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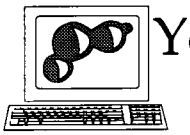

Yeast Functional Analysis Reports

A Set of Vectors with a Tetracycline-Regulatable Promoter System for Modulated Gene Expression in *Saccharomyces cerevisiae*

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A set of *Saccharomyces cerevisiae* expression vectors has been developed in which transcription is driven by a hybrid *tetO-CYC1* promoter through the action of a *tetR*-VP16 (tTA) activator. Expression from the promoter is regulated by tetracycline or derivatives. Various modalities of promoter and activator are used in order to achieve different levels of maximal expression. In the presence of antibiotic in the growth medium at concentrations that do not affect cell growth, expression from the *tetO* promoter is negligible, and upon antibiotic removal induction ratios of up to 1000-fold are observed with a *lacZ* reporter system. With the strongest system, overexpression levels comparable with those observed with *GAL1*-driven promoters are reached. For each particular promoter/tTA combination, expression can be modulated by changing the tetracycline concentration in the growth medium. These vectors may be useful for the study of the function of essential genes in yeast, as well as for phenotypic analysis of genes in overexpression conditions, without restrictions imposed by growth medium composition. © 1997 by John Wiley & Sons, Ltd.

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KEY WORDS — *Saccharomyces cerevisiae*; expression vectors; transcriptional activator; tetracycline-regulatable promoter; function analysis

INTRODUCTION

Yeast expression vectors based on regulatable promoters are currently used for homologous and heterologous protein expression, both in basic research and in biotechnological processes (Rine, 1991; Romanos *et al.*, 1992). In *Saccharomyces cerevisiae*, vectors based on the *GAL1–10* promoter are most commonly used (reviewed by Schneider and Guarente, 1991). Expression from the *GAL* promoter can be induced more than 1000-fold by galactose (Johnston and Carlson, 1992), which makes *GAL*-based vectors very useful for overexpression purposes. However, the fact that the *GAL* promoter is glucose-repressible makes it necessary to carry out induction experiments in the absence of glucose unless specific mutants in the glucose repression pathway are used (Johnston and Carlson, 1992; Ronne, 1995). The required changes in the carbon source may cause pleiotropic effects on cellular metabolism, making these vectors less useful for correlating specific phenotypes with changing levels of gene expression. Besides, modulation of gene expression

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by altering the galactose/glucose ratio in the growth medium is limited (Espinet *et al.*, 1995).

Alternative *S. cerevisiae* expression vectors based on other promoters have also been developed, such as the methionine-regulated *MET3* promoter (Mountain *et al.*, 1991), the phosphate-regulated *PHO5* promoter (Kramer *et al.*, 1984), or the copper-regulated *CUP1* promoter (Macreadie *et al.*, 1991; Hottiger *et al.*, 1994). Although these promoter-based vectors are useful for a range of applications, problems include relatively low induction ratios, drastic nutritional changes required for induction of expression, or possible coinduction of stress responses. An expression system has also been developed in which gene expression is induced up to 100-fold by glucocorticoid hormones in yeast cells expressing the mammalian glucocorticoid receptor (Picard *et al.*, 1990).

A tetracycline operator (*tetO*)-driven expression system has been developed in mammalian cells (Gossen and Bujard, 1992; Gossen *et al.*, 1993), based on the tTA transactivator [VP16 activator domain of herpes simplex virus fused to the tetracycline-inducible repressor (*tetR*) from the Tn10-encoded tetracycline-resistance operon]. In these conditions, genes under *tetO* control are expressed in the absence of tetracycline but not in the presence of the antibiotic, tight control of gene expression being attained in different types of cell lines (Yin *et al.*, 1996). The system has also been adapted for stable expression in transgenic plant cells (Weinmann *et al.*, 1994). More recently, a reverse system based on a tTA activator containing a mutant *tetR* moiety has been engineered (Gossen *et al.*, 1995) in which tetracycline induces *tetO*-driven gene expression. Based on the previous observation that the *tetR* repressor/*tetO* operator system is biologically active in *S. cerevisiae* (Dingermann *et al.*, 1992), we have adapted the original tTA-*tetO* system for yeast cells. The resulting cloning vectors allow modulation of expression of the cloned genes simply by changing tetracycline concentration in the growth medium without the need for metabolic changes. Thus, they are useful for the functional analysis of genes, especially for the study of the terminal phenotype of essential genes, besides allowing high gene overexpression levels.

MATERIALS AND METHODS

Strains

S. cerevisiae wild-type strain CML128 (*MAT***a** *leu2–3,112 ura3–52 trp1–1 his4 can1*^{r}) is a derivative of diploid strain 1788 (Lee and Levin, 1992). Strains CML133, CML177 and CML179 were obtained from CML128 by homologous integration of plasmids pCM87, pCM149 and pCM150 respectively, into the mutated *LEU2* locus of the chromosome. Plasmid DNA was linearized at the single *Eco*RV site internal to the *LEU2* marker. CML195 is a derivative of CML128 with the chromosomal *CLN1* gene deleted by *URA3* disruption and pCM87 inserted at the *LEU2* locus. Strain BMA64–1A (*MAT***a** *leu2–3,112 ura3–1 trp1*-Δ2 his3–11 ade2–1 can1–100) is a haploid segregant from W303. It was employed to obtain CML193 and CML194 by homologous integration of *Eco*RV-linearized pCM87 and pCM150 respectively, into the chromosomal *LEU2* locus. Integrations were checked by Southern blotting. *Escherichia coli* DH5α was employed as bacterial host for plasmids.

