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An activator/repressor dual system allows tight tetracycline-regulated gene expression in budding yeast

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ABSTRACT

We have developed an activator/repressor expression system for budding yeast in which tetracyclines control in opposite ways the ability of *tetR*-based activator and repressor molecules to bind *tetO* promoters. This combination allows tight expression of *tetO*-driven genes, both in a direct (tetracycline-repressible) and reverse (tetracycline-inducible) dual system. Ssn6 and Tup1, that are components of a general repressor complex in yeast, have been tested for their repressing properties in the dual system, using *lacZ* and *CLN2* as reporter genes. Ssn6 gives better results and allows complete switching-off of the regulated genes, although increasing the levels of the Tup1-based repressor by expressing it from a stronger promoter improves repressing efficiency of the latter. Effector-mediated shifts between expression and non-expression conditions are rapid. The dual system here described may be useful for the functional analysis of essential genes whose conditional expression can be tightly controlled by tetracyclines.

INTRODUCTION

Complete sequencing of the *Saccharomyces cerevisiae* genome has revealed the existence of a large number of genes without known function that had evaded previous strategies of study based on the gene function to structure approach (1). About one fifth of these so called orphan genes may be essential for cell growth (2). Systematic analysis of the function of yeast genes requires a number of independent approaches, some of which involve the development of new genetic tools (3). The use of vectors allowing conditional expression of genes is one of these approaches, either for the study of terminal phenotypes in conditions where the gene is not expressed and for the analysis of the effect of gene overexpression on cell physiology.

Recently, we have constructed a set of Tet vectors for tetracycline-regulated conditional expression of genes in *S.cerevisiae* (4), adapted from a previous system developed for mammalian cells (5). The yeast Tet vectors allow modulation of the expression levels of genes cloned under the control of the bacterial Tn10 transposon-derived tetracycline-responsive *tetO* promoter, through the action of a *tetR*-VP16 (tTA) hybrid transactivator. The *tetR* moiety of tTA (from Tn10 as well) is responsible of *tetO* recognition, while VP16 (from herpes simplex virus) is the activator moiety. With this direct Tet system, *tetO*-driven expression occurs in the absence of the effector [tetracycline or other molecules of the same antibiotic family, (4)], while addition of the latter inhibits the tTA activator and swittches off gene expression. We have shown that different levels of expression are achieved depending on the number of *tetO* boxes in the promoter [two ($tetO_2$) or seven ($tetO_7$) boxes]. Compared to other yeast expression vectors (reviewed in 6), gene expression from the Tet vectors does not involve changes in growth medium composition that might cause undesired pleiotropic effects on cell metabolism when carrying out gene function studies. In mammalian cells, a tetracycline-inducible reverse system has also been developed which allows rapid induction of *tetO*-driven gene expression by tetracyclines (7). It is based on a modified tTA molecule (here named tTA′) containing a mutated *tetR* moiety (*tetR*′) that is activated through binding of the antibiotic effector molecule.

Given its possible interest as a tool for the systematic functional analysis of yeast genes, in this work we describe the adaptation of the mammalian Tet reverse system for yeast cells. In order to achieve a tighter regulation of expression (that is, a higher ratio of expression between induced and non-induced conditions), we have also developed a dual system in which a tetracycline-inactivable tTA activator and a tetracycline-activable *tetR*′-Ssn6/Tup1 repressor co-exist in the same cells. The alternative combination (tTA′ activator plus *tetR*-Ssn6/Tup1 repressor) has also been developed. The repressor molecules contain a Ssn6 or Tup1 moiety (actually the repressor moiety) fused in frame to *tetR* or *tetR*′ (acting as the *tetO*-binding domain). In *S.cerevisiae*, complexes of Ssn6 plus Tup1 act as general co-repressors of a wide number of genes including cell-type specific, glucose-repressed, oxygen-repressed or sporulation specific genes (8–11). In these complexes, Tup1 appears to be the active repressor by affecting nucleosome positioning through its interaction with histones H3 and H4 (12), although the Tup1 repression function may also involve direct contact with the RNA polymerase II holoenzyme (13,14). Ssn6 would act as an adaptor between the variable DNA-binding subunit (that determines promoter specifity) of the repressor complex and Tup1 (15,16). Although there are discrepancies from several studies about the size of the Ssn6–Tup1 complexes

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in vitro, there is common agreement that the complexes contain several Tup1 subunits for each Ssn6 subunit (17,18). By employing *tetR* (or *tetR′*) as DNA-binding domain fused to Ssn6 or Tup1, we demonstrate that the tetracycline-regulated activator/repressor dual system allows tightly regulatable expression, with almost undetectable basal levels both when the direct or the reverse tTA activators are tested.

