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Functional Analysis of Yeast Essential Genes Using a Promoter-Substitution Cassette and the Tetracycline-regulatable Dual Expression System

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A promoter-substitution cassette has been constructed that allows one-step substitution of chromosomal gene promoters for the tetracycline-regulatable *tetO* promoter in yeast cells, which uses *kanMX4* as selective marker for geneticin resistance. Oligonucleotides for PCR amplification of the cassette are designed to allow homologous recombination through short flanking regions of homology with the upstream sequences of the chromosomal gene, upon transformation of target cells. By testing three essential genes of chromosome XV (YOL135c, YOL142w and YOL144w), the system causes tetracycline-dependent conditional growth of the cells, being modulatable by intermediate concentrations of the effector. Analysis of terminal phenotypes of the promoter-substituted cells in the presence of the antibiotic may facilitate functional analysis of essential orphan genes. © 1998 John Wiley & Sons, Ltd.

KEY WORDS — *Saccharomyces cerevisiae*; promoter-substitution cassette; tetracycline-regulatable promoter; essential genes; conditional gene expression

INTRODUCTION

A large number of the about 6000 predicted open reading frames (ORFs) characterized during the development of the yeast genome sequencing project (Goffeau *et al*., 1996) correspond to actual genes without known function. These 'orphan' genes (Dujon, 1996) are subject to studies by the EUROFAN network for systematic functional analysis of novel yeast genes (Oliver, 1997). In the frame of this network, we have developed a set of tetracycline-regulatable vectors that allow tetracycline silencing of genes under the control of the bacterial *tetO* promoter, through the action of a tetracycline-responsive tTA activator (Garí *et al*., 1997; Bellí *et al*., 1998). The latter consists of a *tetO*-binding moiety, *tetR*, plus the viral VP16 activator moiety. In combination with a chimeric repressor containing a *tetO*-recognizing moiety plus the *Saccharomyces cerevisiae* Ssn6 repressor, whose activity is regulated by tetracycline in the opposite way to the tTA activator, a tighter control of expression of *tetO*-driven genes is achieved (Bellí *et al*., 1998). In the work reported here, this dual (activator/repressor) system has been tested by us on three essential genes of chromosome XV, for which generation of specific deletant strains for these genes had been carried out during the initial phase of the EUROFAN project. We have constructed a promoter-substitution cassette with *kanMX4* as selectable marker that allows the one-step substitution of the chromosomal ORF promoter for the *tetO* promoter, through the short flanking homology strategy (Wach *et al*., 1994, 1997). We show that *tetO*-directed conditional expression of these essential genes allows the

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Table 1. Characteristics of the six ORFs studied on chromosome XV.

Systematic name	Size (aa)	Coordinates*
YOL144w	484	53096–54547
YOL142w	240	55556–56275
YOL141w	695	56450–58534
YOL138c	1341	65349–61327
YOL137w	497	65620–67110
YOL135c	222	70043–69378

*Coordinates correspond to the nucleotide position (in the direction from 5′ to 3′ ends) in the complete sequence of chromosome XV. For more details, see http://www.mips.biochem.mpg.de

study of terminal phenotypes and thus, to gain information on the cellular roles of the genes.

MATERIALS AND METHODS

Open reading frames

The six ORFs analysed (Table 1) are located in the subtelomeric region of the left arm of chromosome XV, and have been sequenced in our laboratory during the Yeast Genome Sequencing Project (Casas *et al*., 1995; Aldea *et al*., 1996; Dujon *et al*., 1997).

Strains, media and growth conditions

Strains employed in this work (Table 2) derive from *S. cerevisiae* FY1679 (B. Dujon, Pasteur Institute). ORF deletions were also introduced in strain CEN.PK2 (*MAT***a**/α *ura3-52/ura3-52 leu2-3,112/leu2-3,112 his3*Δ*1/his3*Δ*1 trp1-289/trp1-289*; K. D. Entian, Frankfurt University). Yeast cells were usually grown in YPD medium (2% yeast extract, 1% peptone, 2% glucose). YPGly medium contains glycerol at 3% instead of glucose. Glucose minimal medium (SD) contains 0·67% yeast nitrogen base (Difco) plus 2% glucose. When needed, nutritional requirements were added to the SD medium (Kaiser *et al*., 1994). Doxycycline (Sigma) was added at the final concentrations indicated in each experiment, from stock solutions at 5 mg/ml in 50% ethanol. Unless otherwise indicated, cultures were incubated at 30°C. *Escherichia coli* DH5α (Life Biotechnologies) was employed for plasmid amplification and maintenance.

DNA manipulations

Plasmid preparations as well as DNA manipulations and bacterial transformations were carried out by standard methods (Ausubel *et al*., 1989). DNA fragments for subcloning were isolated from agarose (NuSieve GTG, FMC BioProducts) gels and purified using Qiaex (Qiagen).

Construction of single ORF deletant strains using the kanMX4 cassette

The *kanMX4* cassette from pFA6a-kanMX4 (Wach *et al*., 1994) was employed for the construction of FY1679 derivatives carrying single deletions in each of the ORFs described in Table 1, through the short flanking homology approach. Basically, we used the methods described in http://muntjac.mips.biochem.mpg.de (Guidelines for

Table 2. Strains employed.

