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A fast and efficient translational control system for conditional expression of yeast genes

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ABSTRACT

A new artificial regulatory system for essential genes in yeast is described. It prevents translation of target mRNAs upon tetracycline (tc) binding to aptamers introduced into their 5′UTRs. Exploiting direct RNA–ligand interaction renders auxiliary protein factors unnecessary. Therefore, our approach is strain independent and not susceptible to interferences by heterologous expressed regulatory proteins. We use a simple PCR-based strategy, which allows easy tagging of any target gene and the level of gene expression can be adjusted due to various tc aptamer-regulated promoters. As proof of concept, five differently expressed genes were targeted, two of which could not be regulated previously. In all cases, adding tc completely prevented growth and, as shown for Nop14p, rapidly abolished *de novo* protein synthesis providing a powerful tool for conditional regulation of yeast gene expression.

INTRODUCTION

Although the yeast model organism *Saccharomyces cerevisiae* was the first eukaryote to have its genome sequenced (1), the molecular and physiological functions of many of the encoded proteins are still not understood; especially difficult to investigate are essential genes. Since more than one-third of the essential *S. cerevisiae* genes have homologous human counterparts, yeast has become an important model organism for genome functional analysis in eukaryotes. For the investigation of essential gene functions, conditional gene expression is an indispensable tool and several such systems have been developed which regulate either transcription or protein stability (2).

For transcriptional gene regulation natural yeast promoters, such as the *GAL1* and the *MET25* promoters are used. Target genes are expressed either in the presence of galactose (3) or the absence of methionine (4). However, such regulation always interferes with cellular metabolism due to the changes in growth media composition and in many cases regulation is not tight enough to completely prevent growth. A major improvement was the introduction of heterologous tc-regulated promoters which are either inducible or repressible (TetON/OFF) (5–7). A clear advantage of these systems is that tc does not severely interfere with yeast cellular metabolism (8) and nowadays, these strains are commercially available (9).

Alternatively, the stability of essential proteins can be targeted to achieve conditional expression. Most suitable for gene function analyses are temperature-sensitive mutants with the respective proteins loosing their function upon a shift to non-permissible temperatures, which are either increased (temperature-sensitive ts mutants) or decreased (cold-sensitive cs mutants). Unfortunately, ts and cs mutants are isolated in specific strain backgrounds and, thus, cannot be easily transferred to non-isogenic strains. The fusion of heat-inducible degron sequences to essential proteins combined with their expression by the copper-inducible *CUP1* promoter makes the respective target protein proteolytically sensitive upon temperature shift. This provides an alternative method for ts-regulated gene expression (10,11).

Despite many successful applications of the above mentioned systems, an easy adaptable regulatory system for conditional gene expression in yeast is still missing. We developed a new concept for conditional yeast gene expression which regulates translation of the target mRNAs by inserting a tc aptamer with strong binding affinity for tc (12) into the 5′UTRs. In contrast to protein-based systems, such aptamer-based synthetic riboswitches rely on a direct RNA–ligand interaction, are thus strain independent and no interference of heterologous expressed

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regulatory proteins is expected. Because of its small size and its regulatory properties, the aptamer is perfectly suited to control expression of any gene of interest. Here, we describe an easy adaptable PCR-based system, which exploits the principal of direct RNA–ligand interaction for the efficient conditional control of essential genes in yeast.

MATERIALS AND METHODS

Plasmid constructions and genetic manipulations

Established protocols for molecular biology were followed (13). Yeast strains were transformed with the lithium acetate procedure (14).

pADH1-(tc)n-GFP. We used a 108-nt-long 5′UTR from pWHE702 (15) which originates from the *pTEF* promoter and carries an additional 70-nt-long CAA spacer to adjust its length to a 5′UTR which contains one copy of an aptamer. This 108-nt-long fragment was cloned into pWHE602 in front of a *pADH1*-driven *GFP* reporter gene (16) resulting in *pADH1-GFP*. The sequence of the complete 5′UTR with the CAA spacer and all restriction sites is given in the Supplementary Table S1. Overlap-PCR was used to insert one, two and three aptamers, resulting in plasmids *pADH1-tc1-GFP*, *pADH1-tc2-GFP* or *pADH1-tc3-GFP*, respectively (Figure 1A). Sequences for all aptamer-containing 5′UTRs are included in Supplementary Table S1.