MATERIALS AND METHODS

Strains, media and growth conditions

Yeast strains employed in this work (Table 1) derive from *S.cerevisiae* BMA64-1A (19), which is a derivative from the commonly employed W303 strain. Cells were grown at 30°C in SD minimal medium plus 2% glucose and the required amino acids (20). Plasmid transformants were grown in selective conditions depending on the respective auxotrophic requirements. For induction of *GAL1* promoter-driven gene expression, cells were pre-grown in SD medium plus 2% raffinose for at least 10 generations, and induction was achieved by addition of 2% galactose to exponential cells. Induction of *tetO* promoter-driven expression by tetracycline or derivatives was carried out as described in (4). Antibiotic concentration is indicated for each experiment. *Escherichia coli* DH5α was employed as bacterial host for plasmids.

Table 1. Yeast strains

Strain[a]	Additional genotype	Integrative plasmid[b]
CML238	CMV_p(*tetR*)::*LEU2*	pCM218
CML239	CMV_p(*tetR*)::*LEU2*	pCM148
CML240	CMV_p(*tetR-SSN6*)::*LEU2*	pCM244
CML241	CMV_p(*tetR-TUP1*)::*LEU2*	pCM243
CML282	CMV_p(*tetR-SSN6*)::*LEU2*	pCM242
CML283	CMV_p(*tetR-TUP1*)::*LEU2*	pCM240
CML288	$adh1_p$(*tetR-SSN6*)::*LEU2*	pCM245
CML289	$adh1_p$(*tetR-TUP1*)::*LEU2*	pCM246
CML300	$adh1_p$(*tetR-SSN6*)::*LEU2*	pCM247
CML301	$adh1_p$(*tetR-TUP1*)::*LEU2*	pCM248

[a]Strains are derived from *S.cerevisiae* BMA64-1A (MAT**a** *ura3-1 ade2-1 leu2-3,112 his3-11,15 trp1-Δ2 can1-100*) by integration of the corresponding *Eco*RV-linearized plasmid at the chromosomal mutated *LEU2* locus.

[b]Integrative plasmids were constructed as indicated in Materials and Methods. They contain constructions with the *tetO* binding moiety-repressor chimeric protein expressed from the CMV or *adh1* promoters. See the text for details on nomenclature.

Plasmid construction

Plasmids pCM175 and pCM176 are centromeric vectors (*TRP1* as genetic marker for yeast) containing *lacZ* as reporter gene under the control of the $tetO_2$ and $tetO_7$ promoters, respectively, as well as the reverse tTA (tetracycline-inducible) transactivator gene. To construct them, a 1.7 kb *Eco*RI–*Xho*I fragment from pUGH17-1 (7) with the *tetR′* mutated moiety fused in frame to the VP16 activator was integrated respectively in plasmids pCM161 and pCM159 (4). By substitution of *lacZ* for the MCS described in (4), we obtained plasmids pCM251 ($tetO_2$) and pCM252 ($tetO_7$); these are centromeric plasmids with the reverse tTA system suitable for gene cloning using the restriction sites of the MCS region.