Growth and transformation conditions

SD minimal medium containing 2% glucose and the required auxotrophic supplements (Kaiser *et al.*, 1994) was employed for growth of yeast cells at 30°C. For measuring expression from tetracycline-regulatable promoters, cells were grown overnight in the presence of the antibiotic, then diluted into fresh medium and grown exponentially for at least eight generations (by culture dilution into fresh medium when required) under induction (minus antibiotic) or non-induction (plus antibiotic) conditions, before carrying out the expression assays. Tetracycline or derivatives (all of them purchased from Sigma) were added at 1 μg/ml, except when otherwise indicated, from 50% ethanol stock solutions at 5 mg/ml. Bacteria were grown in LB medium (Ausubel *et al.*, 1989) at 37°C. Yeast cells were transformed using the lithium acetate procedure (Gietz *et al.*, 1992). Bacteria were transformed by the standard calcium chloride method (Ausubel *et al.*, 1989).

DNA manipulations and analysis

Standard DNA manipulations were performed according to Ausubel *et al.* (1989). DNA fragments were isolated from agarose gels using Qiaex columns (Qiagen). Southern analyses were carried out with digoxigenin-labeled probes following the instructions provided by the manufacturer (Boehringer). DNA sequencing was done by standard methods (Sanger *et al.*, 1977) in order to check some of the constructions. A

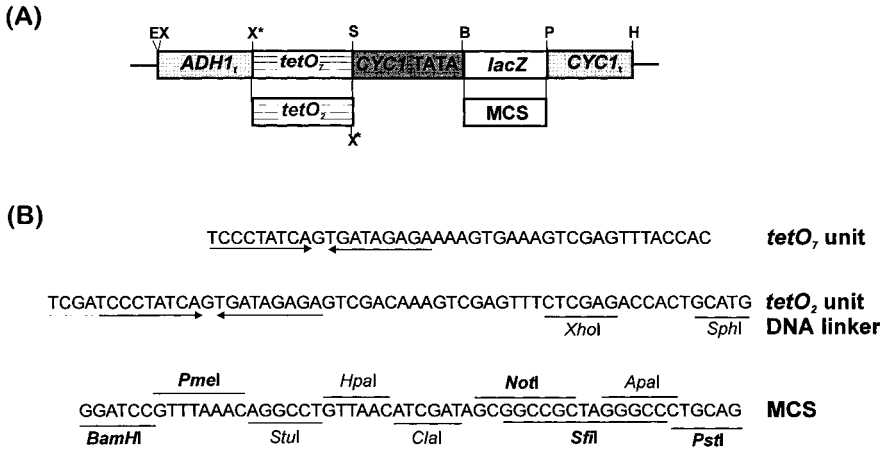

Figure 1. Schematic representation of the *tetO* promoter constructions. (A) Components of the regulatory region (not drawn to scale; see text for details). $ADH1_t$, *ADH1* terminator region; $tetO_2$ and $tetO_7$, promoter region formed respectively by two or seven *tetO* units in tandem (each *tetO* unit corresponds to the palindromic sequence TCCCTATCAGTGATAGAGA); *CYC1* TATA, TATA box from *CYC1*; $CYC1_t$, *CYC1* terminator region. Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sph*I; X, *Xho*I. X* denotes an inactivated *Xho*I site. (B) DNA sequences used in the construction of the *tetO* regulatory region (see Materials and Methods). Arrows underline the palindromic *tetO* unit interacting with each *tetR* repressor molecule. Discontinuous line in the $tetO_2$ unit DNA linker denotes the incomplete *Xho*I site used for subcloning. MCS, multiple cloning site region in the final vector constructions. Restriction sites in bold letters are unique in all the cloning vectors constructed in this work. Other sites in the MCS are unique in plasmids carrying the *TRP1* marker.

digoxigenin-labeled 1·2-kb *Pst*I-*Nco*I *CLN1* internal fragment (Hadwiger *et al*., 1989) was employed as a probe to study *CLN1* expression by Northern blot analysis (to be described elsewhere). Polymerase chain reaction (PCR)-based site-directed mutagenesis was performed as described by Weiner and Costa (1995).

Construction of plasmids harboring the tetO *promoter*

The basic structure of the cassette harboring the *tetO* promoter is shown in Figure 1A. Final plasmids are summarized in Table 1.