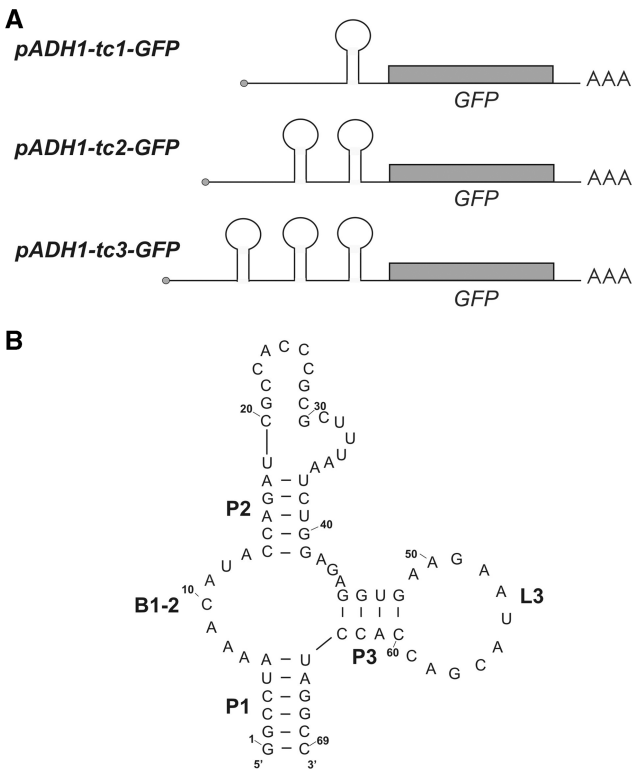

Figure 1. Tc aptamer-mediated translational inhibition. (**A**) Schematic view of the aptamer-containing mRNAs. The cap structure is shown as a ball. (**B**) Secondary structure of the tc aptamer [stem = pedestal (P), bulge (B) and loop (L)]. B1-2 and L3 are important for tc binding as it is supported by structural probing (34) and the crystal structure (35).

pADH1-tc3. The *ADH1* promoter and the tc aptamer containing 5′UTR were PCR amplified from *pADH1-tc3-GFP*. The HA-tag was amplified from the vector pOM10 (17) (kindly provided by EUROSCARF, www.uni-frankfurt.de/fb15/mikro/EUROSCARF) and attached via overlap-PCR. The PCR products were introduced into pUG6 (18) using the two unique restriction sites SalI and BsiWI next to a kanamycin-resistance gene flanked by *loxP* sites resulting in *pADH1-tc3*.

pTDH3-(tc)n. The tc aptamer containing 5′UTRs were PCR amplified from *pADH1-(tc)n-GFP*. The HA-tag was amplified from the vector pOM10 (17) and attached via overlap-PCR. The PCR product was introduced in pPK421 (19) via the unique restriction site BsiWI which is downstream of the transcription initiation site of the *TDH3* promoter and next to a kanamycin-resistance gene flanked by *loxP* sites resulting in *pTDH3-tc1, -tc2 or -tc3*, respectively.

All plasmids, maps and complete sequences are available from EUROSCARF (http://www.uni-frankfurt.de/fb15/mikro/EUROSCARF/index.html).