Name	Genotype	Origin or comments
FY1679	*MAT***a**/α *ura3-52/ura3-52 leu2*Δ*1/+ his3*Δ*200/+ trp1*Δ*63/+ GAL2/GAL2*	B. Dujon (Pasteur Institute, Paris)
CML235	*MAT***a** *ura3-52 leu2*Δ*1 his3*Δ*200 GAL2*	Spore from FY1679
CML276	*MAT***a** *ura3-52 leu2*Δ*1 his3*Δ*200 GAL2* CMV_p(*tetR′-SSN6*)::*LEU2*	From CML235
CML249	*MAT***a** *ura3-52 leu2*Δ*1 his3*Δ*200 GAL2* CMV_p(*tetR′-SSN6*)::*LEU2* $tetO_2$(YOL135c)::kanMX4	From CML276
CML251	*MAT***a** *ura3-52 leu2*Δ*1 his3*Δ*200 GAL2* CMV_p(*tetR′-SSN6*)::*LEU2* $tetO_2$(YOL144w)::kanMX4	From CML276
CML302	*MAT***a** *ura3-52 leu2*Δ*1 his3*Δ*200 GAL2* CMV_p(*tetR′-SSN6*)::*LEU2* $tetO_2$(YOL142w)::kanMX4	From CML276
CML303	*MAT***a** *ura3-52 leu2*Δ*1 his3*Δ*200 GAL2* CMV_p(*tetR′-SSN6*)::*LEU2* $tetO_2$(YOL138c)::kanMX4	From CML276

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Table 3. Oligonucleotides employed for the construction and analysis of single ORF deletant strains.

Name	Sequence	Use
135-S1	5′-(70055)CACAAACTAAAAATGTCCAATGAT CCTGGTAATGAGGTTAGcagctgaagcttcgtacgc	Disruption of YOL135c with kanMX4 casette
135-S2	5′-(69297)GGACTACCACGTGCCTACTATACG CTATACGGTAGTTGAAgcataggccactagtggat	Disruption of YOL135c with kanMX4 casette
135-A1	5′-(70534)GCTGGCATTCAATGCATG	Confirmation of YOL135c disruption at the 5′ end
135-A2	5′-(69457)CATACTTGCTCGATCTCGC	Confirmation of YOL135c disruption at the 5′ end
135-A3	5′-(69475)GCGAGATCGAGCAAGTATG	Confirmation of YOL135c disruption at the 3′ end
135-A4	5′-(68970)TAAGTTGGTTTGCTGTGGC	Confirmation of YOL135c disruption at the 3′ end
142-S1	5′-(55503)CGGATGCTTCAGCAGCTCATCTCC AGCATTTCGACTAACCcagctgaagcttcgtacgc	Disruption of YOL142w with kanMX4 casette
142-S2	5′-(56289)TACATGGTAGTCTACTCCTCCTTG ACCGTAAGTATTTCgcataggccactagtggat	Disruption of YOL142 with kanMX4 casette
142-A1	5′-(54962)ACCAAGGGAAGCCATTCTT	Confirmation of YOL142w disruption at the 5′ end
142-A2	5′-(55580)CACCAGGGAATATGAACGTA	Confirmation of YOL142w disruption at the 5′ end
142-A3	5′-(55561)TACGTTCATATTCCCTGGTG	Confirmation of YOL142w disruption at the 3′ end
142-A4	5′-(56530)AATCGCCAAATCAGCGTAC	Confirmation of YOL142w disruption at the 3′ end
144-S1	5′-(53063)GTACCTGAAGTGAGAACTAGGTA ATATACGACGATGGcagctgaagcttcgtacgc	Disruption of YOL144w with kanMX4 casette
144-S2	5′-(54554)CTCACTATAGAAGAAGCCCGCTCT TTGATTGTTTCCTCgcataggccactagtggat	Disruption of YOL144w with kanMX4 casette
144-A1	5′-(52593)GCAAGTGCAATGGTCAACC	Confirmation of YOL144w disruption at the 5′ end
144-A2	5′-(54014)TGCTCTGGTCCTTCGTCTT	Confirmation of YOL144w disruption at the 5′ end
144-A3	5′-(53996)AAGACGAAGGACCAGAGCA	Confirmation of YOL144w disruption at the 3′ end
144-A4	5′-(54980)AAGAATGGCTTCCCTTGGT	Confirmation of YOL144w disruption at the 3′ end
kan5	5′-GTTCGGATGTGATGTGAG	Confirmation of ORF disruptions at the 5′ end
kan3	5′-TGCCTCGGTGAGTTTTCTC	Confirmation of ORF disruptions at the 3′ end

Numbers in parentheses indicate the nucleotide position in chromosome XV that corresponds to the 5′ end residue of the oligonucleotide. Sequence of the pFA6a-kanMX4 multiple cloning site (Wach *et al.*, 1994) is indicated with lower-case letters.