The initial parts of the cassette were assembled in the multiple cloning site (MCS) region of YCplac22 (Gietz and Sugino, 1988) to construct plasmid pCM145. The original *tetO* promoter is a 300 bp *Eco*RI-*Sma*I fragment from pUHC13–3 containing seven *tetO* boxes (Gossen and Bujard, 1992). Downstream of the *tetO* promoter, a 150 bp *Nde*I-*Sac*I fragment from pEMBLyex4 (Cesareni and Murray, 1987) containing the TATA and leader region of the *S. cerevisiae CYC1* gene was cloned in the *Sma*I site. The *E. coli lacZ* gene flanked by *Bam*HI restriction sites was obtained by PCR using primers 5′GTAC GGATCCATGACCATGATTACGGATT3′ and 5′GTACGGATCCTTATTATTTTTGACACCA GAC3′ (*Bam*HI sites underlined) and plasmid pRS551 (Simons *et al*., 1987) as template. The resulting 3·1-kb fragment was cloned in the *Bam*HI site of the MCS, and then the *Bam*HI site downstream from *lacZ* was inactivated by filling-in 5′ extensions with Klenow enzyme. The termination region of *CYC1* was obtained as a 260 bp *Pst*I-*Hin*dIII fragment by PCR, using primers 5′GATCCTGCAGGAGGGCCG CATCATGT3′ (*Pst*I site underlined) and 5′G ATCAGAAGCTTGGCCGCAAATTAAAGCC3′ (*Hin*dIII site underlined) and pYES2 plasmid DNA (Invitrogene) as template.

Plasmid derived from pCM145 with different numbers of *tetO* boxes in the promoter, either one (pCM140) or two (pCM142), were constructed with a DNA linker that included one *tetO* box (see Figure 1B). The 300 bp *Xho*I-*Sph*I fragment from pCM145 was substituted by the linker (pCM140). Sequence of the linker, with a new *Xho*I restriction site upstream of the *Sph*I restriction site, allowed addition of another box (pCM142).

Table 1. Yeast plasmids containing tetracycline-regulatable promoters constructed in this study.

Plasmid	Type of plasmid, genetic marker[a]	No. of *tetO* boxes[b]	MCS[c]	*lacZ*[d]	tTA[e]
pCM159	Centromeric, *TRP1*	7	−	+	−
pCM161	Centromeric, *TRP1*	2	−	+	−
pCM171	Centromeric, *TRP1*	2	−	+	+
pCM172	Centromeric, *TRP1*	2	−	+	+*
pCM173	Centromeric, *TRP1*	7	−	+	+
pCM174	Centromeric, *TRP1*	7	−	+	+*
pCM180	Centromeric, *TRP1*	7	+	−	−
pCM181	Centromeric, *TRP1*	2	+	−	−
pCM182	Centromeric, *TRP1*	2	+	−	+
pCM183	Centromeric, *TRP1*	2	+	−	+*
pCM184	Centromeric, *TRP1*	7	+	−	+
pCM185	Centromeric, *TRP1*	7	+	−	+*
pCM186	Centromeric, *URA3*	7	+	−	−
pCM187	Centromeric, *URA3*	2	+	−	−
pCM188	Centromeric, *URA3*	2	+	−	+
pCM189	Centromeric, *URA3*	7	+	−	+
pCM179	Episomal, *URA3*	7	−	+	+
pCM190	Episomal, *URA3*	7	+	−	+

[a]Plasmids (Gietz and Sugino, 1988) used as vectors are: YCplac22 (centromeric, *TRP1*), YCplac33 (centromeric, *URA3*) and YEplac195 (episomal, *URA3*).
[b]Number of *tetO* palindromic units (see Figure 1) present in the promoter.
[c]Multiple cloning site, described in Figure 1B. See also legend of Figure 1 for more details.
[d]From *E. coli*, cloned under the control of the *tetO* promoter instead of the MCS.
[e]−, No transactivator; +, tTA with a single VP16ad region immediatly after the *tetR* promoter-binding moiety; +*, *tetR* and VP16ad separated by a cI-λ spacer (see Figure 2).

Plasmids pCM154 and pCM144 derive from pCM145. In pCM154, a 450 bp *Eco*RI-*Bam*HI (see Figure 1A) has been substituted by a 700 bp *Eco*RI-*Bam*HI fragment from YCpGAL (a gift of Alan Boyd, University of Edinburgh) containing the *GAL1* promoter. In pCM144, the same *Eco*RI-*Bam*HI fragment has been replaced by a 460 bp *Eco*RI fragment from pUHC13–3 (Gossen and Bujard, 1992) with the initial *tetO* promoter (seven tetO boxes) and the human cytomegalovirus (CMV) TATA region. In plasmid pCM139, a 100 bp *Eco*RI-*Xho*I fragment from pCM142 was substituted by a 370 bp *Xba*I-*Xho*I fragment from pHAM8 containing the methionine-responsive elements of *MET3* (Mountain *et al.*, 1991).

A 200 bp *Asc*I-*Bgl*II fragment from pFA6a-lacZMT-kanMX3 with the termination region of the yeast *ADH1* gene (Wach *et al.*, 1994) was cloned in the *Eco*RI site upstream of the *tetO* promoter of plasmids pCM142 and pCM145. Then, the *Xho*I restriction sites in the *tetO* cassette (see Figure 1) were inactivated by filling-in the 5′ extension using Klenow, followed by cloning of a new *Xho*I restriction site [using a DNA linker (5′AATTCAGTGTTAGCTCACGATGT CATCTCGAGT3′, 5′AATTACTCGAGATGA CATCGTGAGCTAACACTG3′)] in the *Eco*RI site upstream of the *tetO* promoter, resulting in pCM159 (from pCM145) and pCM161 (from pCM142). These latter plasmids were used as recipients to clone the 1·7-kb and a 1·8-kb *Xho*I-*Eco*RI fragments from plasmids pCM168 and pCM169 respectively, containing the tetracycline transactivator genes, into the *Eco*RI-*Xho*I restriction sites of the cassette (resulting in plasmids pCM171 to 174). The episomal plasmid pCM179 was constructed by cloning a 5·7-kb *Eco*RI-*Hin*dIII fragment from pCM173 in YEplac195 (Gietz and Sugino, 1988). To obtain the desired cloning vectors, the *lacZ* gene was substituted by