Acc.-No	Plasmid
p30595	pADH1-tc1-GFP
p30596	pADH1-tc2-GFP
p30597	pADH1-tc3-GFP
p30598	pADH1-tc3-3xHA
p30599	pADH1-tc3-6xHA
p30600	pTDH3-tc3-3xHA
p30601	pTDH3-tc3-6xHA
p30602	pTDH3-tc2-3xHA
p30603	pTDH3-tc2-6xHA
p30604	pTDH3-tc1-3xHA
p30605	pTDH3-tc1-6xHA

Strains

CEN.PK122	*MATa/MATα MAL2-8^c/MAL2-8^c SUC2/SUC2* (9)
RS453α	*MATα ade2-1 trp1-1 can1-100 leu2-3 his3-1 ura3-52*

GFP measurements

S. cerevisiae strain RS453α transformed with the respective constructs was grown at 28°C for 48 h in 5 ml of minimal medium [0.2% (w/v) yeast nitrogen base, 0.55% ammonium sulfate, 2% (w/v) glucose, 12 μg/ml adenine, MEM amino acids, Gibco BRL] in the absence or presence of 250 μM tc. Cells were harvested by centrifugation and resuspended in 2 ml phosphate-buffered saline (PBS). For each construct, three independently grown cultures were analyzed. Fluorescence measurements were carried out at 25°C on a Fluorolog FL3-22 (Horiba Jobin Yvon) with the excitation wavelength set to 482 nm and an emission wavelength of 510 nm. Optical density (OD_{600}) was determined to ensure homogeneous cell growth. The vector pVT102-U (20) without the *GFP* gene was analyzed in parallel as a blank and its value was subtracted from all data.

Genomic integration of the aptamer-containing integration cassettes

All genetic modifications of the *S. cerevisiae* genome were performed via homologous recombination using PCR products with 40-nt-long flanking homologies to the target genes (21). The insertion cassettes were PCR amplified from the corresponding vectors *pADH1-tc3* and *pTDH3-(tc)n* using the primer pairs x-Tc1 and x-Tc2. Primer pairs x-Tc1B and x-Tc2 can be used for amplification of the integration cassettes without HA-tag. Sequences of all primers used for the amplification of the integration cassettes are given in Supplementary Table S2. After transformation of *S. cerevisiae* strain CEN.PK122 (22) with the PCR products, correct integration in G418 resistant strains was confirmed by PCR using primer pairs x-A1/K2 and x-A2/ADH1-A5 and TDH3-A7, respectively (promoter-specific primer, see Figure 2B and Supplementary Table S2) and by sequencing. Diploid transformants were subsequently analyzed by tetrad dissection.

Serial dilution growth assay

Yeast cells were grown overnight in 1% yeast extract, 2% peptone supplemented with 2% glucose (YPD) or 2% fructose (YPF) to an OD_{600} of 1-2. Cells were diluted in normal saline to an OD_{600} of 1 followed by 3-fold 1:10 serial dilution. From the diluted cultures, 5 µl were spotted onto YPD plates in the absence (control) and presence of tc. Growth differences were recorded following incubation of the plates for 2–3 days at 30°C.

Western blot analysis

Protein extracts were prepared from HA-epitope tagged strains using glass beads. Total protein concentration was determined with the micro-biuret method (23,24). Equivalent amounts of protein were separated with 10% SDS polyacrylamide gels and blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore) by electrotransfer. Membranes were blocked with 5% nonfat dry milk and HA-tagged proteins were detected with 1:5000 dilutions of the 12CA5 anti-HA monoclonal antibody (Roche), followed by a second reaction with a 1:30 000 dilution of anti-mouse IgG-conjugated horseradish peroxidase (BioRad) for the use with ECL chemoluminescence protocol (GE Healthcare).

Northern blot analysis

RNA was prepared as previously described (25); 18.4 µg total RNA was separated on 1% agarose gels (in 1xTAE) supplemented with 6.66% formaldehyde and transferred to a positively charged nylon membrane (Hybond N+, GE Healthcare) *via* capillary blotting. 10 pmol oligonucleotides NB_HA (hybridizes to the HA-tag) and NB_ACT1_1 (hybridizes to *ACT1*-mRNA) were radioactive labeled at the 5′ end using 6 µl γ-^{32}P-ATP (~3.3 pmol/µl, Hartmann-Analytik) and 1 µl T4 polynucleotide kinase (Roche) in the supplied buffer for 1 h at 37°C and purified with Illustra Microspin G-25 columns (GE Healthcare); 23 µl-labeled oligonucleotides (~600.000 c.p.m/µl) were used as probe for one membrane. Hybridization was done in 12 ml RotiHybriQuick (Roth) overnight at 42°C. Signals were visualized by phosphoimaging using a Typhoon 9100 (GE Healthcare).

