introduction

The yeast *Saccharomyces cerevisiae* is widely used as a host for the expression of heterologous proteins. Expression vectors based on the use of either constitutive or inducible strong yeast promoters have been developed. Regulated systems are generally favoured since they allow the development of processes that can be controlled by simple experimental strategies. Strong inducible promoters suitable for high-level regulated expression have been derived from the galactose-regulated genes (Guarente et al. 1982). In yeast, the GAL1–10 upstream activating sequence (UAS_{GAL}) directs the transcription of 0.25 to 1% of total poly(A^+)RNA (Johnston 1987). When cloned in high copy number plasmids these sequences are able to drive very high levels of gene expression (Velati-Bellini et al. 1986; Schultz et al. 1987; Lotti et al. 1988; Porro et al. 1991; Broker et al. 1991).

The transcription of galactose-regulated genes in *S. cerevisiae* has been shown to depend on the balance between two major regulatory proteins encoded by *GAL4* and *GAL80* genes (for a comprehensive review see Johnston 1987). The system is repressed by glucose and induced by galactose with an induction ratio of about 1000. Increasing evidence has suggested that additional regulatory mechanisms exist. Operator-like sequences having a negative control on this system have been described (West et al. 1987) and researches done in our laboratory have shown that UAS_{GAL}-driven expression is tightly related to the growth phase of the yeast culture, reaching its maximum level in a transient physiological phase corresponding to the early stationary phase of batch growth (Lotti et al. 1988; Vanoni et al. 1988) and to a specific growth rate in continuous cultures (Porro et al. 1991).

The GAL4 activator protein might be a limiting factor since it is usually expressed at low levels; its moderate overexpression has indeed been shown to have a small but positive effect (Johnston and Hopper 1982; Lotti et al. 1988). However, problems related to the different genetic and physiological constraints of each yeast host strains (Broker et al. 1991), the long lag time (up to 5 h) following induction (Adams 1972) and the requirement of large amounts of inducer, have not yet been solved. In the present paper we describe the effect of a strong overexpression of the *GAL4* gene obtained by cloning the *GAL4* gene and the UAS_{GAL}/*CYC1*-lacZ expression cassette in a unique expression vector occurring at high copy number in yeast cells.

Materials and methods

Strains, media and growth conditions. The following yeast strains were used: X4004-3A (*MATa lys5 met2 ura3 trp1*), MVY49.35 (*MATα leu2 ura3*), GRF18 (*MATα leu2 his3*). Wild-type yeast cells were grown by shaking at 30° C in YPD medium (1% yeast extract, 2% peptone, 2% glucose), whereas transformants were grown in minimal media containing 0.67% (w/v) Yeast Nitrogen Base (YNB) without amino acids (Difco, West Mosley, Survey, UK) supplemented with 50 μg/ml of the required supplements and 2% (w/v) glucose, galactose or raffinose as a carbon source. For the nutritional shift experiments, cells were grown in YNB-glucose medium to which galactose was then added at 0.5% (w/v) final concentration. Growth was monitored by counting the number of

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cells per millilitre with a Coulter Counter ZBI (Lotti et al. 1988). *Escherichia coli* HB101 was grown in LB medium (1% bacto-peptone, 0.5% yeast extract, 0.5% NaCl) at 37° C. Selection of transformants was performed by adding 50 μg ampicillin/ml. Solid media contained 2% agar.

Recombinant DNA. Plasmid pLGSD5 (Guarente et al. 1982) was kindly supplied by L. Guarente (MIT, Cambridge, Mass., USA) and YEpG525 (Johnston and Hopper 1982) by R. Driscoll (University of Utah, Salt Lake City, Utah, USA). Recombinant DNA manipulations were done as described by Maniatis et al. (1982). Total yeast RNA was extracted as previously described (Lotti et al. 1988). Plasmid pLA41 was obtained by subcloning the *LEU2*-d gene into pLGSD5 as described (Porro et al. 1991). pM1 was obtained by cloning a 3.7-kb *GAL4* DNA from YEpG525 in the *Hin*dIII site of pLA41 (a partial digestion was required). Enzymes were from Boehringer (Mannheim, FRG). Yeast cells were transformed by the LiCl procedure (Ito et al. 1983).

β-Galactosidase assay. Yeast cells (10^8) were harvested from the culture medium and the specific activity of β-galactosidase was detected as previously described (Lotti et al. 1988). The specific activity of purified *E. coli* enzyme was assumed to be 300000 units/mg (Miller 1972).