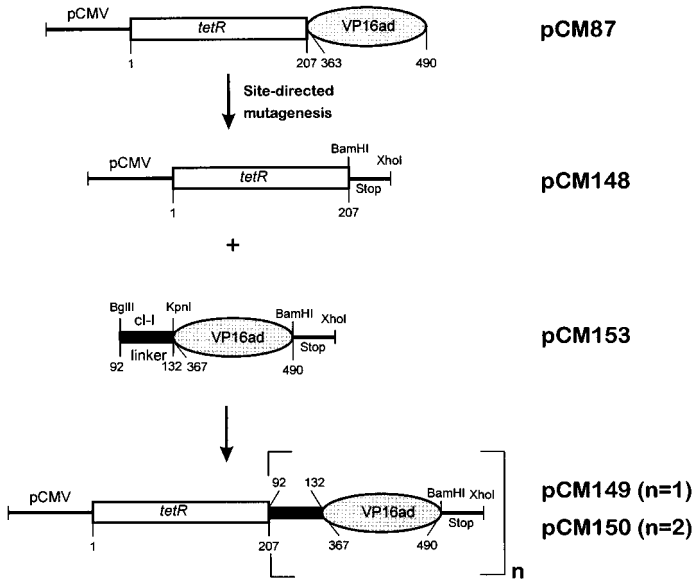

Figure 2. Scheme of the construction of the tTA transactivators used in this work. The original construction (in plasmid pCM87) contained the human cytomegalovirus promoter IE (pCMV) directing expression of the hybrid tTA activator gene consisting of *tetR* (tetracycline repressor) fused in frame to the activator domain of herpes simplex virus VP16 (VP16ad). From this basal tTA construction, derivatives were obtained in the indicated plasmids (see Materials and Methods for more details) in which a number (n) of spacer regions from the lambda bacteriophage cI gene (cI-λ) in frame to VP16ad were repeated in tandem fused to *tetR* as C-terminal extensions. Numbers correspond to the the amino acid residue in the original proteins.

an MCS DNA linker as a final step (Figure 1B). The plasmids thus resulting, as well as those with constructions placed in the YCplac33 vector (Gietz and Sugino, 1988), are described in Table 1.

Construction of the plasmids harboring the tTA gene

The integrative plasmid with the original tTA, pCM87, consists of a 1·79-kb *Xho*I-*Bam*HI fragment from pUHD15–1 (Gossen and Bujard, 1992) containing the human CMV promoter and the entire coding region for tTA cloned into the *Sal*I-*Bam*HI restriction sites of YIplac128 (Gietz and Sugino, 1988). The procedure to construct the pCM87 derivatives with either one or two VP16 activation domains separated from each other by the bacteriophage lambda cI repressor linker region (Ohashi *et al*., 1994) is depicted in Figure 2. Plasmid pCM148 was constructed by substitution of the pCM87 VP16 transactivation domain by a DNA region with the *Bam*HI and *Xho*I restriction sites flanking two stop codons, by PCR-based site-directed mutagenesis using primers 5′GAG CTACTAGGATCCACTTTCACATTTAAG3′ and 5′GAGGTACCGAGCTCGAATTCAC3′.

The cI linker region was isolated by PCR from lambda DNA, using primers 5′CGATGAATT CAGATCTGTTAGTATGCAGCCGTCA3′ and 5′CGATGGTACCGCTTACCCATCTCTCCG3′, which introduced *Eco*RI and *Bgl*II sites at the 5′ end, and a *Kpn*I site at the 3′ end. Also, the VP16 transactivator domain was isolated by PCR from pUHD15–1. Primers used were 5′GTTGGGT ACCGCGCGTACGAAAAACAAT3′ and 5′G TTGTCTAGACTCGAGCTACTAGGATCCCC CACCGTACTCGTCAAT3′, introducing a *Kpn*I restriction site in the 5′ end and three other restriction sites, *Xba*I, *Xho*I and *Bam*HI, at the 3′ end. In order to obtain plasmid pCM153, the 120 bp *Eco*RI-*Kpn*I fragment containing the lambda cI linker region and the 385 bp *Kpn*I-*Xba*I fragment with the VP16 transactivator domain were cloned in YCplac22. Plasmids pCM149 and pCM150 were constructed by cloning the 507 bp *Bgl*II-*Xho*I