Plasmid pCM148 (4) is a derivative of the integrative plasmid YIplac128 (21), with the *tetR* gene under the control of the cytomegalovirus promoter (CMV_p). Plasmid pCM218 was constructed from pCM148, and contains the mutated *tetR′* moiety from pUGH17-1 instead of the wild-type one. By linking the lambda cI spacer (4) to the C-terminal ends of *tetR* or *tetR′* from pCM148 or pCM218, plasmids pCM217 and pCM223 resulted, respectively. The latter four plasmids were the basis for additional plasmid constructions with the Ssn6 or Tup1 repressor moieties fused in frame to *tetR* or *tetR′*. Characteristics of these derivatives are summarized in Table 1. The *SSN6* and *TUP1* regions were isolated from yeast genomic DNA (strain BMA64-1A) by PCR with the Expand Plus System of Boehringer (using the optimal conditions specified by the purchaser) and <20 amplification cycles. For *SSN6*, the following oligonucleotides were employed: 5′-AGGA**AGATCTATG**AATCCGGGCGGTGAAC-3′ (a cloning *Bgl*II site and the *SSN6* initiation codon are shown in bold letters) and 5′-TCCG**CTCGAG**GTAGATACACAATGAAGGAT-3′ (in bold letters is a *Xho*I site used for cloning). The use of the amplified fragment results in an in-frame fusion from the first codon of *SSN6* to the C-terminus of *tetR* (or *tetR′*) through an RS dipeptide bridge, plus 306 bp downstream of the *SSN6* stop codon. For *TUP1*, the following oligonucleotides were employed: 5′-GTAAG**GGTACCTAC**GAAGCAGAGATCAAGC-3′ (a *Kpn*I cloning site and codon number 73 of the *TUP1* open reading frame are in bold letters), and 5′-AGGAAT**GGCGCC**TTGATC-ATCAAAGAATAATGAACCGCAA-3′ (a *Nar*I site used for cloning is in bold letters). When the amplified fragment was cloned in the adequate plasmids (Table 1), this resulted in an in-frame fusion of *tetR* (or *tetR′*) plus the lambda cI spacer followed by the Tup1 product begining at amino acid 73 of the original protein. The construction also included 212 bp at 3′ of the *TUP1* stop codon. The *Schizosaccharomyces pombe adh1* promoter ($adh1_p$) from pART1 (a gift of Avelino Bueno, University of Salamanca, Spain) was employed to replace the CMV_p promoter in some plasmids (Table 1). Plasmid pCM250 contains the *CLN2* gene with a C-terminal 3× HA epitope under $tetO_2$ promoter control; it derives from pCM188 (4) by cloning a PCR-generated blunt-ended fragment containing the tagged *CLN2* gene (to be described elsewhere) in the *Pme*I site of the vector. The same PCR-generated fragment was cloned in the *Pme*I site of pCM252 to obtain plasmid pCM254 (thus containing the tagged *CLN2* gene under $tetO_7$ promoter control).

Further details on constructions and maps of the plasmids described here will be given by the authors upon request.

DNA manipulations for plasmid construction and analyses were performed by standard methods (22). DNA fragments were isolated from agarose gels using Qiaex columns (Qiagen).

Yeast transformation

Transformation of yeast cells was carried out by the lithium acetate procedure (23). For integrative transformation of YIplac128 derivatives at the chromosomal *LEU2* locus, plasmid DNA previously linearized by digestion at the single *Eco*RV locus inside the plasmid *LEU2* gene was employed. Integrations were

checked by Southern analysis using digoxigenin-labeled *LEU2* DNA probes.

Northern and western blot analysis

Samples of total RNA for northern analysis were processed (RNA purification, electrophoresis and blotting to positively-charged nylon membranes) as described in (22). UV-crosslinked membranes were washed twice in washing buffer (1% SDS, 20 mM Na_2HPO_4, pH 7.2, 1 mM EDTA) at 65°C, pre-hybridized for 1 h at 65°C in 20% SDS, 0.5% blocking reagent (Boehringer), 250 mM Na_2HPO_4, pH 7.2, 1 mM EDTA and hybridized overnight in the same conditions with a labelled probe (internal to the *CLN2* open reading frame) at 2 ng/ml. Labelling had been performed by random-priming PCR with digoxigenin-dUTP labelling mixture (Boehringer). Membranes were washed twice at 65°C with washing buffer, and immunodetection steps were carried out as described by the manufacturer (Boehringer) using CDP* (Tropix) as chemi-luminiscent substrate. Signals were detected and quantified with a Lumi-Imager equipment (Boehringer).