RESULTS

The tc aptamer has been used to control gene expression on different levels, including translation initiation (16,26) and pre-mRNA splicing (27). The use of two tc aptamer-regulated introns or combination of translational and splicing control significantly increased efficiency of regulation (27). Therefore, we questioned if the insertion of multiple copies of the tc aptamer into the 5′UTR also increases regulation.

As shown for an *ADH1* promoter-driven *GFP* reporter gene, one tc aptamer (*pADH1-tc1-GFP*) reduced basal GFP expression to ~28% and constructs with two (*pADH1-tc2-GFP*) or three tc aptamers (*pADH1-tc3-GFP*) had 24 and 21% residual activity, respectively (Table 1). In all cases, addition of tc resulted in a further strong reduction of GFP fluorescence with 8-fold for a construct with one, 21-fold with two and 37-fold with three tc aptamer copies, respectively.

To test the potential of tc aptamer inhibition for conditional regulation of essential genes in yeast we developed an easy, adaptable and flexible PCR-based strategy for genomic tagging. Homologous flanking regions were used for all constructs to integrate different promoters with tc aptamer-containing 5′UTRs directly in front of the ATG start codons of the selected open reading frames, thereby uncoupling them from their endogenous promoters and 5′UTRs. This allows the use of only a single pair of gene-specific PCR primers to amplify all available promoter-aptamer constructs (Figure 2). To compensate for the decreased translation efficiency, the glycolytic *TDH3* and *ADH1* promoters with different expression strengths were used. The insertion cassette also harbours an HA-tag for detection of the proteins by western blots and a kanamycin resistance gene flanked by *loxP* sites, as selection marker for chromosomal integration. Five essential genes (*NEP1*, *NOP8*, *NOP14*, *PGI1*

Table 1. Regulatory properties of tc aptamer insertion

Construct	Relative fluorescence (%) no tc[a]	Relative fluorescence (%) 250 µM tc[a]	Regulatory factor[b]
pADH1-tc1-GFP	28.0	3.6	8
pADH1-tc2-GFP	23.8	1.1	21
pADH1-tc3-GFP	20.5	0.6	37

[a]Gfp fluorescence expressed by the vector without an aptamer (*pADH1-GFP*, Figure 1A) was set to 100% and corresponds to 1.1×10^6 counts per second (cps) without and 1.3×10^6 cps with 250 µM tc, respectively, with background level subtracted (8×10^3 cps without and 16×10^3 cps with 250 µM tc, respectively); values are mean of three independently grown cultures with standard deviation below 7%.
[b]Efficiency of regulation is given as the ratio of respective values with and without tc.