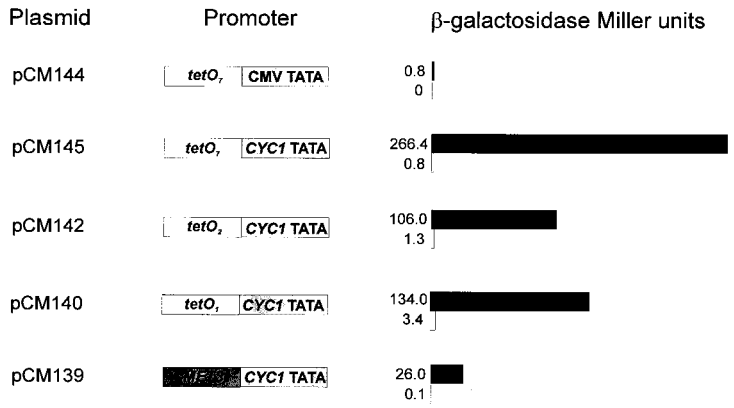

Figure 3. β-Galactosidase activities in the absence (black boxes) or presence of 1 μg/ml doxycycline (white boxes) from exponential cultures of strain CML133 transformed with the respective plasmids containing the indicated promoter constructions. For the *MET3* promoter plasmid transformants, methionine (300 μg/ml) was added instead of doxycycline to achieve repression conditions. CMV TATA derives from the human cytomegalovirus IE promoter. Numbers denote enzyme activity values. Results are from a representative experiment.

fragment from pCM153 in the *Bam*HI-*Xho*I restriction sites from pCM148 and pCM149, respectively.

Plasmids pCM168 and pCM169, harboring the original tTA and the tTA with the cI lambda linker region (from pCM149) respectively, are derivates of pUHD15–1. These plasmids have the *Eco*RI restriction site inactivated by filling-in 5′ extension with Klenow. Also, they have a linker (5′GATCG TAATAAGAATTCTTATTAC3′) inserted in the *Bam*HI restriction site, which inactivates this site and introduces two stop codons and a new *Eco*RI site.

Determination of β-galactosidase activity

β-Galactosidase activity was determined (as Miller units) in whole yeast cells from exponential cultures (Zhang *et al.*, 1989).

RESULTS AND DISCUSSION

*Expression of tetracycline operator (*tetO*)-based promoters*

To test expression driven by *tetO*, the *lacZ* gene, under the control of different *tetO* promoters, was used as a reporter system. Plasmids with the *tetO-lacZ* constructions were transformed into strain CML133, which contains the original tTA transactivator gene (Gossen and Bujard, 1992) inserted into the *LEU2* locus. No expression was seen with the promoter consisting of seven *tetO* boxes ($tetO_7$) and the human CMV TATA region (Figure 3). Since the latter might be non-functional in yeast, it was substituted by the *S. cerevisiae CYC1* TATA region. Expression of *lacZ* from the resulting construction was regulated by doxycycline (a tetracycline derivative; Degenkolb *et al.*, 1991), with a 200-fold induction factor (Figure 3). In the presence of the antibiotic, expression turned off to almost negligible levels. Maximum levels of expression were 10-fold higher than with the *MET3*-based promoter.

In order to characterize better the *tetO* system, we studied the effect of changing the number of *tetO* boxes. The construction with two boxes ($tetO_2$) gave the same number of enzyme units as the one-box construction ($tetO_1$), and in both cases the values without doxycycline were only two-fold lower compared with $tetO_7$ (Figure 3). These results indicate that there is not a properly additive effect when increasing the number of *tetO* boxes, maybe due to low levels of tTA (whose expression is controlled by a CMV promoter) in the cell. Expression driven by $tetO_1$ and $tetO_2$ also turned off efficiently in the presence of doxycycline.

tTA derivates improve expression of the tetO *promoter*

Ohashi *et al.* (1994) have described that the transcriptional activation protein composed by the

Table 2. Effect of the tTA transactivator structure on expression of a *tetO-lacZ* reporter system.

tTA characteristics	β-Galactosidase activity (Miller units)			
	$tetO_2$ promoter		$tetO_7$ promoter	
	− Dox	+ Dox	− Dox	+ Dox
No tTA	0·3	ND	0·2	ND
No spacer (tTA)	150	0·4	323	0·5
One spacer-VP16ad unit (tTA*)	315	0·5	515	1
Two spacer-VP16ad units (tTA**)	516	0·3	629	0·1

Strains CML128 (without tTA activator), CML133 (containing a chromosomally-integrated construction coding for a tTA without cI spacer), CML177 (tTA with one spacer between *tetR* and VP16ad) and CML179 (*tetR* fused to two cI spacer-VP16ad units in tandem) (see Figure 2 for details on the tTA structure) were transformed with plasmids pCM161 (*lacZ* expression driven by $tetO_2$) or pCM159 (*lacZ* expression driven by $tetO_7$). Numbers indicate enzyme activity in exponential cultures grown in the absence or presence of doxycycline (Dox) at 1 μg/ml. ND, not determined.

Gal4p DNA binding domain and the VP16 activation domain can increase the transcriptional potency *in vitro*, either by the multimerization of the VP16 activation domain or by the addition of the lambda cI linker region between the VP16 activation domain and the Gal4p binding domain. We studied whether the same effect occurred with the tTA activator, which, in its basic form, is composed of a *tetR* DNA binding domain and a VP16 activation domain. If this were the case, the transcriptional potency of the Tet system could be improved without increasing the amount of the tTA protein in the cell and, thus, expression still could be turned off with the same efficiency as described above using low amounts of antibiotic. In this study, the $tetO_2$ and $tetO_7$ promoters, which give the lowest expression levels under non-inducing conditions (Figure 3), were used. To avoid possible readthrough from plasmid sequences into the *tetO* promoters, the *S. cerevisiae ADH1* transcription terminator sequence was placed upstream of the promoters, resulting in plasmids pCM161 ($tetO_2$) and pCM159 ($tetO_7$).