For western blot analysis, protein extracts were prepared from 5 OD_{600} of exponentially growing cells, by resuspension of cell pellets in 15 µl of 5 M urea and boiling for 2 min. An equivalent volume of glass beads was then added and cells were broken by vortexing (8 min at room temperature), followed by addition of 50 µl of 2% SDS in 0.125 M Tris–HCl buffer, pH 6.8, vortexing for 1 min more, boiling for 2 min and centrifugation. Equivalent amounts of protein (quantified by the Micro DC protein assay of BioRad) were separated in SDS–polyacrylamide gels, blotted to PVDF by electrotransference and immunodetected using a Super Signal CL HRP-based method (Pierce) and the Lumi-Imager equipment for signal detection. HA epitope-tagged proteins were detected with a 1:2500 dilution of the 12CA5 anti-HA monoclonal antibody. Software of the equipment was employed for determination of relative signal intensities.

Determination of β-galactosidase activity

β-galactosidase activity (as Miller units) was determined in toluenized cells as described in (4). Samples were taken from cells that had been growing exponentially for at least 10 generations in the conditions (plus or minus antibiotic) specified.

RESULTS AND DISCUSSION

Development of a reverse tTA system for yeast

Gossen *et al.* (7) have developed a reverse tTA (that is, tetracycline-inducible) system for mammalian cells, based on a mutated *tetR′* moiety of the transactivator protein that only recognizes the *tetO* promoter boxes when interacting with the antibiotic effector molecules. Adaptation of this system for yeast cells could provide conditions for overexpressing proteins without the need to change cells to glucose-minus growth conditions as occurs with galactose-driven expression. Maintaining growth conditions as constant as possible may be desirable when correlations are made between overexpression of a particular protein and physiological effects. For that purpose, the reverse system may be advantageous over the direct one since: (i) the antibiotic should not be required for basal (non-overexpressing) growth conditions, and (ii) overexpression would not require the previous dilution of the effector molecules as would occur with the direct system, therefore allowing more rapid induction of the system.

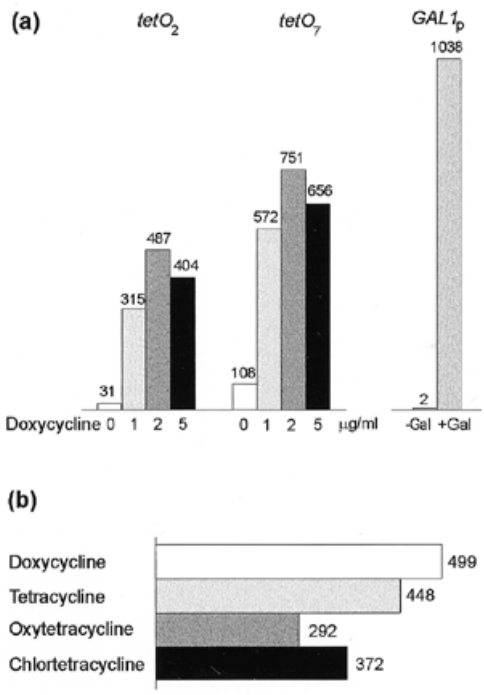

Figure 1. Characterization of the reverse tTA system. As reporter gene, *lacZ* was employed under the $tetO_2$ promoter [plasmid pCM171, (4)] or the $tetO_7$ promoter (plasmid pCM173). (**a**) Effect of doxycycline concentration on inducibility of the system. BMA64-1A cells transformed with the corresponding plasmid were grown exponentially for 20 h in the conditions indicated. Numbers above the bars show the β-galactosidase activity (Miller units) in the cultures from a representative experiment. For comparison, enzyme activity in BMA64-1A cells transformed with pCM154 [containing *GAL1-lacZ* as reporter gene, (4)] is also shown, after exponential growth in SD-raffinose medium (–Gal) or after 24 h (in exponential conditions) of galactose addition to raffinose-grown cells (+Gal). (**b**) Effect of several members of the tetracycline antibiotic family on inducibility of the $tetO_7$-*lacZ* reporter gene. pCM173-transformed BMA64-1A cells were grown for 24 h in the presence of the antibiotic at 1 µg/ml, before determination of β-galactosidase activity (Miller units, shown by the numbers).