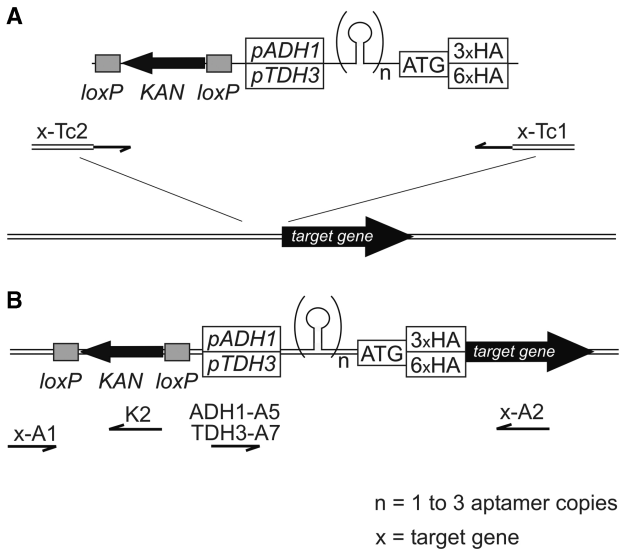

Figure 2. Universal insertion cassettes for tc aptamer-mediated regulation. (**A**) Schematic representation of the insertion cassette with a kanamycin-resistance gene (*KAN; kanMX4 modul*) flanked by *loxP* sites. The promoters *pADH1* or *pTDH3* are fused to a synthetic 5′UTR with n copies of the tc aptamer ($n = 1$, 2 and 3, respectively) followed by an HA-tag (either 3xHA or 6xHA) for protein quantification. All plasmid-encoded insertion cassettes can be PCR amplified with a single pair of target gene specific primers (x-Tc1 and x-Tc2). x-Tc2 shares 40-nt homology with the promoter and x-Tc1 40-nt homology with the open reading frame of the target gene. The double line represents the target chromosomal locus. (**B**) Chromosomal situation after integration. The gene of interest is expressed by a heterologous promoter (either *pADH1* or *pTDH3*) and controlled by n copies of the tc aptamer in its 5′UTR. Primers to control successful integration are indicated.

and *SEC1*) were chosen as proof of concept for tc-dependent translational regulation. For two of them, *NOP8* and *SEC1*, attempts to establish conditional regulation using other systems had failed so far (8,28).

Transformation of wild-type yeast with PCR amplified insertion cassettes containing three tc aptamers led to colony formation on medium containing G418. The correct insertion of the *pTDH3-tc3*- and *pADH1-tc3*-cassettes in front of all five genes was confirmed by an analytic PCR of genomic DNA and by sequencing. This shows that the PCR-based strategy is easily adaptable and efficient enough to target any gene within the yeast genome.

In the absence of tc, all five constructs driven by the *TDH3* promoter fully complemented the endogenous promoters and 5′UTRs. For *NEP1, SEC1, NOP8* and *NOP14*, the *TDH3-tc3* constructs provided normal growth to the respective insertion strains. In contrast, after tetrad analysis of heterozygous asci, the *pTDH3-tc3-PGI1* constructs showed reduced growth when compared to wild-type segregants (Figure 3A). For the highly expressed *PGI1* gene, *pTDH3-tc3* promoter strength was obviously not sufficient to permit normal growth in glucose-containing media. However, with fructose as carbon source growth of *TDH3-tc3-PGI1* segregants was like wild type (data not shown). Expression mediated by the less efficient *pADH1-tc3* promoter was not sufficient to complement the substitution of the endogenous *NEP1* and *PGI1* promoters (Figure 3A).

Serial dilution growth assays showed that increasing concentrations of tc (Figure 3B) abolished growth of all *pTDH3-tc3*-constructs. However, while 100 μM tc was sufficient to prevent growth of the *pTDH3-tc3-NEP1* and *pTDH3-tc3-PGI1* strains, 500 μM tc were needed to abolish growth of the *pTDH3-tc3-NOP8* and *pTDH3-tc3-SEC1* strains. The less active *ADH1* promoter was more suitable for controlling *NOP8* (*pADH1-tc3-NOP8*) and *SEC1* (*pADH1-tc3-SEC1*) expression where 100 μM tc completely prevented growth.

Taken together, our data clearly show that both the expression strength of the promoter and the tc concentration influence the efficiency of regulation. This allows the system to be easily adjusted to the individual expression level of the target gene.

The number of tc aptamers inserted also contributed to tc-dependent translational control. As exemplified for *NEP1*, no regulation was achieved with only one aptamer present in the 5′UTR, whereas addition of a second tc aptamer provided efficient regulation. Although a third tc aptamer copy further improved the strength of regulation in the *GFP* system (Table 1), no difference with respect to growth regulation was observed for constructs with two or three copies (Figure 3C).

The efficiency of tc aptamer inhibition was shown for *pTDH3-tc3-NOP14* which encodes an essential protein needed for ribosome biogenesis. Addition of tc to liquid medium results in a reduced growth rate within 4 h. Furthermore, Nop14 protein levels decreased to <50% 2 h after tc addition and almost completely disappeared within 6 h (Figure 4).

