Short Technical Reports

Tightly Regulated, β -Estradiol Dose-Dependent Expression System for Yeast

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ABSTRACT

We have refined the regulated expression of $UAS_{GAL1,10}$ -driven genes in yeast by modifying a vector encoding the β -estradiol inducible activator, $GAL4.ER.VP16$ (GEV). The expression of GEV was placed under the regulation of the low-level, constitutive *MRP7* promoter, and β -estradiol-regulated expression was monitored by the expression of an integrated UAS_{GAL10} -lacZ reporter and by immunoblot analysis of a UAS_{GAL1} -regulated gene product. Target gene expression regulated by low levels of GEV has several advantages over the standard galactose-inducible expression systems. (i) Most importantly, the target gene expression is undetectable in the absence of hormone; (ii) target gene expression is β -estradiol dose-dependent, and variable levels of target gene expression from low to several hundred-fold induction can be achieved; and (iii) induction or depletion studies can be conducted independent of carbon source in *gal4* Δ strains. In addition, any $UAS_{GAL1,10}$ expression construct can be used without modification of the target gene or many *gal4* Δ host strains, and GEV vectors are compatible with other inducible yeast expression systems. This method may be useful to researchers investigating the functions of essential genes, dominant negative mutants, mitochondrial genes, and viral, plant, and mammalian genes in yeast assay systems.

INTRODUCTION

The yeast *Saccharomyces cerevisiae* is a powerful model system for ectopic gene expression and for studying a wide array of biological processes. Co-expression of interacting components at physiologically relevant levels is essential for understanding structural and catalytic complexes. Critical to these kinds of studies are regulated levels of ectopically expressed target genes and a tight

off state. We describe here a β -estradiol-regulated expression system that permits a broad range of dose-dependent target gene expression levels and a tightly regulated off state that is carbon source independent in *gal4* Δ strains. This system is designed for functional studies of gene products that are not amenable to the standard galactose induction protocols, such as proteins normally expressed only under respiratory conditions or induced by anaerobic growth or other environmental stress conditions. This $GAL4.ER.VP16$ (GEV) system for yeast is analogous to the steroid-inducible, Gal4p-directed expression systems available for transgenic plants (2) and mammalian cells (20).

A standard method for the ectopic expression of genes cloned in yeast makes use of the highly inducible, galactose-responsive promoter ele-

ment, $UAS_{GAL1,10}$, and its specific transcriptional activator, Gal4p (21). This method has two major drawbacks that limit its application. First, the induction requires a shift in carbon source from glucose (repressed) to galactose (induced), and cell metabolism subsequently converts from fermentative to largely respiratory. Second, the induction is all or nothing, although various modifications have been made to reduce either the magnitude of Gal4p induction (1,13) or the stability of the target gene product by coupling expression to degradation by the degron system (3).

Louvion et al. (11) developed, and have subsequently improved (15), a yeast gene expression system with β -estradiol as a gratuitous inducer of Gal4p-regulated genes by constructing a chimeric activator pHCA/GEV that

Figure 1. The structure of pGEV-HIS3. The GEV expression plasmids were constructed in pRS313, 314, and 315 vectors to yield pGEV-HIS3, pGEV-TRP1 and pGEV-LEU2, respectively. The GEV chimera contains the following domains: the N-terminal 93 amino acids (aa) of Gal4p, aa 282–576 of the human β -estradiol α -receptor, and the C-terminal 424–490 aa of VP16 (11). The numbers in parentheses indicate the positions of *FspI* sites in pGEV-HIS3, the sizes of the DNA fragments encoding the *MRP7* promoter, and the domains of the chimeric activator in base pairs.

