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Use of a restriction endonuclease cytotoxicity assay to identify inducible *GAL1* promoter variants with reduced basal activity

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Abstract

Inducible promoter fusions are commonly employed to study the biological functions of genes as well as to investigate mechanisms of transcription regulation. A concern for many studies of heterologous gene expression is that steady state transcription may be too high under non-inducing conditions, producing undesired phenotypes prior to induction. Fusions containing the galactose-inducible *GAL1* promoter joined to *Pvu*II, a bacterial DNA endonuclease gene, are toxic to yeast cells even under non-inducing conditions, i.e., in glucose media. This toxicity was utilized in conjunction with PCR-based mutagenesis of the *GAL1* regulatory region to isolate mutant promoters that retained high inducibility but exhibited reduced basal level expression. The Mig1 repressor binding and putative TATA box regions were unchanged among four mutant promoters examined in detail. However, each promoter contained one or more mutations within previously identified binding sites for the Gal4 activator protein. Genetic assays developed to monitor *GAL1p::I-SceI* endonuclease-induced recombination demonstrated that basal expression from two of the new promoters (designated *GAL1-V4* and *GAL1-V10*) was strongly reduced. These experiments and additional quantitative luciferase reporter gene assays demonstrate the utility of the approach for identifying promoters that permit more tightly controlled gene expression.

Keywords: Nuclease; Activator; Repressor; Expression vector

1. Introduction

Many gene promoters transcribed by RNA polymerase II exhibit variable expression in response to environmental stimuli. These promoters are typically subject to complex regulatory mechanisms involving the actions of activator proteins, repressors and interconnected processes such as nucleosome remodeling (Lohr et al., 1995; Kleinjan and van Heyningen, 2004; Smith and Peterson, 2005). Knowledge of such regulatory networks has led to the generation of promoter-containing expression vectors that permit regulated expression of genes in many organisms. Inducible promoters have proven particularly useful for heterologous gene expression as they typically exhibit low levels of transcription under normal cellular growth conditions, but can be induced to higher levels by changing the cellular growth environment or by addition of exogenous agents. An additional advantage of such systems is that they can often be used to investigate consequences of either underexpression or overexpression of cloned genes.

A variety of systems have been developed for conditional expression of genes in the budding yeast *Saccharomyces cerevisiae*. Many approaches have involved regulation of gene expression via media supplementation with exogenous chemicals such as tetracycline, copper or beta-estradiol (Louvion et al., 1993; Hottiger et al., 1995; Mascorro-Gallardo

Abbreviations: bp, base-pairs; nt, nucleotides; wt, wildtype.

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et al., 1996; Belli et al., 1998; Gao and Pinkham, 2000; Patel et al., 2003). Other methods have involved manipulation of organic nutrients, e.g., maltose or methionine, or have involved more exotic approaches such as elevation of protein expression using pulses of light or manipulation of mRNA translation (Yao et al., 1993; Mimran et al., 2000; Finley et al., 2002; Mao et al., 2002; Shimizu-Sato et al., 2002).

The most commonly used and widely studied promoters are *GAL1* and *GAL10*, whose activities can be induced by transferring cells to media containing galactose (e.g., Hovland et al., 1989; Porro et al., 1992; Mitchell et al., 1993; Foreman and Davis, 1994; Sil et al., 2000). *GAL1* is often employed for studies requiring high expression since its activity is increased up to three orders of magnitude when cells are switched from glucose to galactose media (Johnston and Davis, 1984; Hovland et al., 1989; Lohr et al., 1995; Li et al., 2000). An advantage of this system is that many of the components involved in regulation of transcription of *GAL1* and other *GAL* structural genes have been identified. For example, expression is positively regulated by the dual-domain transcriptional activator protein Gal4, which binds to four UAS (upstream activator sequence) sites in the *GAL1* promoter, in association with the SAGA coactivator complex (Lohr et al., 1995; Bhat and Murthy, 2001; Papamichos-Chronakis et al., 2002; Larschan and Winston, 2005). Negative regulation is accomplished through the actions of Gal80, an inhibitor of Gal4, and also by the DNA-binding Mig1 repressor protein acting in conjunction with the Cyc8–Tup1 complex (Reagan and Majors, 1998; Papamichos-Chronakis et al., 2002). Another advantage associated with the use of *GAL* promoters is that it is often possible to vary the level of induction by altering galactose concentration in the presence of another carbon source such as raffinose which, unlike glucose, does not inhibit induction (e.g., Hovland et al., 1989; Inga et al., 2002).