Northern blot analyses of the *TDH3-tc3-NOP14* strains showed that the *NOP14* mRNA was stable for at least 6 h after tc addition (Figure 5A). This indicates that the *TDH3-tc3-NOP14* gene was further transcribed after tc addition and that the tc-bound mRNA was not degraded. Remarkably, the *ACT1* mRNA signal, which was used as an internal control, strongly decreased after 6 h in the *TDH3-tc3-NOP14* strain. This degradation is probably the result of a diminished number of ribosomes due to the Nop14-mediated arrest of ribosome biogenesis. For the *TDH3-tc3-PGI1* gene, which encodes the glycolytic phosphoglucose isomerase enzyme, *PGI1* and *ACT1* mRNAs stayed stable (Figure 5B).

Taken together, our results impressively show that tc aptamer-induced inhibition causes an immediate translational response upon tc addition.

DISCUSSION

Conditional regulation is an indispensable tool for the functional analysis of essential genes. Most commonly target gene expression is addressed at the transcriptional level. However, not all essential genes can be controlled by transcriptional regulation and in some cases these systems give false-positive results due to enhancing or silencing effects, such as the recently reported *trans* activation (29). This study, therefore, introduces an alternative regulatory system that targets translation. Previously, we have shown that the ligand-bound form of the tc aptamer