Table 1. Effects of β -Estradiol and GEV Levels on Growth Rate and Target Gene Expression

Strain	β -Estradiol Concentration (μ M)	Generation Time (min)	β -Gal Units ^a	Terminal Culture Density ^b
YGY13, no plasmid ^c	0	162	ND ^d	2.45
	0.1	162	ND	2.47
	1.0	162	ND	2.46
YGY13, pHCA	0	186	5	2.54
	0.1	216	442	1.56
YGY13, pGEV-HIS3	0	186	0.2	2.47
	0.1	186	315	2.50

^a12-h induction.
^bA₆₀₀ after 24 h growth at 30°C.
^c0.004% histidine added to medium.
^dND, not determined.

contains the DNA binding and dimerization domain of Gal4p, the hormone binding domain of the human β -estradiol α -receptor, and the C-terminus of the herpes virus transcriptional activator VP16. In the original system, GEV is expressed constitutively from the high-

ly active *ADHI* promoter. However, the high-level expression of GEV results in an β -estradiol-independent, basal expression of a UAS_{GAL10}-*lacZ* target gene (11). Our strategy was to lower the expression of GEV to a level that prevents any basal expression of our target

Figure 2. Expression of the integrated UAS_{GAL10}-*lacZ* reporter in response to β -estradiol concentration. Cells were grown in the presence of β -estradiol at the indicated concentrations for 14 h (see Materials and Methods). YGY13, pGEV-HIS3 in SD (stippled bars) and SR (filled bars) media; YGY20, pGEV-HIS3 in SD medium (white bars). β -gal assays are reported as the average of two independent pGEV-HIS3 transformants in duplicate. The assay variability ranged from less than or equal to 1%–20%; error bars indicated greater than or equal to 1% variation.

Short Technical Reports

genes in the absence of hormone. We show that this β -estradiol-regulated expression system permits a wide range of dose-dependent target gene expression levels for physiological studies and a tightly regulated off state for depletion studies. In *gal4 Δ* strains, these features are independent of carbon source.

MATERIALS AND METHODS

Strains and Plasmids

E. coli strain DH5 α (*F* Φ 80*lacZ* Δ *M15* [*lacZYA-argF*] *U169 endA1 recA1 hsdR17 [rk-mk-]* *deoR thi1 supE44 λ -gyrA96 relA1*) was used for plasmid propagation. Strain YGY13 (*ade1 leu2-3, 2-112::UAS_{GAL10}-lacZ-LEU2 his3 Δ gal4 Δ ura3-52*) and the isogenic Gal4⁺ strain, YGY20, were constructed from Sc252a (*ade1 leu2-3, 2-112 ura3-52*) by the integration of the UAS_{GAL10}-*lacZ* reporter on pBM266 (20) and the sequential deletion of the coding sequences of *GAL4* and *HIS3* using pBM2436 (9) and pPC101 (14), re-

spectively. Plasmid pJPG11 encoding the mitochondria-targeted T7 RNA polymerase (RNAP), its UAS_{GAL1} regulation, and importation into mitochondria have been described elsewhere (16). Plasmid pHCA encodes the GEV activator transcribed from the *ADHI* promoter (11). Yeast transformations were performed as described by Elble (4).

Media and Culturing Conditions

Standard yeast media (18) were supplemented with carbon sources at 2%, and adenine, uracil, and required amino acids were supplemented at 0.004%. Precultures were grown from single colonies at 30°C. For dose-response experiments, cells were precultured in either rich glucose (YPD) or rich raffinose (YPR) for 18–30 h, then diluted to an A₆₀₀ less than or equal to 0.03 in minimal glucose (SD) or minimal raffinose (SR) plus β -estradiol at varying concentrations for 14 h. For depletion experiments, control strain YGY20 was precultured in YPGal, diluted to A₆₀₀ = 0.015 in SGal medium, and grown for 13 h to an A₆₀₀ = 0.5 before the shift to SD. YGY13, pGEV-HIS3 cells were precultured and diluted as described for the dose-response experiments, then grown with 100 nM β -estradiol (Sigma, St. Louis, MO, USA) in SD or SR for

13 h to an A₆₀₀ = 0.1–0.3. At the shift, cells were centrifuged at 4°C, washed with 10 mL cold hormone-free media, and suspended in either SD or SR media warmed to 30°C. β -estradiol was freshly dissolved and diluted in 95% ethanol for each experiment.