We constructed the centromeric plasmids pCM175 and pCM176, which contain the reverse tTA transactivator, as well as the *lacZ* gene as reporter system under the control of a $tetO_2$ (pCM175) or a $tetO_7$ (pCM176) promoter. Transformants with any of both plasmids exhibited a marked induction of *lacZ* expression by doxycycline (Fig. 1a), with enzyme activity levels that in the case of the $tetO_7$ promoter are ~70% of those reached with a $GAL1_p$-*lacZ* system. Although no large differences in maximal expression were observed in the range of doxycycline concentration between 1 and 5 µg/ml, highest levels were consistently obtained at 2 µg/ml (Fig. 1a). Concentrations >5 µg/ml were somewhat toxic for the yeast cells (data not shown). Doxycycline and tetracycline were the most effective inducers among the tetracycline family members tested (Fig. 1b), in accordance with their most effective role in switching-off the direct tTA system (4) and in correlation with their highest association equilibrium constant to the Tet repressor of Tn10 (24).

The expression levels achieved in yeast cells with the reverse tTA system may be adequate for overexpression physiological studies, and for other types of studies requiring high amounts of

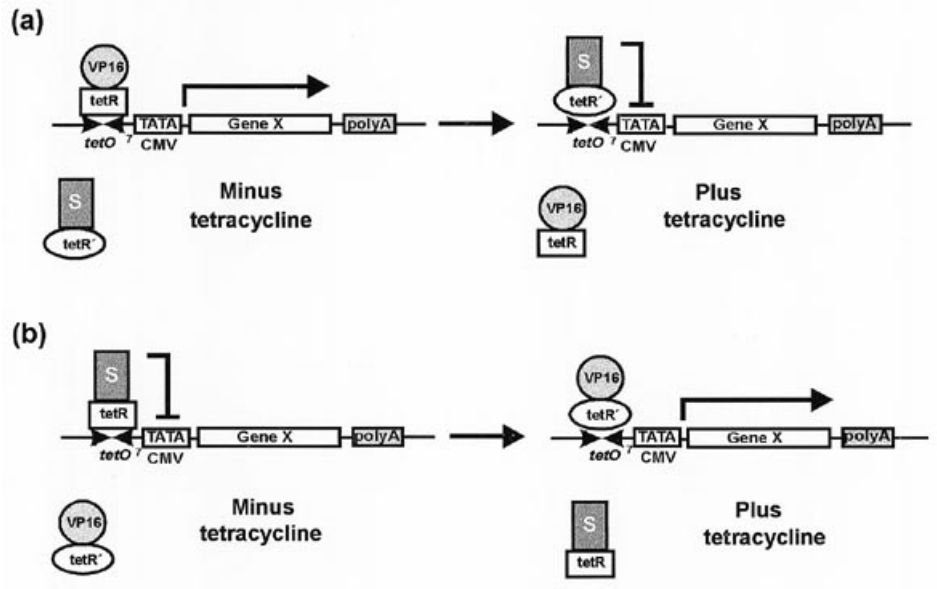

Figure 2. Tetracycline-regulatable dual (activator/repressor) system for *S.cerevisiae*. The tetracycline-repressible direct (**a**) and tetracycline-inducible reverse (**b**) systems are depicted. S corresponds in this work to the entire Ssn6 or the truncated (minus the N-terminal 72 amino acids) Tup1 silencer moieties of the repressor protein. See the text for more details.

protein. However, these advantages may be obscured by the high basal levels observed, especially in the case of the $tetO_7$ promoter (Fig. 1a). The incomplete switching-off of the promoter probably reflects the partial ability of *tetR′* to interact with the *tetO* boxes in yeast cells even in the absence of effector molecules. This leakiness, that appears not to occur in mammalian cells (7), has also been observed by us with other *tetO*-driven genes such as the *S.cerevisiae CLN2* and *CLN3* genes (data not shown, and see below).

Construction of tetracycline-regulatable dual (activator/repressor) systems for yeast cells

Taking advantage of the opposite effect of tetracycline molecules on the ability of the *tetR* and *tetR′* moieties of the respective tTA activators to interact with the *tetO* boxes, we have developed two modalities of a tetracycline-regulatable dual system, that is, a system in which a *tetO*-binding activator and a *tetO*-binding repressor (both of them regulated by tetracycline in opposite ways) co-exist in the same cell. The dual system might allow a tighter regulation of *tetO*-driven expression, since the presence of the repressor could lower the basal levels in silencing conditions. Paralleling the direct and reverse tTA activators, two different types of *tetO*-recognizing chimeric repressors have been constructed: the tetracycline-activable one (co-existing with the direct tTA activator) consists of *tetR′* fused to the silencer moiety, while the tetracycline-inactivable one (co-existing with the reverse tTA activator) consists of *tetR* fused to the silencer moiety. Figure 2 depicts the two modalities of the dual system, that are respectively switched off and on by the effector antibiotic.