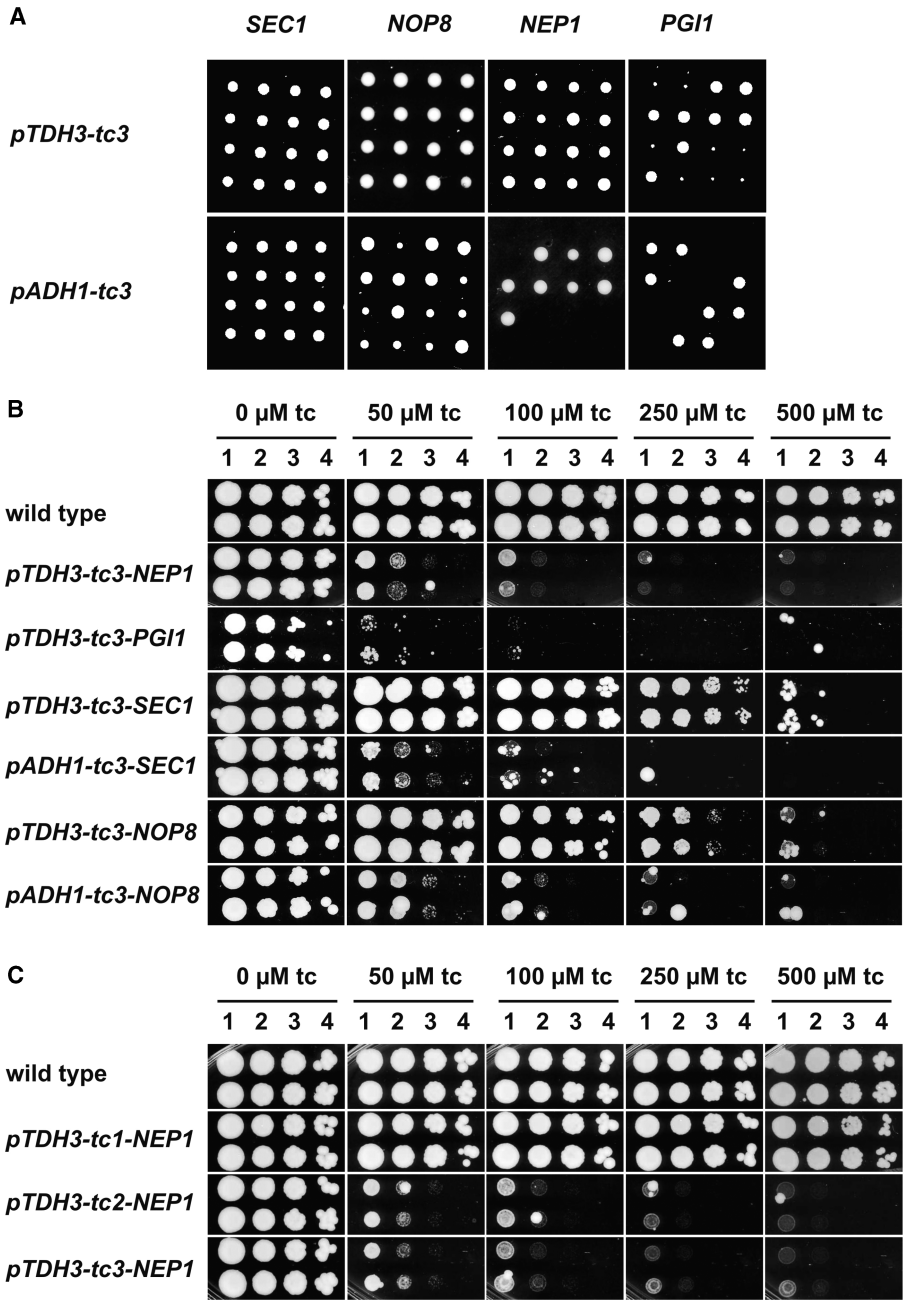

Figure 3. Tc aptamer-regulated expression of essential genes. (**A**) Segregation pattern of tc aptamer-regulated genes after tetrad analysis. Diploid strain CEN.PK122 was transformed with the tc aptamer insertion cassettes resulting in a heterozygous strain carrying a wild type and a tc aptamer modified allele of the target gene. After meiosis, the viability of the tc-regulated target genes was followed by tetrad dissection. For the *pTDH3*-driven constructs, four viable segregants were obtained for all target genes (upper four lanes). No viable segregants were obtained for the *pADH1*-driven *PGI1* and *NEP1* (2:2 segregation). (**B**) Growth dilution assays were performed for wild-type and insertion mutants. Cultures were grown overnight in YPD medium and diluted to a final density of OD_{600} of 1.0 (1). Ten-fold serial dilutions (corresponding to 2, 3 and 4) were spotted on YPD medium with increasing concentrations of tc. Colonies were grown for three days at 30°C. (**C**) Regulation of a *pTDH3*-driven *NEP1* gene with one (*pTDH3-tc1-NEP1*), two (*pTDH3-tc2-NEP1*) or three (*pTDH3-tc3-NEP1*) copies of the tc aptamer.

when inserted into the 5′UTR inhibits binding of the small ribosomal subunit to the cap structure and the formation of the 80S ribosome without any effect on the mRNA steady state level (15). In contrast, insertion of the aptamer into the 3′UTR leads to no regulation (unpublished data). For all five cases studied here, conditional translational regulation was successfully achieved, including two genes for which conditional regulation at the level of transcription had failed so far.

The simple and efficient technique using PCR to tag genomic loci with tc-dependent aptamers allows fast and efficient regulation of any yeast gene of interest. The promoter strength is of major importance for successful conditional regulation. Here, we provide several insertion