EUROFAN B0 Program: ORF deletants, plasmid tools, basic functional analysis). Oligonucleotides employed for construction of the respective deletion cassettes by PCR amplification from pFA6a-kanMX4 are described in Table 3 (S1 and S2 series). Expand High Fidelity enzyme mixture (Boehringer) was employed for the amplification reactions, and the amplified DNA was purified (Qiaquick PCR Purification columns, Qiagen) before transformation. Yeast cells were transformed as described in Gietz *et al.* (1992). After 4 h of phenotypic expression, cells transformed with DNA molecules carrying the kanMX4 cassette were plated on YPD agar plates containing G418 (geneticin, Sigma) at 200 µg/ml. Deletions were confirmed by PCR amplification of DNA [obtained as described by Kaiser *et al.* (1994), rapid preparation method] from the geneticin-resistant transformants. The A1-A2 and A1-kan5 combinations of oligonucleotides (Table 3) were used for confirmation of disruptions at the 5′ end, and A3-A4 plus A3-kan3 for the 3′ end. DNA from the confirmed single ORF-disrupted strains was employed for PCR amplification and cloning of the kanMX4-based disruptant cassettes. For the amplification reactions, the A1 plus A4 oligonucleotides and the Pwo DNA polymerase (Boehringer) were used following the instructions of the manufacturer. The disruptant cassettes amplified in this way were closed in the single *Eco*RV site of plasmid pUG7 (a gift of P. Philippsen, University of Basel).

Construction of cognate gene clones

Cognate genes were cloned in pRS416 (Sikorski and Hieter, 1989). For YOL135c, a pBluescript SK+-based clone containing a 5·4 kbp *Pvu*II-*Bam*HI fragment from cosmid pEOA461 in the *Eco*RV site of the vector [constructed during

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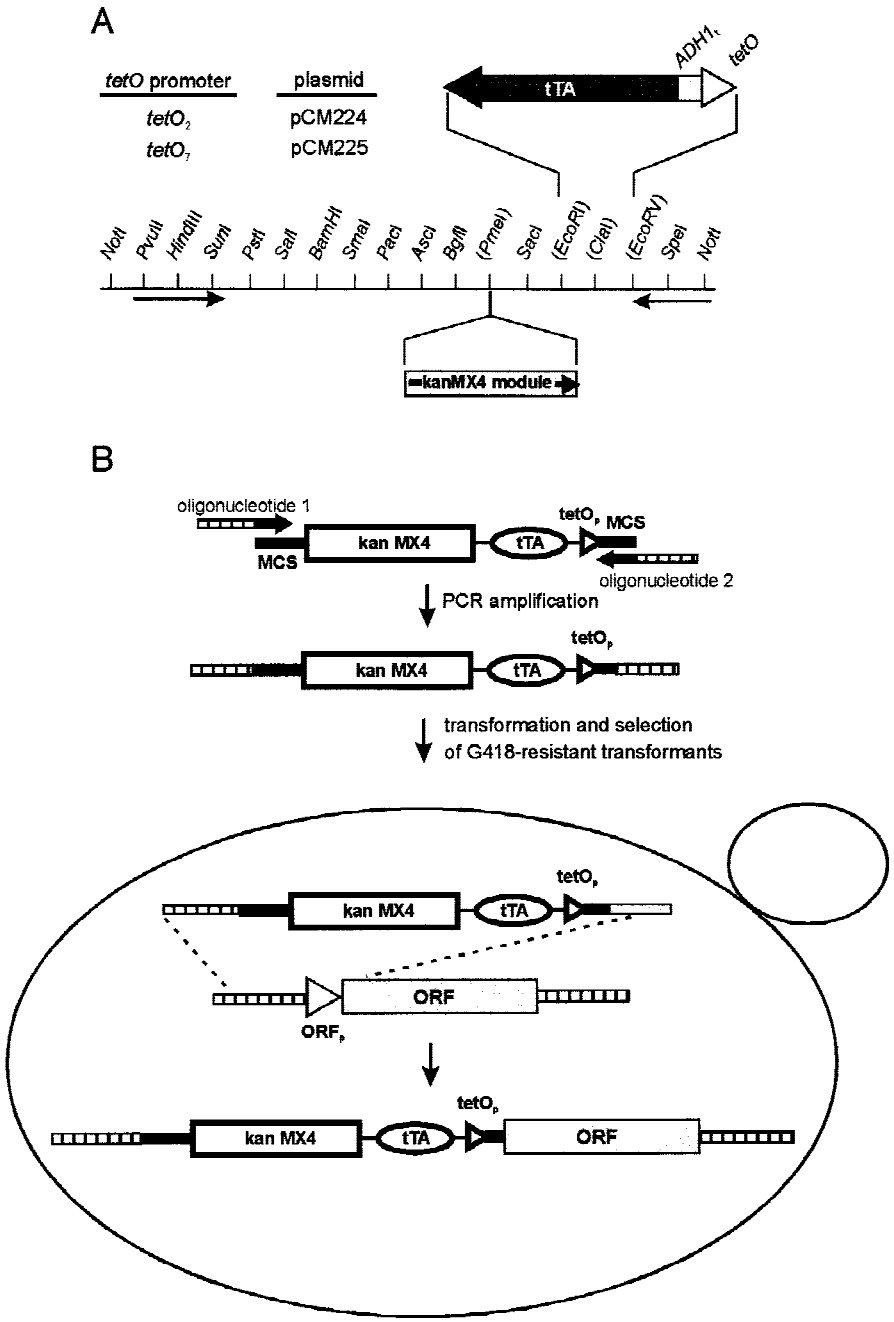

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the sequencing project (Aldea *et al*., 1996)] was the source of a 1492 bp *Avi*II-*Sca*I fragment that included YOL135c. This was cloned in the *Sma*I site of pRS416. For YOL142w, a 3324 bp *Bam*HI fragment from cosmid pEOA179 (Casas *et al*., 1995) was cloned in the *Bam*HI site of pRS416; the resulting plasmid was digested with *Ava*I, followed by Klenow fragment treatment and religation. The cognate gene clone thus obtained has a 2013 bp insert including YOL142w. For YOL144w, a pBluescript SK+ derivative containing a ca. 11 kbp *Bam*HI fragment from cosmid pEOA179 (Casas *et al*., 1995) was the source of a *Nhe*I-*Bam*HI fragment of 2561 bp that included YOL144w. The latter was cloned into *Xba*I plus *Bam*HI-digested pRS416.