Strains CML177 (containing a tTA with the lambda cI linker region; described as tTA* in Tables 2 and 3) and CML179 (containing a tTA with the lambda cI linker region-VP16 domain duplicated in tandem and fused to the *tetR* binding domain − tTA**) were constructed (see Materials and Methods) and transformed with plasmids pCM159 and pCM161. β-Galactosidase levels were determined from cultures growing either in the presence or absence of 1 μg/ml of doxycycline (Table 2). Comparison with strain CML133 (harboring the original tTA without cI linker) indicated that the lambda cI linker region in the tTA was sufficient to increase two-fold the expression levels irrespective of the *tetO* promoter used. Expression was further raised using the tTA with the cI linker region-VP16 activation domain duplicated in tandem. The increase was slight with the $tetO_7$ promoter, probably reflecting a situation where the tTA-mediated activation is saturating. These *in vivo* data agree with the previous *in vitro* results (Ohashi *et al.*, 1994), indicating that the multimerization of the VP16 activation domain and the increased flexibility between the domains are able to raise the transcriptional activation potency. In all cases, the system was efficiently switched off to less than one Miller unit, and the expression levels obtained under inducing conditions could be adequate for overexpression experiments.

Construction of plasmids harboring the tetO *promoter and the tTA transactivator*

In order to improve the Tet system, plasmids harboring the *tetO* promoter and the tTA transactivator together were constructed. Plasmids pCM161 and pCM159 were the basis to clone two versions of the transactivator [tTA (no cI spacer) and tTA* (*tetR* and VP16 separated by a single cI spacer)] upstream of the *ADH1* terminator sequence (see Materials and Methods). The plasmids obtained (pCM171 to 174, Table 1) contain *lacZ*

Table 3. Effect of different *tetO* and tTA combinations on expression levels and regulation of the *lacZ* reporter system.

Plasmid	Strain	Configuration of *tetO* in plasmid	Configuration of tTA in		β-Galactosidase activity (Miller units)	
			plasmid	chromosome	+ doxycycline	− doxycycline
None	BMA64–1A	—	—	—	ND	<0·1
pCM161	BMA64–1A	$tetO_2$	—	—	0·2	0·2
pCM161	CML193	$tetO_2$	—	tTA	0·3	161
pCM161	CML194	$tetO_2$	—	tTA**	0·7	620
pCM159	BMA64–1A	$tetO_7$	—	—	0·2	0·2
pCM159	CML193	$tetO_7$	—	tTA	0·3	398
pCM159	CML194	$tetO_7$	—	tTA**	0·3	655
pCM171	BMA64–1A	$tetO_2$	tTA	—	0·3	202
pCM172	BMA64–1A	$tetO_2$	tTA*	—	0·3	281
pCM173	BMA64–1A	$tetO_7$	tTA	—	0·4	357
pCM174	BMA64–1A	$tetO_7$	tTA*	—	0·8	417
pCM179	BMA64–1A	$tetO_7$	tTA	—	25 (0·3)	998

Numbers indicate the enzyme activity in exponential cultures of the indicated strains transformed with the corresponding plasmids, grown in the presence or absence of doxycycline at 1 μg/ml (value in parentheses for pCM179 corresponds to enzyme activity in cultures with doxycycline at 5 μg/ml). The characteristics of the *tetO* promoter/tTA activator system are indicated for each plasmid. — denotes the absence of transactivator. tTA, tTA* and tTA** correspond respectively to transactivators without cI linker, with a single cI linker between the *tetR* and VP16ad moieties, or with two cI spacer-VP16ad units in tandem fused to the *tetR* moiety. Values are the mean of at least two experiments. ND, not determined.

as reporter gene, and were transformed into the BMA64–1A strain (a standard strain used in the project for the systematic functional analysis of yeast genes) and the β-galactosidase levels were determined from the cultures growing either in the presence or absence of 1 μg/ml of doxycycline. Assays were also performed with cell cultures in which plasmids pCM161 and pCM159 were in the BMA64–1A-derivative strains containing the tTA genes integrated at the *LEU2* locus. Results are shown in Table 3.

The levels of expression and the regulation pattern in the strain BMA64–1A background were essentially the same as in strain CML128 (compare data of Tables 2 and 3). The $tetO_2$ and $tetO_7$ promoters produced higher β-galactosidase levels in strain CML194, containing a tTA with the lambda cI linker region-VP16 domain duplicated in tandem, compared with strain CML193 containing the original tTA activator.

Plasmids harboring together the original tTA and the $tetO_2$ or $tetO_7$ promoters (pCM171 and pCM173 respectively) transformed in the BMA64–1A strain gave the same β-galactosidase levels as plasmids pCM161 ($tetO_2$, no transactivator) or pCM159 ($tetO_7$, no transactivator) transformed in strain CML193 with the tTA gene chromosomally integrated. Turning off expression was efficiently achieved with 1 μg/ml doxycycline (Table 3).