Although *GAL* promoter activity in haploid yeast cells is much lower in glucose media than in galactose (i.e., in 2% glucose versus 2% galactose), previous experiments have indicated that basal levels of expression of several cloned genes (including *YFH1*, homologue of the human frataxin gene, and the endonucleases *Pvu*II, *Eco*RV and I-*Sce*I) are high enough in glucose media to produce undesirable phenotypes (e.g., Plessis et al., 1992; Karthikeyan et al., 2003; and see below). These results are in contrast to past studies involving, for example, *GAL1* fusions to the *Eco*RI and HO endonucleases, which produce cohesive-ended double-strand breaks (DSBs) within chromosomal DNA. Basal level expression of *Eco*RI or HO in non-inducing media (2% glucose) does not greatly affect any phenotypes tested, though induction in galactose produces DSBs and elevated recombination (Herskowitz and Jensen, 1991; Lewis et al., 1998).

*Pvu*II is a type II bacterial restriction endonuclease that generates blunt-ended DSBs after cleavage at its recognition sequence CAG^CTG. In the current study we have taken advantage of the toxicity caused by low-level expression of *Pvu*II in yeast cells to identify mutant *GAL1** promoters that are strongly inducible but have reduced basal expression relative to the wildtype promoter.

2. Materials and methods

2.1. Strains, enzymes and laboratory techniques

The yeast strain VL6α (*MATα ura3-52 trp1-Δ63 lys2-801 his3-200 met14 ade2-101*) was used for transformation studies with *Pvu*II gene-containing plasmids. Yeast cells were propagated using rich or synthetic dropout media as described (Sherman, 1991) and yeast–*E. coli* shuttle plasmids were propagated in either DH5α or Top10 bacterial cells. Restriction endonuclease, T4 DNA ligase and Klenow fragment DNA polymerase enzymes were obtained from New England Biolabs. Shrimp alkaline phosphatase was purchased from USB Corporation. DNA sequencing was performed by using the ABI dRhodamine Terminator Cycle Sequencing Kit and an ABI Prism 377 DNA sequencer. Transformation of DNA into yeast cells was accomplished using a modification of the lithium acetate procedure (Gietz and Woods, 2002). PCR reactions were performed using TaqPlus Long (Stratagene Cloning Systems) unless specified otherwise.

2.2. PCR mutagenesis of the GAL1 regulatory region and isolation of promoter mutants

A centromeric plasmid containing the *GAL1* promoter fused to the *Pvu*II gene was created in two steps. The *Pvu*II gene was amplified by PCR using the *Pvu*II R and M gene plasmid pPvuRM3.4 (Blumenthal et al., 1985) with 5′ end primer PvuBam (GCTCGGATCCTCTCACTTAAAAATGAGTCACCCA-GATCTAAATAA) and 3′ end primer *Pvu*III (TGATTGAGCT-CACTTAGTAAATCTTTGTCCCATG) containing artificial *Bam*HI and *Sac*I (*Ecl*136II) sites, respectively (underlined). The 0.5 kb *Pvu*II gene PCR product was cut with *Bam*HI plus *Sac*I and cloned into vector pRS316Gal (Lewis et al., 1998) that had also been cut with *Bam*HI and *Sac*I. pRS316Gal contains a 0.7 kb *Eco*RI–*Bam*HI *GAL1/10* promoter fragment cloned into yeast vector pRS316 (*CEN/ARS URA3*; Sikorski and Hieter, 1989). The resulting plasmid, pJW6, contains the *Pvu*II gene under the control of the wt *GAL1* promoter, but contains two endogenous *Pvu*II recognition sites. In the second step, a new plasmid was created by removing the 1.2 kb *GAL1p::PvuII* cassette from pJW6 by cleavage with *Xho*I and *Ecl*136II and filling in the *Xho*I sticky ends using the Klenow fragment of DNA Polymerase I. This blunt-ended fragment was inserted into the cloning vector pRS316 that had been cut with *Pvu*II and *Nae*I. The resulting plasmid, pJW8 (*CEN/ARS URA3 GAL1p::PvuII*) contains no sites for cleavage by *Pvu*II. Sequencing confirmed that the *Pvu*II gene sequence was unaltered.

To generate *GAL1* promoter fragments containing multiple random mutations, the *GAL* regulatory region within pJW8 was amplified using primers that annealed just upstream and downstream of the *GAL1/10* promoter region (primers J8LysC and UVP6; sequences available upon request) using conventional Taq DNA polymerase (Roche, Indianapolis, IN) in the presence of 0.25 mM $MnCl_2$ as described (Muhlrad et al., 1992). The resulting mutagenized 0.7 kb *GAL1/10* promoter fragment retained the original *Eco*RI and *Bam*HI sites near each end. Concurrently,