As the silencer moiety of the repressors, we have tested the entire Ssn6 protein or the truncated Tup1 product lacking the N-terminal 72 amino acids [that is, the region of Tup1 required for interaction with Ssn6 (15,16)] of the native protein. In both cases, the silencer was C-terminal to the *tetO*-binding moiety. In the native Ssn6–Tup1 general repressor, Ssn6 acts as a bridge between the promoter-binding subunit and the Tup1 subunits that actually perform the silencing role (15,16). Thus, to increase the flexibility of the final chimeric protein and to separate the DNA-binding moiety from the silencer one, a lambda cI linker region (4) was added as spacer between both moieties in the Tup1-based repressors. Although in the native general repressor Ssn6 and Tup1 are differentiated subunits from the promoter-recognizing one, previous studies (8,9,15,16) had shown that Ssn6 and Tup1 have also repressor activity when fused in frame to promoter-binding regions forming a single chimeric molecule.

The *tetO* binding domain-silencer chimeric constructions (initially under the control of the viral CMV_p promoter) were chromosomally integrated at *LEU2*, and the resulting strains (Table 1) were transformed with three $tetO_7$-driven expression plasmids with *lacZ* as reporter gene. Plasmids pCM173 (centromeric) and pCM179 (episomal), both of which contain the direct tTA activator (4), were used to transform strains with the integrated *tetR′*-silencer constructions, while pCM176 (centromeric) carrying the reverse tTA activator was tested on strains with the *tetR*-silencer constructions. Measurements of β-galactosidase activity in induction and non-induction conditions (Table 2) showed that in the presence of the repressor, basal expression is lowered to almost undetectable levels when the direct tTA transactivator is in a centromeric plasmid, both the Ssn6 and Tup1-based repressors being similarly efficient. Basal levels are also decreased by the repressors when expression depends on a direct tTA activator in a multicopy plasmid or in the case of the reverse tTA activator, although in these situations the Ssn6-based repressor is more efficient. In fact, basal expession is decreased by the Ssn6 chimeric molecules to levels under detectability in the case of the reverse system (Table 2). In none case maximal expression is affected by the presence of the repressor, and growth rate is also not modified (data not shown).

Table 2. Expression of a *tetO-lacZ* reporter construction in different modalities of the activator/repressor dual system

Plasmid[a]	tTA activator	Repressor[b]	β–galactosidase activity (Miller units ± SD) in[c]	
			Induction conditions	Repression conditions
None	–	–	≤0.10	≤0.10
pCM173	Direct	CMV_p(tetR′)	413.1 ± 50.0	0.45 ± 0.25
(centromeric)		CMV_p(tetR′–Ssn6)	459.0 ± 68.5	0.16 ± 0.12
		CMV_p(tetR′–Tup1)	392.8 ± 95.1	0.28 ± 0.18
		$adh1_p$(tetR′–Ssn6)	487.2 ± 32.9	0.11 ± 0.09
		$adh1_p$(tetR′–Tup1)	501.0 ± 80.1	0.18 ± 0.13
pCM179	Direct	CMV_p(tetR′)	1013.6 ± 71.7	21.0 ± 9.7
(episomal)		CMV_p(tetR′–Ssn6)	1014.5 ± 164.1	1.0 ± 0.43
		CMV_p(tetR–Tup1)	1120.5 ± 101.4	5.6 ± 1.3
pCM176	Reverse	CMV_p(tetR)	506.0 ± 163.7	127.2 ± 44.7
		CMV_p(tetR–Ssn6)	556.5 ± 79.8	≤0.10
		CMV_p(tetR–Tup1)	378.7 ± 109.8	0.16 ± 0.08
		$adh1_p$(tetR–Ssn6)	765.8 ± 183.9	0.11 ± 0.09
		$adh1_p$(tetR–Tup1)	557.8 ± 117.9	0.14 ± 0.09

[a]The plasmids employed to transform host cells contain the *$tetO_7$-lacZ* reporter construction.
[b]The moieties of the chimeric protein and the promoter under which it is expressed are indicated. Host strains with these constructions are shown in Table 1.
[c]Measurements (from at least three independent experiments) were done in cells grown exponentially during 20 h in induction or repression conditions; when required, doxycycline was employed at 2 μg/ml. SD, standard deviation.