Construction of the promoter-substitution cassettes

Two *tetO*-based promoter-substitution cassettes were constructed containing kanMX4 (Wach *et al*., 1994) as the selectable marker. For this aim, plasmids pCM188 and pCM189 (Garí *et al*., 1997) were digested with *Eco*RI plus *Pme*I. The smaller of the two resulting fragments contained the tetracycline-responsive tTA activator gene plus the *tetO* promoter [two *tetO* boxes ($tetO_2$) in pCM188 or seven *tetO* boxes ($tetO_7$) in pCM189]. This fragment was cloned in the *Eco*RI plus *Eco*RV-digested pFA6a-kanMX4 vector. The structure of the resulting plasmids (pCM224 and pCM225, respectively with $tetO_2$ or $tetO_7$) is shown in Figure 1A. They are integrative plasmids that contain the kanMX4 module added with the tTA activator and the *tetO* promoter next to the right region of the *kanMX4* multiple cloning site (MCS). Therefore, the MCS of these promoter-substitution cassettes only differs from the original *kanMX4* MCS because it lacks the *Eco*RI to *Eco*RV sequences (Figure 1A).

Promoter substitutions

Plasmid pCM224 DNA served as template for PCR amplification [using the *Pwo* DNA polymerase (Boehringer) as recommended by the vendor] of the promoter-substitution cassette (Figure 1B). Oligonucleotides employed (Table 4) contained about 40 bp homologous to the target yeast DNA followed by sequences of the cassette MCS (see Figure 1A). They were devised such that oligonucleotide 1 (Figure 1B) was homologous to regions about 100 to 200 bp upstream of the ORF initiation codon (depending on the location of the upstream ORF putative promoter or transcription termination regions) while oligonucleotide 2 included the ORF initiation codon. Transformation with the amplified product, transformant selection strategy and testing for correct integration (with the A1 plus A4 combination of oligonucleotides, Table 3) were carried out as for integration of the kanMX4 module.

Northern analysis

RNA purification, electrophoresis, probe labelling with digoxygenin, hybridization and signal detection were carried out as described (Gallego *et al*., 1997). The following probe fragments generated by restriction enzyme digestion from the plasmids containing the respective cognate genes were employed: for YOL135c, a 450 bp *Ava*I-*Xmn*I fragment; and for YOL144w, a 802 bp *Hae*II-*Xba*I fragment. For YOL142w, a fragment from bp 55511 to 56278 was PCR-amplified from the cognate gene clone, and used as probe after purification from agarose gel and labelling. All probes are internal to the respective ORFs.

Protein sequence analyses

Analysis of the sequences of the predicted ORF products was made using the information retrieved

Figure 1. (A) Structure of the one-step promoter-substitution cassettes. The MCS from the original pFA6a plasmid (Wach *et al*., 1994) is shown, although the map of the remaining plasmid regions is omitted. Sites in parentheses have been eliminated or inactivated during the cloning processes. Arrows below the MCS line mark the sequences employed for cassette amplification in this work (see Table 4 for details). Lengths of the kanMX4 module as well as of the components of the tTA-$ADH1_t$-*tetO* box inserted in the MCS are proportional to their sizes in base pairs, except for the *tetO* promoter, which has different lengths depending on the number of repeat units. As a consequence, the tTA-$ADH1_t$-*tetO* module has 2298 and 2516 bp respectively in pCM224 and pCM225. The arrows in the modules mark the direction of transcription. (B) Strategy for promoter substitution. Components of the substitution cassette are not shown to scale. Short-flanking homology PCR-amplified DNA is employed for transformation. About 40 bp of the 5′ end of oligonucleotide 1 used for amplification are homologous to the intergenic sequence 100–200 bp upstream of the ORF initiation codon, while the 3′ end corresponds to the MCS sequence of the substitution cassette. At its 5′ end, oligonucleotide 2 is homologous (about 40 bp) to the region immediately 5′ to the ORF initiation codon (including the latter), the 3′ end corresponding to the MCS sequence of the cassette. Homologous recombination of the linear transformant DNA results in substitution of the chromosomal ORF promoter (ORF_p) for the *tetO* promoter ($tetO_p$).

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Table 4. Oligonucleotides employed for promoter substitution.