We intended to achieve maximum expression levels in strain BMA64–1A under induction conditions by constructing plasmid pCM174 (haboring the $tetO_7$ promoter and the tTA* activator gene). Expression levels in the pCM174 transformants were somewhat lower than expected (Table 3), considering the levels reached in cells of the same genetic background where the $tetO_7$ promoter and the tTA gene (in this case coding for an activator with two cI-VP16 domains in tandem) reside in the

plasmid and chromosome respectively (CML194 cells transformed with pCM159; Table 3).

Expression could be further increased in BMA64–1A cells transformed with an episomal plasmid (pCM179) harboring the $tetO_7$ promoter and the tTA activator (Table 3). However, doxycycline at 1 μg/ml could not completely eliminate *lacZ* expression and in these conditions, significant expression from the $tetO_7$ promoter remained. Higher concentrations of the antibiotic reduced basal expression and at 5 μg/ml, this was comparable to that observed with centromeric plasmids at 1 μg/ml. The highest expression levels attained with the pCM179 plasmid are comparable to those obtained with a *GAL1–10* promoter (pCM154). In fact, a *lacZ* reporter system under the later promoter in BMA64–1A cells gave 1201 Miller units under induction conditions (galactose medium) versus 0·1 units under repression conditions (glucose medium).

A slight reduction of the growth rate was seen under induction conditions in strains containing the tTA version with the two cI-VP16 activation domains integrated in tandem at the *LEU2* locus (CML179 and CML194), as well as in strains harboring the episomal plasmid pCM179 with the basal tTA activator (data not shown). This effect was not observed with the tTA constructions in centromeric plasmids here described, or with the chromosomally integrated tTA and tTA* versions. The negative effect on growth rate also occurred in cells transformed with the episomal plasmid pCM190 (not producing β-galactosidase), and it was suppressed by the addition of doxycycline (not shown). Since the *tetR* moiety alone does not have any deleterious effect (Dingermann *et al*., 1992), these results suggest that the basal VP16 activator in high amounts, or the derivative activator with two VP16 moieties in tandem, may have some negative effect on the transcriptional machinery of the cell.

Derivatives of the above vectors were constructed where the *lacZ* reporter gene is replaced by an MCS region (Figure 1). These plasmids (carrying *TRP1* or *URA3* as markers) are described in Table 1, and they can be used for regulated expression of foreign genes cloned in the MCS.

tTA inactivation by tetracycline

Several antibiotics of the tetracycline family (tetracycline, doxycycline, oxytetracycline and chlortetracycline) were examined for their ability to inactivate the tTA function in yeast cells. Doxycycline (up to 2 μg/ml in the growth medium) was the most effective in switching off the expression of the *lacZ* reporter system under different *tetO* promoters. This result agrees with the fact that doxycycline shows the highest association equilibrium constant to the Tet repressor in comparison with the other derivatives tested (Degenkolb *et al*., 1991).

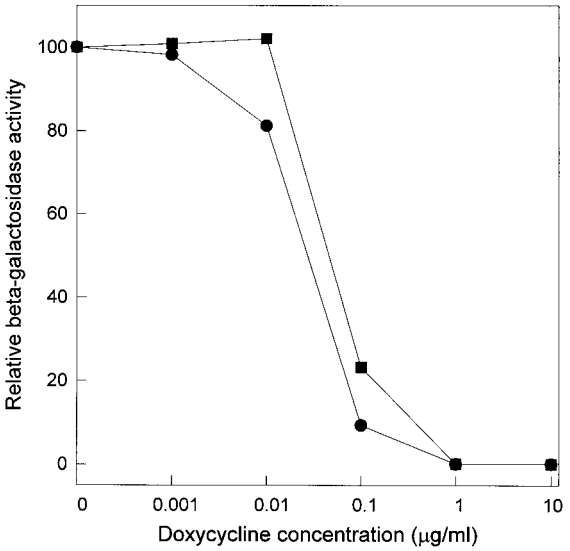

Figure 4. Relative β-galactosidase activity (Miller units) in exponential cultures of BMA64–1A cells transformed with plasmid pCM171 (circles) or pCM173 (squares), growing in the presence of different amounts of doxycycline. One hundred per cent activity corresponds to 205 and 454 Miller units respectively for pCM171 and pCM173.

The dependence of the tTA function on antibiotic concentrations was tested in the BMA64–1A strain transformed with plasmids pCM171 ($tetO_2$, tTA) and pCM173 ($tetO_7$, tTA). Overnight cultures growing with 1 μg/ml of doxycycline were diluted in the same medium with different concentrations of doxycycline and after eight or nine generations, the β-galactosidase levels were determined (Figure 4). Maximal inactivation of the tTA was reached at 1 μg/ml of doxycycline, while concentrations below 1 ng/ml were ineffective to switch off *tetO* promoter expression. Between 1 ng/ml and 1 μg/ml, expression of the reporter gene followed a sigmoidal curve, indicating that the *tetO*-tTA system can be efficiently modulated by modifying the concentration of the antibiotic. We