Northern analyses. Total yeast RNA was separated on 1.2% denaturating gel (Maniatis et al. 1982) and transferred to Hybond N-membrane (Amersham, UK). Radiolabelled single-strand RNA probes were generated by in-vitro transcription of DNA fragments inserted into the multiple cloning site of pGEM-blue plasmid using the Riboprobe system from Promega Biotec and [α-^{32}P]UTP (Amersham).

Electrophoresis and immunoblotting. Total yeast proteins were fractionated by 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, electrophoretically transferred to nitrocellulose filters and immunodetected with polyclonal anti-β-galactosidase antibodies (Vanoni et al. 1989).

Results and discussion

We have previously studied the regulated expression of heterologous proteins in yeast using plasmids pLGSD5 (Lotti et al. 1988) and pLA41 (Porro et al. 1991), which contain the *E. coli* lacZ gene under the control of the inducible promoter $UAS_{GAL}/CYC1$. These 2 μ-based vectors are maintained in yeast at about 10 and 100 copies per cell, respectively (data not shown). Availability of the GAL4 protein could be one of the factors limiting expression since a tenfold increase in its gene dosage induced a doubling in β-galactosidase production (Lotti et al. 1988).

To further increase the level of GAL4 protein available to the expression system, we cloned a 3.7-kb DNA fragment spanning the whole *GAL4* sequence in pLA41, thus obtaining the high copy number plasmid pM1 (Fig. 1). The level of specific GAL4 transcript was evaluated by Northern blot hybridization. In Fig. 2 the GAL4 mRNA content in MVY49.35 cells bearing pLA41 (only chromosomal *GAL4*), YEpG525 (*GAL4* at ten copies/cell) (Lotti et al. 1988) or pM1 (about 50–100 copies/cell) is compared, showing a dramatic increase in the latter sample.

Table 1 shows heterologous protein production by three different yeast strains bearing pLGSD5, pLA41 or

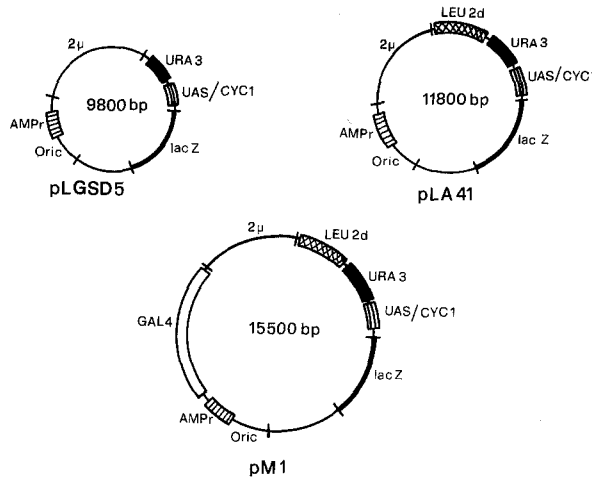

Fig. 1. Structural diagram of the plasmids used. pLGSD5 (Guarente et al. 1982) contains the hybrid $UAS_{GAL}/CYC1$ yeast promoter fused with the *Escherichia coli* lacZ gene. pLA41 (Porro et al. 1991) was derived from pLGSD5 by replacing a 700-bp 2-μ fragment with a 2000-bp fragment spanning the *LEU2*-d gene and part of the 2-μ sequence. pM1 was obtained by subcloning the *GAL4* gene in pLA41

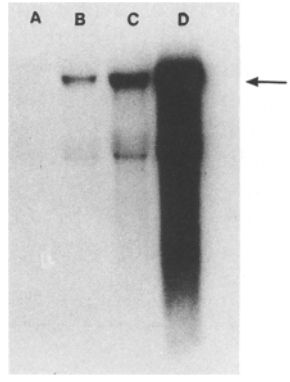

Fig. 2. Northern blot analysis of GAL4 transcripts. Twenty micrograms of RNA extracted from MVY49.35 carrying different plasmids exponentially growing on glucose- or galactose-based media were hybridized with a labelled RNA *GAL4* probe. The specific GAL4 transcripts are marked by an *arrow*. **A** [pLA41] cells grown on glucose. **B** [YEpG525] cells grown on glucose. **C** [YEpG525] cells grown on galactose. **D** [pM1] cells grown on glucose. Levels of specific GAL4 transcript (*arrow*) were higher on galactose growth media (compare **B** and **C**), as recently suggested (Griggs and Johnston 1991)

pM1 during batch growth in galactose minimal media. The increase in plasmid copy number had little effect on enzyme expression, since it induced at best a doubling of heterologous protein (MVY49.35 strain), whereas in the case of X4004 and GRF18 no increase at all was observed. These results provided a clear indication that the genetic and physiological background, more than the cloned gene dosage, influence the expression of genes controlled by UAS_{GAL}.