In the above conditions, repressor expression (as well as that of tTA) is directed by the cytomegalovirus promoter (4,5). Therefore, levels of repressor are probably low due to inefficient expression from the heterologous promoter in yeast cells. We reasoned that increasing the amount of repressor by expressing it from a moderately strong promoter in *S.cerevisiae* cells such as $adh1_p$ from *S.pombe*, might further reduce expression from *tetO* in non-inducing conditions. However, combination of an $adh1_p$(*tetR′*-Ssn6) or $adh1_p$(*tetR′*-Tup1) repressor with a direct tTA activator in a multicopy plasmid was partially deletereous for the cells (data not shown). Although no clear explanation exists for this fact (since it is observed either in induction and non-induction conditions), other results from us discard the possibility of repressor interference with replication of 2 μ based plasmids (not shown). On the other hand, expression of the chimeric repressors from $adh1_p$ did not reduce levels of *lacZ* expression in a clear fashion when this gene was in centromeric plasmids, both with the direct and the reverse systems (Table 2).

Next we analysed regulability of the direct dual system on the expression of an homologous *S.cerevisiae* gene such as *CLN2*, coding for the G_1 cyclin Cln2. For this purpose, we employed a *$tetO_2$–CLN2* construction in a centromeric plasmid (pCM250), in which *CLN2* is C-terminus tagged with three copies of the HA epitope. Results (Fig. 3) confirmed those previously obtained with the heterologous *lacZ* gene. Essentially, the presence of the tetracycline-activable chimeric repressor (expressed from the CMV promoter) decreased the basal levels of expression to almost undetectability when Ssn6 was the silencer constituent of the repressor moiety. To achieve similar results with Tup1, the chimeric molecules had to be expressed from the stronger $adh1_p$ promoter.

The higher performance of Ssn6 when fused to *tetR* or *tetR′* may be explained by its ability to recruite Tup1 subunits, therefore forming multimeric complexes estructurally similar to those naturally occurring in the cell. On the contrary, the excessive proximity of the silencer moiety to the DNA-binding

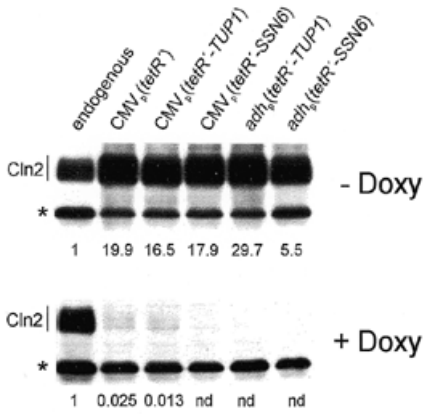

Figure 3. Regulation of *CLN2* expression under *$tetO_2$* promoter control using the dual direct system. Western blots showing the effect of different chimeric repressor molecules (with the Ssn6 or the Tup1 moieties) expressed from the CMV or the *adh1* promoters in pCM250-transformed cells growing in non-repression (–Doxy) or repression (+Doxy, 24 h with doxycycline at 2 μg/ml) conditions. The respective host strains are those shown in Table 1. The Cln2 product was 3× HA-tagged at its C-terminus, and the protein was detected in the western blots with anti-HA antibodies. Heterogeneous mobility of the Cln2 molecules is due to differences in phosphorylation levels (26). Signal levels corresponding to Cln2 were divided by those of a higher-mobility band (marked with an asterisk) cross-reacting with the 12CA5 antibody that maintained constant levels of labelling, and then made relative to the signal in extracts from cells of strain CML204 (as BMA64-1A but containing the endogenous chromosomal HA-tagged *CLN2* gene, left column), which was given the unit value. Numbers under the photographs represent the values calculated in this way. nd: non-detectable (relative values < 0.005).