Name	Sequence	Use
135p-1	5′-(70107)ATGACATAAAAGAGGTTTCATATCC CAAATTTAAGCTAGTcagctgaagcttcgtacgc	One-step substitution of the YOL135c promoter
135p-2	5′-(70004)GGTACAAGGAGCTAACCTCATTACC AGGATCATTGGA**CAT**ataggccactagtggatctg	One-step substitution of the YOL135c promoter
142p-1	5′-(55353)GAAGTTTCGTGGAAACTAGTTCCAT CCACCTCACTTAAAGcagctgaagcttcgtacgc	One-step substitution of the YOL142w promoter
142p-2	5′-(55595)CTACAGGAAAGCTATCACCAGGGA ATATGAACGTAGA**CAT**ataggccactagtggatctg	One-step substitution of the YOL142w promoter
144p-1	5′-(53028)ACAAAGTATACAATAGGCCCATATC ATTTTAGATTGTACCcagctgaagcttcgtacgc	One-step substitution of the YOL144w promoter
144p-2	5′-(53135)TATTTCCGACAAAAATTCTTTTTTG AATTACACTATC**CAT**ataggccactagtggatctg	One-step substitution of the YOL144w promoter

Series 1 and 2 correspond to oligonucleotides 1 and 2 respectively in Figure 1B. Numbers in parentheses indicate the nucleotide position in chromosome XV that corresponds to the 5′ end residue of the oligonucleotide. Sequence of the multiple cloning site of the pCM224 template is indicated with lower-case letters. Position of the initiation codon of the respective ORFs is indicated with bold letters.

from http://www.mips.biochem.mpg.de/yeast, and the PEDANT software system (Frishman and Mewes, 1997) available at http://pedant.mips.biochem.mpg/de.frishman/pedant/htlm.

Quantification of plasmid segregation

Transformants carrying a *URA3*-based plasmid previously grown in selective SD medium were inoculated in YPD medium, and kept growing exponentially by successive redilutions in these non-selective conditions for 48 h. To determine the percentage of cells still carrying the plasmid, equivalent inocula from the cultures were extended in parallel on SD plates with or without 5-fluororotic acid (Kaiser *et al.*, 1994), and the number of colonies forming on to both types of plates was compared.

Other methods

Standard genetic methods were as described (Kaiser *et al.*, 1994). Cell number was determined from formaldehyde-fixed samples, using a haemocytometer. Percentage of budded cells was determined from the same samples by observing at least 200 cells with a phase-contrast microscope. Fixed cells were photographed under a Zeiss LSM microscope. Fluorescence microscopy was employed for observation of DAPI-stained nuclei in ethanol-fixed cells (Kaiser *et al.*, 1994). FACS analysis for DNA content measurements was carried out as described (Gallego *et al.*, 1997).

RESULTS

Growth phenotypes of the deletant strains

Mutant FY1679 derivatives carrying deletions in each of the six chromosome XV ORFs described in Table 1 were constructed using the kanMX4 module in the course of the EUROFAN project. All the heterozygous diploids thus obtained were viable, as well as *MAT***a** and *MAT*α haploid derivatives with disruptions in YOL137w, YOL138c and YOL141w. Haploid single mutants in YOL137w and YOL141w did not show any growth defect compared with the wild-type strains at different temperatures (15°C to 37°C) or growth media (YPD, YPGly or SD), while YOL138c disruptant haploid mutants grew poorly in SD medium at 15°C. This growth defect was suppressed by the plasmid containing the cognate gene. No morphology alterations, mating or sporulation defects were observed in the haploid disruptants for the above ORFs. On the contrary, spores from FY1679 heterozygous disruptants for YOL135c, YOL142w and YOL144w did not give rise to visible colonies (microcolonies up to about 20 cells were microscopically observed for YOL135c and YOL144w). Instead, haploid *MAT***a**

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and *MATα* disruptants for each of these three ORFs were viable (showing normal growth and morphology) when transformed with the cognate gene in the pRS416 plasmid. When a heterozygous diploid for YOL135c, YOL142w or YOL144w transformed with the respective cognate gene clone was sporulated, Ura^+ G418-resistant segregants could be obtained, but not Ura^-, confirming that growth inability is exclusively caused by the ORF disruption. The respective disruptant cassettes (see Materials and Methods) for the six ORFs were employed to transform the diploid CEN.PK2 strain. In this genetic background YOL135c, YOL142w and YOL144w were also revealed to be essential in haploid cells of both mating types, in contrast to the other three ORFs. Further studies described here concentrate on the three essential ORFs in the FY1679 background.

Substitution of the promoters of the essential ORFs for the $tetO_2$ *promoter*

To adapt the previously developed *tetO*-regulatable system (Garí *et al.*, 1997; Bellí *et al.*, 1998) for the study of essential genes in yeast, we have constructed a cassette containing kanMX4 as a selectable marker for G418 resistance, the tetracycline-inactivable tTA transactivator and the *tetO* promoter (Figure 1A). This cassette allows one-step substitution of chromosomal promoters based on a rationale (Figure 1B) similar to ORF disruption by the kanMX4 cassette with short flanking regions of homology to the targeted locus (Wach *et al.*, 1994). Strains were constructed in this way that have the respective chromosomal promoters of YOL135c, YOL142w and YOL144w substituted for the $tetO_2$ promoter (Table 2). The strains also contain the doxycycline-regulatable Ssn6-based repressor (Bellí *et al.*, 1998) to achieve lower basal levels in the presence of the effector. While the promoter-substituted cells grew normally in the absence of doxycycline, growth was largely inhibited in the presence of the antibiotic at 2 μg/ml (Figure 2A). Partial growth was observed at intermediate antibiotic concentrations, confirming that modulability of the *tetO* promoter-directed expression at intermediate effector concentrations (Garí *et al.*, 1997) is also valid for growth phenotypes. Northern analysis (Figure 2B) showed that ORF expression was turned off to near-undetectable levels in the case of YOL135c and YOL142w, although for YOL144w some residual expression level remained that may explain the residual growth observed on YPD plates with the antibiotic at high concentration (Figure 2A).