The kinetics of expression was dependent on the growth phase, as is characteristic for heterologous genes cloned under UAS_{GAL} control (Vanoni et al. 1988). Growth in glucose medium fully repressed lacZ

Table 1. β-Galactosidase production by pLGSD5-, pLA41- or pM1-transformed yeast cells during batch growth in galactose minimal media

Strain	Copy number[a]	% β-Gal[b]	Sta/Exp[c]
X4004[pLGSD5]	10	9	25
X4004[pLA41]	100	10	25
X4004[pM1]	50/100	9	20
MVY49.35[pLGSD5]	10	2	15
MVY49.35[pLA41]	100	4	15
MVY49.35[pM1]	50/100	—[d]	—[d]
GRF18[pLGSD5]	10	8	20
GRF18[pLA41]	100	8	20
GRF18[pM1]	50/100	8	15

[a] Plasmid copy number/cell, as determined with Southern analysis

[b] Percentage of active heterologous enzyme on total cell proteins. Values of expression reported were the maximal levels of production found

[c] Ratio of specific enzyme activity determined in the early stationary and in the exponential phases of growth, respectively

[d] MVY49.35[pM1] cells failed to grow if directly inoculated in yeast nitrogen base-galactose medium

expression by pLGSD5 transformed cells, whereas pLA41 cells produced very low amounts of enzyme (0.0003–0.0005% of the total yeast proteins). For cells transformed with pM1 growing on glucose an enzyme activity ranging between 1.2 and 1.6% of total proteins was detected throughout growth.

A constitutive expression of UAS_{GAL}-regulated genes in glucose medium as a consequence of an increased *GAL4* gene dosage, has been previously reported (Johnston 1987; Johnston and Hopper 1982). This effect is generally explained on the basis of a titration of the GAL80 protein by the GAL4 excess. Surprisingly, we observed that exponential X4004[pM1] cells grown in the absence of inducer in both glucose (repressing medium) and raffinose (non-repressing and non-inducing source) based media, constitutively synthesized β-galactosidase at comparable levels (1.2% and 1.8% of total cell proteins, respectively).

In spite of the *GAL4* overexpression supported by pM1 (Fig. 2), the level of enzyme obtained did not increase over the values previously obtained by the same strains bearing pLA41. MVY49.35[pM1] transformants were not able to grow on galactose; however, they accumulated active enzyme after addition of galactose to cells growing on glucose (see below). This inhibitory effect may be related to the negative effect of a strong *GAL4* overexpression as observed also by others (Gill and Ptashne 1988; Laughon et al. 1984; Schultz et al. 1987).

The different enzyme activities measured in the experiments described above, were consistent with the absolute protein levels (Fig. 3A) and were related to the presence of specific lacZ transcripts (Fig. 3B). It is interesting to note an increase in specific lacZ transcripts in the presence of galactose for transformed pM1 cells (Fig. 3B, compare lane E with lane F).