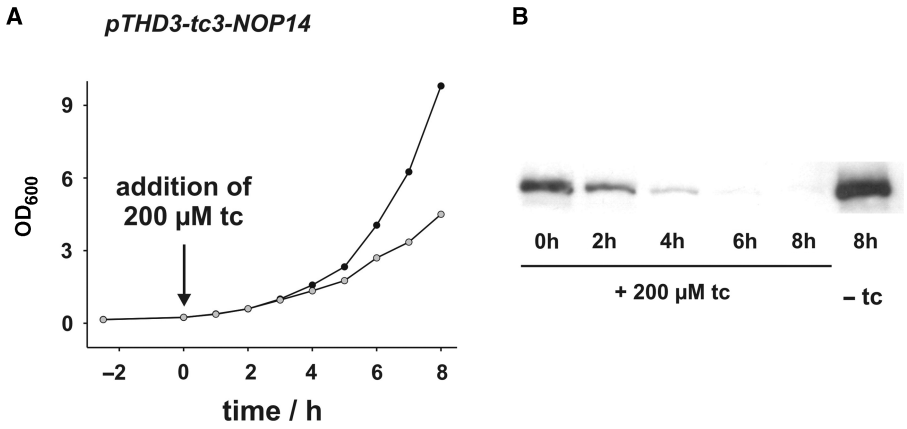

Figure 4. Repression kinetic of tc aptamer-controlled NOP14 expression. (**A**) Growth curves of a strain with NOP14 regulated by the tc aptamer (*pTHD3-tc3-NOP14*). A culture was diluted to an OD_{600} of 0.1 in YPD media and incubated for 2.5 h at 30°C. After splitting, one aliquot was treated with 200 µM tc. Growth was followed for 8 h (black dots, no tc, gray dots, 200 µM tc). (**B**) Samples were taken at indicated time points after treatment with tc and Nop14-HA protein amounts determined by western blot analysis.

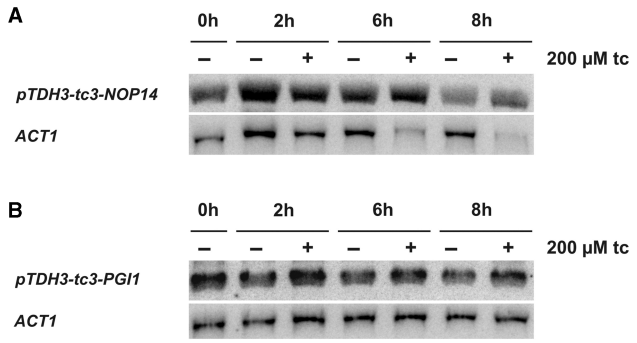

Figure 5. Northern blot analysis of tc aptamer-controlled *NOP14* and *PGI1* expression. Strains with (**A**) *pTDH3-tc3-3HA-NOP14* and (**B**) *pTDH3-tc3-3HA-PGI1* were grown in YPD media (*NOP14*) and YPF (*PGI1*), respectively. Overnight cultures were grown to an OD_{600} of 0.4 at 30°C. After splitting, one aliquot was treated with 200 µM tc. Samples were taken at indicated time points after treatment with tc and total RNA was prepared. mRNA amounts of *NOP14* and *PGI1* were determined by northern blot analysis. Actin mRNA (*ACT1*) was used as internal control.

cassettes, which allow adjustment to the individual expression level of the target gene *via* a combination of two different promoters and the number of tc aptamers. For easy handling all integration cassettes can be amplified with a single pair of target specific primers.

The striking advantage of our system is that no further auxiliary protein factors are necessary so that no interference of heterologous expressed regulatory proteins is expected. Furthermore, the application is completely strain independent. The effector tc is pharmacologically well characterized, with good cell permeability and without any influence on mRNA stability, protein expression and growth (8,15). Our experiments with *NOP14* have shown that protein synthesis is switched off immediately upon tc addition. The relative amount of Nop14p decreased by 50% after one generation time. This inversely corresponds to the 2-fold increase in overall protein amount after one cell cycle.

Our work greatly expands the applicability of aptamer-controlled gene expression systems. For the first time we demonstrate the control of essential, endogenous genes by an RNA aptamer-based regulation system. This is possible since the system is tight and independent of positioning effects. Until now only three examples for endogenous (but not essential) genes regulated by aptamers have been reported. A synthetic riboswitch, which responds to theophylline engineered by the Gallivan group, shows robust increase in gene expression upon theophylline addition. However, this system is restricted to gene regulation in bacteria since regulation involves sequestration of the bacterial ribosomal binding site (30,31). Conditional gene expression by RNAi controlled by aptamers has also been shown (32,33). However a specific design of the regulating elements is needed for each new gene. So far, our system is applicable in *S. cerevisiae* and possibly other yeasts. Attempts to regulate genes in human cell lines and plants failed so far probably due to stronger helicase activities (unpublished data).

Taken together, we established a tight tc-dependent translational control system, which allows regulated expression of yeast genes with one single PCR step. Therefore, it is a highly suitable method to study the physiological function of essential and nonessential yeast proteins.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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