Terminal phenotypes caused by arrested expression of YOL135c, YOL142w and YOL144w

Strains where the promoters of the above-mentioned essential genes had been substituted for the $tetO_2$ promoter were analysed for their terminal phenotypes after several hours of doxycycline exposure in YPD liquid medium. CML249 cells (YOL135c promoter-substituted) grew normally in the absence of the antibiotic (doubling time about 100 min, Figure 3A), although $tetO_2$-driven expression led to considerably higher ORF mRNA levels than in conditions where YOL135c expression is driven by the original promoter (Figure 2B). In the presence of doxycycline, growth became affected after about 6 h, and cell division (but not biomass increase) was totally arrested some 8 h later (Figure 3A). Microscopic analysis indicated that cells with arrested YOL135c expression were larger than control cells, with a considerable proportion of them showing elongated buds (Figure 3B), which were mostly mono- or binucleated. Morphologically abnormal cells were already observed at 12 h after doxycycline addition.

Repression of YOL142w expression in CML302 cells by doxycycline affected growth in YPD liquid medium also after about 6 h in the presence of the effector, and this was followed by parallel arrest of cell division and biomass increase (Figure 4A). In these conditions, cells displayed uniform 1 N DNA content as determined by FACS analysis (Figure 4B), with almost the whole of the population in an unbudded state (data not shown). Although the doubling time of CML302 cells in conditions of full $tetO_2$-driven expression of YOL142w was longer than in wild-type CML235 cells (150 and 95 min respectively), this was not reflected in abnormalities in morphology, DNA content or percentage of budded cells (data not shown).

Growth of cells where YOL144w expression is driven by $tetO_2$ was normal in YPD liquid medium in the absence of doxycycline (doubling time about 100 min). When the antibiotic was added, cells abruptly changed cell division rate after 8 h to reach a doubling time of 210 min (data not shown). However, cells did not stop dividing even after successive redilutions in fresh medium plus doxycycline. This slow although continuous

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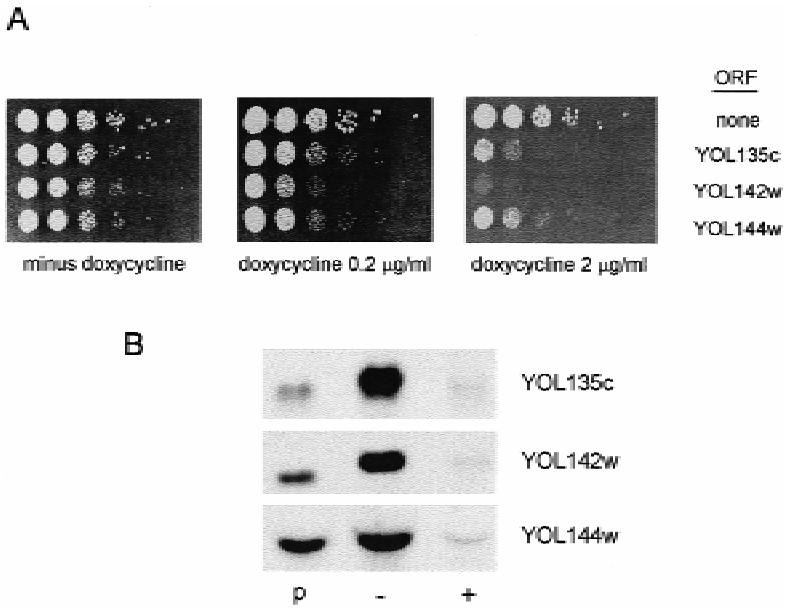

Figure 2. Regulation of expression of essential ORFs under $tetO_2$ promoter control. (A) Doxycycline-dependent growth of cells from strains in which the promoter of the indicated ORF has been substituted for $tetO_2$ by means of the pCM224 replacement module amplification. Serial sevenfold dilutions of exponentially growing cultures were spotted on to YPD plates plus the indicated concentration of doxycycline, and incubated at 30°C for 2 days. Strains (Table 2) derive from CML276, which is employed as control. (B) Northern analysis of $tetO_2$-driven ORF expression in the absence (−) or after 6 h in the presence (+) of doxycycline at 2 µg/ml in YPD liquid medium. Cells from the same strains as above were employed. Expression from the respective endogenous promoters in CML276 cells growing exponentially is also shown (p). Equivalent amounts of RNA, as determined by rRNA staining, were run per sample.

growth was also observed in experiments on solid medium, as shown in Figure 2A. No abnormalities in cell morphology were observed in these conditions (not shown). Since this observation could contradict with the fact that YOL144w is defined as an essential ORF from the inability of heterozygous-derived spores carrying the disrupted allele to form macroscopic colonies, we analysed whether growth restriction did not affect disrupted haploid cells once these had germinated. For this aim, a heterozygous diploid for YOL144w transformed with the cognate gene clone (in a plasmid with *URA3* as marker) was sporulated, and spore-derived wild-type or mutant colonies carrying the cognate gene were grown in non-selective conditions. After 48 h of exponential growth no mutant cells had been able to segregate the cognate gene plasmid, while wild-type cells had lost the plasmid efficiently. This confirmed that YOL144w is essential for vegetative growth.