Plasmid Construction

Standard methods for DNA manipulations were used (17). The promoter including the transcriptional initiation sites for the mitochondrial ribosomal protein *MRP7* was isolated on a 1066-bp *Bgl*III-*Hind*III fragment from plasmid pKF5 (6). The GEV chimeric gene was isolated as a 1591-bp *Hind*III-*Not*I fragment from plasmid pHCA/GEV (11). These fragments were ligated into pRS313 (19) cleaved with *Bam*HI and *Not*I to create pGEV-HIS3 (Figure 1). The DNA sequence of the junction at the *MRP7* promoter and GEV cassette was determined. Plasmids pGEV-TRP1 and pGEV-LEU2 were constructed by ligating the 4264-bp *Fsp*I fragment from pGEV-HIS3 with *Fsp*I-cleaved vector sequences of pRS314 and pRS315, respectively.

β -Galactosidase Assays

β -galactosidase (β -gal) activity is

Figure 3. Expression of mitochondria-targeted T7 RNAP in response to β -estradiol concentration. Cultures were grown in SR media in the presence of β -estradiol at the indicated concentrations for 15 h, and β -gal activity was determined. Total cell lysates were electrophoresed on a 10% SDS-polyacrylamide gel. Immunoblots were prepared and probed as described in Materials and Methods. Lane 1, YCY13, pGEV-HIS3 containing YCp50 control; lanes 2–7, YCY13, pGEV-HIS3 with pJPG11 grown with the indicated β -estradiol concentrations. The lower band in lanes 1–3 is a cellular protein that contains an epitope weakly recognized by the polyclonal antibody against T7 RNAP; when high levels of T7 RNAP are present (lanes 4–7), this protein does not bind antibody. Degradation of the T7 RNAP is evident in lanes 6 and 7 and has been observed in cells that expressed high levels of T7 RNAP (16).

expressed in Miller units (12), and assays were performed exactly as described by Guarente (10) with SDS-chloroform permeabilized cells. X-Gal plates (10) were supplemented with 10 or 100 nM β -estradiol.

Immunoblot Analysis

Total yeast lysates were prepared by glass bead lysis (7). The proteins were separated by SDS-PAGE and transferred to nitrocellulose as described previously (16). Immunoblots were probed with a mouse polyclonal primary antibody against T7 RNAP diluted 1:1000 (16), a secondary rabbit anti-mouse antibody conjugated with horseradish peroxidase diluted 1:500, and the complexes were visualized by chemiluminescence as suggested by the supplier (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

RESULTS

Growth Characteristics of GEV-Bearing Strains

Growth arrest of yeast cells bearing pHCA cultured in the presence of β -estradiol has been shown to be caused by sequestration of essential transcription factors by the VP16 moiety of hormone-bound GEV (8). To demonstrate that neither hormone-bound GEV expressed from the pGEV-HIS3 construct nor β -estradiol at experimentally relevant concentrations inhibits cell growth, we compared the generation times and induction of the integrated UAS_{GAL10}-*lacZ* reporter in strain YGY13 bearing either pGEV-HIS3 or pHCA with and without β -estradiol (Table 1). Although 1 μ M hormone did not inhibit cell growth in the absence of either plasmid, the strain containing pHCA showed a

14% increase in generation time with 0.1 μ M hormone and a reduced cell density after 24 h compared to strains grown without hormone. It is significant that the level of GEV expressed from pGEV-HIS3 is sufficient to activate UAS_{GAL10}-*lacZ* expression to 70% of that induced by pHCA in the presence of 0.1 μ M β -estradiol, yet the hormone-bound GEV activator does not affect the generation time or terminal culture density. We conclude that the *MRP7* promoter provides GEV expression at a low level such that no hormone-independent GEV activity is detected, and in the presence of β -estradiol, there is ample activator for maximal induction without cell growth inhibition.

Carbon Source-Independent β -Estradiol Dose Response

The induction of an integrated UAS-

Short Technical Reports

GAL10-lacZ reporter as a function of hormone concentration was quantified in strains YGY13 (*gal4Δ*) grown in SD and SR media and YGY20 (*Gal4⁺*) in SD medium (Figure 2). A greater than 500-fold induction can be achieved after 14 h with 500 nM β -estradiol, and similar profiles are observed in either SD or SR media. This is the expected result from constitutive GEV expression from the *MRP7* promoter and confirms that *MRP7*-promoted GEV expression is sufficient for high-level target gene induction. YGY20 grown in SD medium has glucose-repressed, wild-type *Gal4* protein, and it retained undetectable target gene expression in the absence of hormone but showed only half the induction with 50–5000 nM β -estradiol. A second notable feature is the low to moderate expression of the reporter gene at 5–10 nM hormone. The level of target gene induction can be manipulated either with low (1–10 nM) concentrations of hormone over a 10–15 h growth period or by a short-term induction with higher (0.1–1 μ M) concentra-