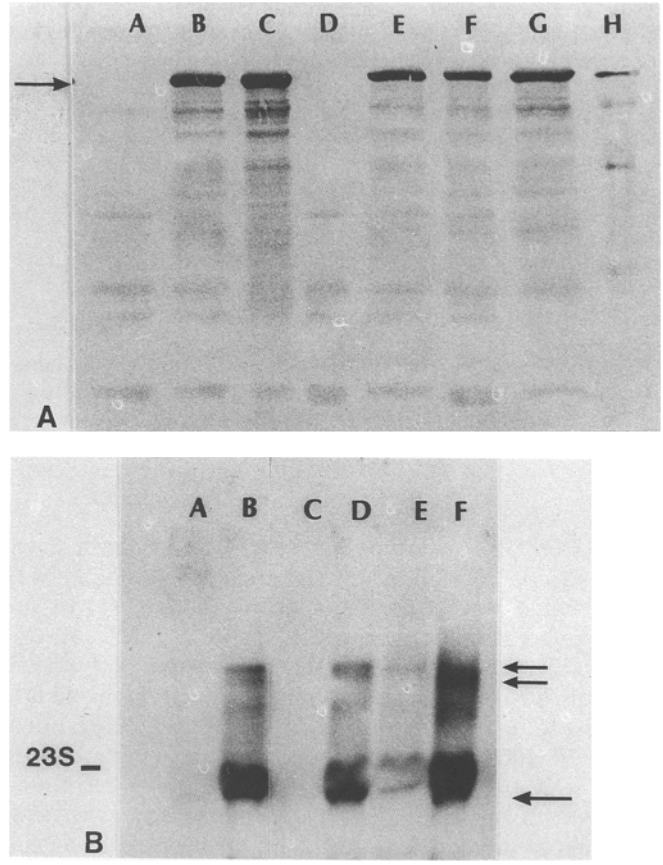

Fig. 3 A, B. Levels of β-galactosidase protein and lacZ mRNAs. **A** Western analysis of heterologous proteins. Total cell proteins (10 μg) were loaded in each lane and probed with anti-β-galactosidase polyclonal antibodies. The major product (*arrow*) accumulated was indentified as a 116-kDa protein. Other immunoreactive species of lower molecular mass are likely to represent degradation products of the mature enzyme, since their levels were proportional to enzyme activity. *Lane A*, X4004[pLGSD5] grown on glucose (exponential phase); **B**, X4004[pLGSD5] - galactose - (exponential phase); *C*, X4004[pLGSD5] - galactose - (stationary phase); *D*, X4004[pLA41] - glucose - (exponential phase); *E*, X4004[pLA41] - galactose - (exponential phase); *F*, X4004[pM1] - glucose - (exponential phase); *G*, X4004[pM1] - galactose - (exponential phase); *H*, commercial β-gal (250 ng). **B** Effect of carbon source on the transcription of the lacZ gene cloned in different plasmids. RNA (10 μg) extracted from exponentially growing cultures were separated in each lane and hybridized with a labelled RNA lacZ probe. lacZ mRNAs (indicated by the *arrows* on the *right side*) were identified by comparison between transformed strains expressing varying levels of specific transcripts. The position of 23S yeast ribosomal RNAs is indicated on the left margin. *Lane A*, total RNA from X4004[pLGSD5] grown on glucose; *B*, X4004[pLGSD5] - galactose; *C*, X4004[pLA41] - glucose; *D*, X4004[pLA41] - galactose; *E*, X4004[pM1] - glucose; *F*, X4004[pM1] - galactose

In another set of experiments, galactose at low concentration (0.5% w/v) was added to pM1-transformed cells exponentially growing in YNB-glucose (5×10^6 cells/ml). High levels of enzyme were obtained within a very short time and after 5 h β-galactosidase accounted for 6% of the total protein in the cells, and reached 12% of total proteins later (Table 2). A very high level of

Table 2. Heterologous enzyme production by pLA41- or pM1-transformed yeasts after addition of galactose (0.5% w/v) to exponentially growing batch cultures on YNB-glucose based medium

Strain:	Time (h)[a]				
	0.0	0.5	1.0	5.0	18.0
X4004[pM1]	1.2	1.5	2	6	12
X4004[pLA41]	<0.001	N.D.	N.D.	N.D.	<0.001
MVY49.35[pM1]	1.6	N.D.	N.D.	N.D.	12
GRF18[pM1]	1.4	N.D.	N.D.	N.D.	11

Data in the table represent the percentage of active heterologous β-galactosidase on the total cell proteins: N.D., not determined
[a] Time after addition of galactose at which β-galactosidase specific activity was determined

expression was sustained for several hours with each host strain, including transformed MVY49.35[pM1] cells, which were not viable during batch growth on YNB-galactose media (compare Table 1 and Table 2). Results in Table 2 indicate that the expression system can still be induced by galactose even though a high glucose concentration (about 15–16 g/l; concentrations above 50–100 mg/l are considered as repressing conditions) was still present in the medium. Since the induction was also obtained in a short time and with low amounts of inducer, we consider plasmid pM1 as a "superinducible" vector.

All data reported indicate that the regulatory mechanism controlling the GAL system appears to be partially modified. However, two facts deserve to be further underlined: (i) the system is still inducible and an increase in the specific lacZ transcript is also evident (Fig. 3B), and this is hard to explain on the basis of the simple GAL4/GAL80 interaction mechanism, confirming the existence of additional regulatory mechanism acting on UAS_{GAL}; (ii) similar levels of β-galactosidase were obtained both in glucose and raffinose media, suggesting a strong attenuation of glucose repression related to the overexpression of the *GAL4* gene.

In view of biotechnological applications, the results reported in this work suggest that the use of UAS_{GAL} promoters associated with the overexpression of the *GAL4* gene could contribute to solving three important issues hampering the full exploitation of inducible systems. In fact, the use of the vector pM1 allows obtaining high and comparable levels of production with any yeast strain used, in spite of different genetic and physiological backgrounds. Furthermore, the superinducible system can be rapidly induced by simple addition of the inducer; during the induction processes, the main carbon and energy source is glucose, allowing the utilization of low concentrations of galactose. As a consequence, the withdrawal of the repressing nutrient or of the transformed biomass to be shifted to inducing conditions, as well as a preliminary growth on a derepressing carbon source are not longer required, greatly simplifying the achievement of two-step fermentation procedures.

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