DISCUSSION

More than 10% of the *S. cerevisiae* ORFs may correspond to essential orphan genes. Traditionally, the study of the terminal phenotype of conditional mutants in the non-permissive condition has shed light on the function of essential genes. The yeast temperature-sensitive cell cycle *cdc* mutants are an example of the latter. Since abrupt shift from permissive to non-permissive conditions causes a rapid inactivation or modification of the activity of the mutated gene product, phenotypic alterations become manifested rapidly after the shift. However, introduction of conditional mutations in a gene using a mutagen or PCR-based approaches may be limited by the specific structural and/or functional characteristics of the gene product. Thus, it is not always possible to produce conditional mutants for a determined gene, or only mutations affecting some regions of the gene may

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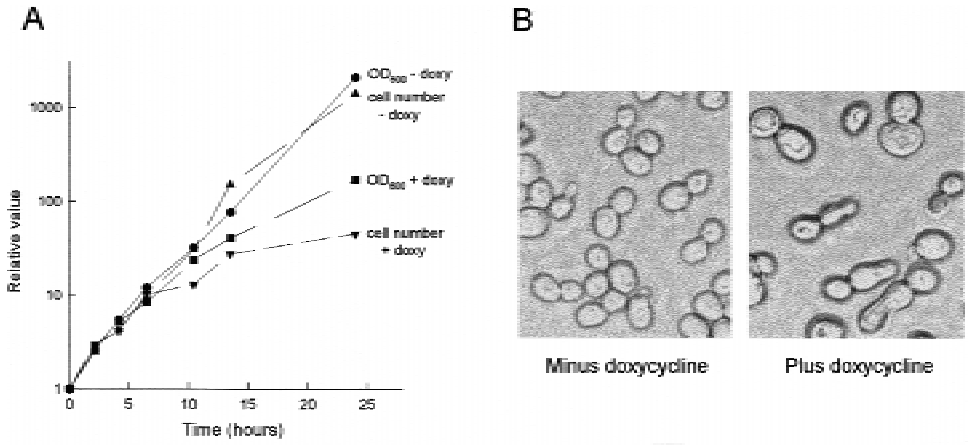

Figure 3. Effect of doxycycline on growth of CML249 cells. (A) Kinetics of cell growth, as determined by optical density at 600 nm (OD_{600}) and cell number measurements, in YPD liquid medium at 30°C in the absence or presence of doxycycline (2 µg/ml) added at time 0. The unit value was given to the respective measurements at time 0. (B) Phase contrast photographs of CML249 cells grown in YPD liquid medium at 30°C in the absence of doxycycline or after 24 h in the presence of doxycycline at 2 µg/ml.

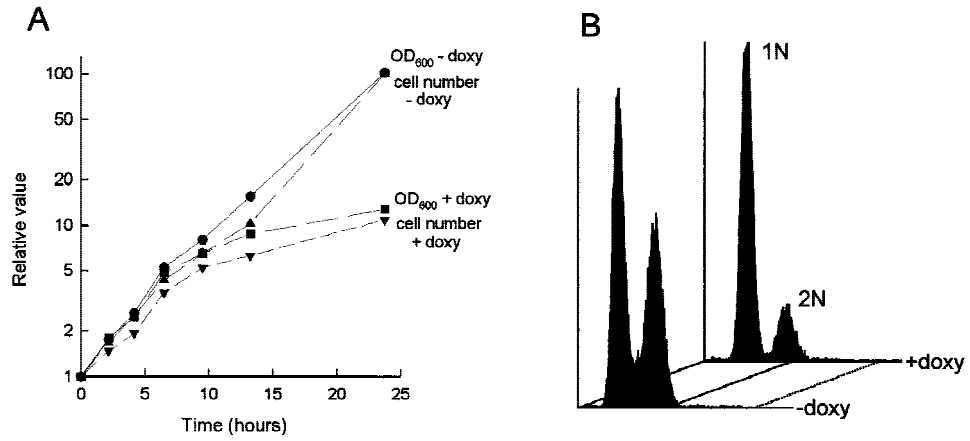

Figure 4. Effect of doxycycline on growth of CML302 cells. (A) Kinetics of cell growth, as determined by optical density at 600 nm (OD_{600}) and cell number measurements, in YPD liquid medium at 30°C in the absence or presence of doxycycline (2 µg/ml) added at time 0. The unit value was given to the respective measurements at time 0. (B) DNA content distributions of CML302 cells from YPD cultures in the absence of doxycycline or after 24 h in the presence of doxycycline at 2 µg/ml.

lead to conditional functionality. This situation could introduce bias into functional analyses based on terminal phenotypes, especially when multi-functional proteins are involved. Leakiness of many conditional mutations may also restrict their usefulness. Although conditional mutants continue to be a powerful tool for functional genomics in yeast, studies based on them should be complemented with those based on conditional expression of the wild-type gene. In this second approach, experimental conditions with near to null activity of the essential product can be attained due to lack of the latter, provided that a tightly-regulatable expression system is employed and high stability of the protein does not invalidate the approach. With this aim, we have constructed a promoter-substitution cassette based on the *kanMX4* geneticin-resistance gene as selectable marker for yeast (Wach *et al.*, 1994) together with the tetracycline-regulatable tTA system and the *tetO*

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promoter (Garí *et al.*, 1997; Bellí *et al.*, 1998). Our previous studies had shown that this system is exquisitely regulated, and that extremely low basal expression levels in repression conditions are reached with different reporter genes. A system based on the same rationale has also been developed by other authors (Nagahashi *et al.*, 1997), although the method that we describe here based on the kanMX4 module as the selectable marker allows this system to be systematically used for promoter substitutions.