moiety may cause some esteric hindrance to the Tup1-based repressors (lacking Ssn6 as adaptor). This fact may be important when relatively high basal expression must be lowered. Assuming that activity of Ssn6 or Tup1-based repressors affects nucleosome positioning in the sensitive promoter regions (12), our results support the existence of nucleosomal structure in plasmid

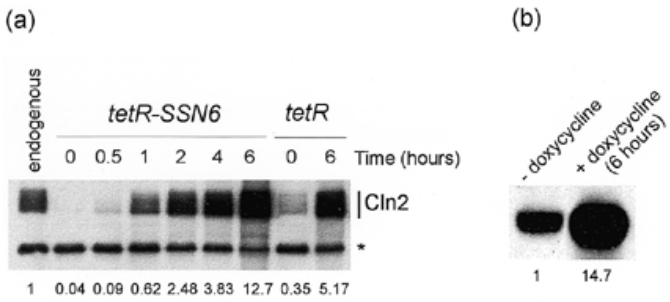

Figure 4. Kinetics of overexpression of *CLN2* using the reverse dual system. Strains CML238 [CMV_p(*tetR*)] or CML282 [CMV_p(*tetR-SSN6*)] transformed with pCM254 growing in SD minimal medium were added with doxycycline (2 μg/ml) at time 0, and samples were taken at the indicated times. As a control, a sample was also taken from exponentially-growing cultures of strain CML204 (HA-tagged *CLN2* expressed from its endogenous promoter). (**a**) Western blot analysis of Cln2. Relative levels of Cln2 expressed from the *tetO*$_7$ promoter (with respect to those from the endogenous promoter) were calculated as indicated in the legend of Figure 3, and are shown by the numbers under the respective runs. The asterisk marks the band that cross-reacts with the 12CA5 antibody. (**b**) Northern blot analysis of *CLN2* mRNA levels cells growing in the absence or in the presence of doxycycline (2 μg/ml). Numbers indicate the relative levels of the signal.

promoters (25) and show that this structure is still sensitive to the general Ssn6–Tup1 repressor.

Kinetics of induction of the reverse dual system

Once shown that the presence of the *tetR*–Ssn6 repressor decreased basal levels of the reverse system below detectability (Table 2), we measured the kinetics of induction of *CLN2* expression under *tetO*$_7$ promoter control in cells containing the CMV_p(*tetR–SSN6*) construction, which also allowed us to compare final overexpression steady state levels with those cells that express *CLN2* exclussively from its endogenous promoter. Previously we had seen (using the *tetO*$_7$*–lacZ* reporter system) that the Tup1-based chimeric repressor caused a slower induction kinetics than the Ssn6-based one, although final overexpression β-galactosidase levels did not differ significantly beween both types of repressor (data not shown). The Cln2 protein began to accumulate at detectable levels shortly (30 min) after antibiotic addition, and after 6 h it accumulated ~13-fold with respect to steady-state levels in exponentially-growing cells expressing Cln2 from its own promoter (Fig. 4a).

Cln2 is a rather unstable protein (half-life of ~10 min) in exponentially-growing cells expressing normal levels of the former (26,27). Since the observed half levels of Cln2 after 6 h in induction conditions could be influenced by changes in the half-life of the protein in these overexpressing cells, the above reported numeric values might not reflect the relative strength of the *tetO*$_7$ promoter compared with the *CLN2* own one. Therefore, we compared *CLN2* mRNA levels between induction and non-induction conditions in cells that could direct *CLN2* transcription from both promoters (Fig. 4b). In the presence of doxycycline (both promoters being active), *CLN2* mRNA levels are almost 15-fold those of non-induced cells (*CLN2* expressed only from its own promoter). That is, the relative strength of *tetO*$_7$ when expressing *CLN2* parallels the relative level of the product.

In summary, tetracyclin-regulated expression of *tetO*-driven genes in cells that constitutively express a Ssn6- or Tup1-based chimeric repressor allows tigh control of product levels between induction and non-induction conditions. When this is applied to the direct system, it may be useful for the analysis of terminal phenotypes of essential genes that otherwise could not be studied through this approach, and also for functional analysis of mutations in non-essential genes which cause detectable phenotypes. The dual reverse system permits rapid passage from a tightly-controlled non-expression situation to high expression conditions without introducing externally-induced side effects in cell physiology, therefore being an adequate tool for overexpression phenotypic analyses.

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