tions of hormone (data not shown). Furthermore, we have monitored pGEV-HIS3-dependent *UAS_{GAL10}-lacZ* expression under hormone-free conditions for up to 30 h in both SD and SR and do not detect any β -gal activity, indicating that the stringency of the off state is independent of the growth phase of the culture. Finally, β -estradiol added to X-Gal plates induced the *UAS_{GAL10}-lacZ* reporter in proportion to the hormone concentration (data not shown).

The expression of a *UAS_{GAL1}*-regulated, mitochondria-targeted T7 RNAP as a function of β -estradiol concentration was tested in SR media that stimulates mitochondrial biogenesis (Figure 3), and the results are similar to those observed for *UAS_{GAL10}-lacZ* expression. The immunoblot of T7 RNAP shows first that there is no detectable T7 RNAP in the absence of hormone, but at 1 nM β -estradiol, a discernible level of T7 RNAP accumulates, although β -gal activity remains at or below the sensitivity of the assay. T7 RNAP accumulation with 5 and 10 nM

β -estradiol is approximately proportional to hormone concentration. The hormone concentration required to give detectable β -gal activity is five times greater than that required to yield detectable T7 RNAP. Thus, very low levels of a single polypeptide such as T7 RNAP can be achieved with low concentrations of hormone. However, the expression profile for a specific target gene must be determined empirically.

Depletion of β -Estradiol-Induced Target Genes

We compared the depletion profile of β -gal activity, a relatively stable enzyme, after hormone withdrawal to that after a shift from galactose to glucose in a *Gal4⁺* strain. Figure 4 shows the depletion profile of reporter activity after a 13-h induction with 100 nM β -estradiol in SD or SR media and the profile of the same reporter in the *Gal4⁺* strain YGY20 after a 13-h induction in SGal followed by a shift to SD medium. The depletion at 2 h post-shift in SR (58%) is nearly identical to that of the *Gal4⁺* control (57%), and by 4 h post-shift, the depletion in SD (76%) is slightly greater than that of the *Gal4p*-regulated system (69%). The most dramatic depletion profiles are observed when the hormone withdrawal is done while the induced cells are in early logarithmic growth.

DISCUSSION

This work extends and complements the previous characterization of the GEV chimeric activator by investigating the profiles of β -estradiol dependence in media containing glucose or the semi-fermentable carbon source, raffinose. Further, we characterized the expression of a mitochondria-targeted protein and show a depletion profile for a cytoplasmic β -gal. The advantages of GEV system for ectopic gene expression in *S. cerevisiae* are first, its tight carbon source-independent off state in a *gal4Δ* strain. Second, it can be combined with other commonly used inducible yeast promoters such as *MET25* (13) and *CUP1* (5) to study protein complexes. Third, it allows functional studies of gene products that are directly or indirectly regulated by carbon source and

Figure 4. β -estradiol depletion profiles of *UAS_{GAL10}-lacZ* expression. Cells were cultured as described in Materials and Methods with 100 nM β -estradiol for 13 h before the media shift, and samples were withdrawn every 2 h after the shift for β -gal assays. Plotted values are the average of two independent cultures. The assay variability ranged from less than or equal to 1%–20%; error bars indicate variability greater than or equal to 7%. YGY20, white bars; YGY13, pGEV-HIS3 grown in SD, stippled bars, and in SR, filled bars.

oxygen availability, such as mitochondrial components, cell wall and plasma membrane proteins, and stationary phase proteins. This system may be appropriate for ectopic expression of heterologous proteins that are lethal or that inhibit cell growth at high levels and has potential for ectopic gene expression in *Schizosaccharomyces pombe* or other yeast species. Most of these applications in *S. cerevisiae* require only a standard UAS_{GAL1,10} target gene construct in a *gal4Δ* strain and the introduction of one of the pGEV plasmids.

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