One-step substitution of the original chromosomal ORF promoter for the *tetO* promoter as described here has the primary advantage of simplicity, such that physiological studies with the resulting strains can be carried out in non-selective rich medium. This compares favourably with conventional approaches where conditional growth of haploid cells carrying an inactivating disruption of the chromosomal gene copy depends on a plasmid-carried *tetO*-regulated copy of the gene. We have repeatedly observed that the frequency of transformants raised with the substitution cassette for promoters of essential genes and carrying the correct substitution, does not differ significantly between haploid and diploid cells, with values from 5 to 20 transformants per microgram DNA. Although two different cassettes have been made with the $tetO_2$ and $tetO_7$ promoters respectively, in this study on three essential ORFs of chromosome XV the $tetO_2$-based cassette has been used exclusively, reasoning *a priori* that the strength of the $tetO_2$ promoter might be at least comparable with the original ORF promoters. Northern analysis demonstrated that expression levels from $tetO_2$ were even higher than from the original promoters, without this affecting significantly the growth of the promoter-substituted strains. In any case, modulability of expression from the *tetO* promoters (Garí *et al.*, 1997; and this work) may be useful for those situations where *tetO*-driven overexpression might negatively affect cell physiology.

Sequence analysis of the predicted products of YOL135c, YOL142w and YOL144w does not permit us to ascribe them to known protein families. They also do not exhibit putative transmembrane domains. Analysis of gene expression using the DNA microarray technique (DeRisi *et al.*, 1997) indicates that the three ORFs are expressed constitutively during the population growth cycle, excluding obvious regulatory effects on their expression caused by glucose or nutritional stress conditions.

Conditional abolition of expression of YOL135c had clear effects on cell growth after three to four generations. This rapid phenotypic effect may be explained because high amounts of protein could be required for normal cell function, or because high turnover rates of transcript and/or product would cause low levels of protein and thus cause rapid depletion of the latter upon transcription repression. Cells not expressing YOL135c become enlarged, many of them consisting of a round nucleated mother cell with a single long mononucleated bud, although a small proportion of buds contained two nuclei. This defect is characteristic of some morphogenetic mutants affected in cytoskeletal proteins (Botstein *et al.*, 1997; Cid *et al.*, 1995). Since the phenotypic manifestation of YOL135c expression inhibition occurs not very long after effector addition, this allows us to exclude major effects of prolonged cell death on morphology. However, the YOL135c protein does not exhibit significant homology with other proteins in data banks, so there is no evidence in support of its role in cell morphogenesis.

Phenotypic effects of inhibition of YOL142w expression are also manifest three to four generations after antibiotic addition. Cell proliferation ceases with uniform arrest at the G1 stage of the cell cycle. In these conditions cells do not become enlarged, as corroborated by the parallel kinetics of optical density and cell number evolution. This suggests that the observed growth inhibition is not an active G1 arrest by direct inhibition of cell cycle regulators (Lew *et al.*, 1997), but an inhibition of proliferation secondarily causing uniform G1 arrest. This is comparable with the effect caused by rapamycin when inhibiting translation initiation through interference with *TOR1* and *TOR2* functions (Barbet *et al.*, 1996). It may also be reminiscent of the homogeneous arrest of stationary phase cells upon nutrient starvation with an unbudded morphology and 1 N DNA content (Werner-Washburne *et al.*, 1993). Although the YOL142w protein does not share homology with proteins with known or predictable function, it is 38% identical and 61% similar over a stretch of 229 amino acids with the hypothetical product of the *Schizosaccharomyces pombe* chromosome I SPAC22A12.12c gene. Forty-one of the identical residues are also shared with the *Caenorhabditis elegans* F59C6.4 product (Wilson *et al.*, 1994). A KRYIP motif (residues 62 to 66 in YOL142w)

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is present in all three products, although no correlations with putative functions have been made.

Although doxycycline-mediated alteration of YOL144w expression affects growth kinetics of the promoter-substituted strain, growth does not become completely arrested. The observations that (i) heterozygous diploid-derived kanMX4-disrupted spores are able to form microcolonies that arrest growth after a few generations, and (ii) the cognate gene clone is necessary for vegetative growth of YOL144w-disrupted haploids, demonstrate that YOL144w function is not necessary for spore germination but instead for vegetative growth. In the case of this ORF the residual gene expression in the promoter-substituted construction may be sufficient to provide enough product for cell growth. Sequence data do not contain clues for YOL144w function, although the protein has a very acidic central region, with 50 aspartic and glutamic acid residues from amino acid 225 to 394 (Casas *et al*., 1995).

The method described here for the conditional expression of essential genes could be extended to the study of non-essential genes with clear phenotypes upon inactivation. Although we show that phenotypic alterations become rapidly visible for the genes studied in this work, for those cases where a more immediate effect would be desirable, doxycycline-induced transcriptional repression could act simultaneously with induction of specific degradation of the product in the new conditions. For that aim, the promoter-substitution cassette could be combined with such systems as a heat-inducible degron in-frame fused to the N-terminus of the *tetO*-driven protein (Dohmen *et al*., 1994), or the *tetO*-directed induction of the *UBR1* protein involved in the ubiquitin degradation pathway specifically targeted against the N-end of the studied protein (Moqtaderi *et al*., 1996).

The promoter-substitution cassettes described here are deposited at EUROSCARF (Institute for Microbiology, University of Frankfurt, Germany) for distribution among the scientific community.

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