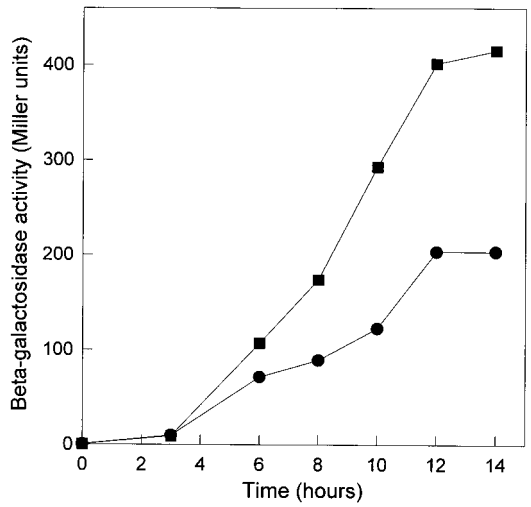

Figure 5. Kinetics of induction of β-galactosidase activity in a *tetO-lacZ* reporter system. BMA64–1A cells transformed with pCM171 (circles) or pCM173 (squares) growing exponentially in the presence of doxycycline (1 μg/ml) were deprived of the antibiotic (time 0) and enzyme activity (Miller units) was subsequently measured at the indicated times. Cultures were diluted into fresh medium when needed to maintain cells growing exponentially.

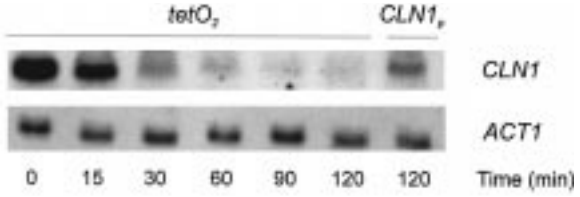

Figure 6. Northern analysis of repression of *tetO*-driven *CLN1* expression. Exponential cultures of CML195 transformed with plasmid pCM124 (which contains *CLN1* under the $tetO_2$ promoter) grown in the absence of tetracycline were added with the antibiotic at 1 μg/ml (time 0), and samples were taken at the indicated times to determine *CLN1* expression. This was also determined in exponential cultures of CML195 cells transformed with plasmid pCM108 [containing *CLN1* under its own promoter ($CLN1_p$) cloned in YCplac22 (Gietz and Sugino, 1988; to be described elsewhere)] after 120 min of tetracycline addition. Northern analysis of *ACT1* was carried out in parallel.

have confirmed this fact by being able to modulate levels of the Cln3 *S. cerevisiae* G_1 cyclin in a 50-fold range between the same range of doxycycline concentrations (data not shown). The range of antibiotic concentrations allowing modulation of gene expression and its sigmoidal pattern are similar to those described in mammalian systems (Gossen *et al.*, 1995; Yin *et al.*, 1996). Moreover, we tested the doxycycline effects on growth of yeast cells and, at the concentrations used (up to 10 μg/ml in the growth medium), no changes in growth rate were detected (data not shown).

Kinetics of tetO-*tTA-driven expression*

The analysis of induction kinetics was carried out in strain BMA64–1A transformed with pCM171 ($tetO_2$, tTA) and pCM173 ($tetO_7$, tTA). Cultures growing under non-induction conditions with 1 μg/ml of doxycycline were washed and diluted in the same medium without doxycycline. Samples were taken at intervals to determine β-galactosidase activity (Figure 5). Accumulation of β-galactosidase began to increase at 3 h, although maximal levels were attained 12 h after doxycycline elimination.

To study the time required to switch off expression of the *tetO* promoters after tetracycline-mediated tTA inactivation, a Northern analysis of *CLN1* expression after antibiotic addition was carried out. Cultures of the CML195 strain (deleted in the *CLN1* chromosomal locus and containing a chromosomally-integrated tTA gene) transformed with pCM124 ($tetO_2$-*CLN1*) or pCM108 (*CLN1* under its own promoter) were used for this purpose. The $tetO_2$-driven expression was turned off almost completely 30 min after tetracycline addition, whereas the *CLN1* promoter-driven expression maintained physiological levels of mRNA after 2 h under the same conditions (Figure 6). These results show that tetracycline molecules are rapidly transported into the nucleus in order to completely inhibit tTA activity.

Conclusions

The *tetO*-tTA expression system as developed for yeast cells is comparable to other regulatable expression systems such as that based on the *GAL* promoter, with respect to the induction ratio and the basal levels under non-inducing conditions. Besides its modulability, it does not require any changes in nutrient composition of the growth medium to induce expression of the regulated genes. Although vectors could still be improved to attain higher overexpression levels, the already constructed versions can be used to study phenotypic effects of gene overexpression. On the other hand, by choosing *tetO*-tTA

combinations expressing physiological levels of the desired gene product, it is possible to engineer the conditional expression of essential genes in haploid cells carrying a disruption in the chromosomal locus, as a way of studying the terminal phenotype of the mutant cells after tetracycline addition. Alternatively, product levels can be modulated with appropriate tetracycline concentrations. Thus, these vectors may be useful for the functional analysis of yeast genes.

Vectors described in Table 1 are deposited at EUROSCARF (Institute of Microbiology, University of Frankfurt) for distribution among the scientific community.

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