Regulated expression of proteins in yeast using the *MAL61-62* promoter and a mating scheme to increase dynamic range

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Abbreviations: XGal, 5-bromo-4-chloro-3-indolyl-b-D-galactoside; DBD, DNA-binding domain; AD, transcription activation domain; NLS, nuclear localization signal; ORF, open reading frame.

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Abstract

The ability to express heterologous genes in yeast has become indispensable for many biological research techniques. Expression systems that can be regulated are particularly useful because they allow an experimenter to control the timing and levels of gene expression. Despite their many advantages, however, surprisingly few conditional expression systems are available for yeast. Moreover, of those that have been described, many are not ideal either because they have high background expression levels, low induced levels, or because they require restrictive growth conditions. Here we describe a conditional expression system that takes advantage of the yeast *MAL62* promoter (*MAL62*p), which can be controlled by adding maltose or glucose to the growth medium to induce or repress transcription, respectively. In addition, we use a mating scheme to dramatically increase the dynamic range of expression levels possible. We show that *MAL62*p background activity can be effectively eliminated by maintaining expression constructs in a *mal*$^-$ yeast strain. High-level expression can be induced in diploids formed by mating the *mal*$^-$ strain with a *MAL*$^+$ strain. A similar mating scheme may be useful for other conditional expression systems as well. Among other uses, this approach should aid high throughput yeast two-hybrid assays, which rely on maintaining large libraries of expression strains, which are eventually mated to conduct assays for protein interactions. We demonstrate a two-hybrid system in which *MAL62*p is used in conjunction with the yeast *GAL1* promoter to independently regulate expression of both hybrid proteins, and to allow detection of interactions involving toxic proteins.

1. Introduction

The yeast *Saccharomyces cerevisiae* has been a valuable tool for biological research, both as a model organism for understanding fundamental processes and as a system in which to express and study proteins from other organisms. A key to many studies in yeast is the ability to introduce and control the expression of transgenes using heterologous promoters. Among the most useful promoters for this purpose are those which are regulated by growth conditions. These conditional promoters allow an experimenter to control the timing and often the level of expression of the transgene. They also allow controlled, transient expression of proteins that are not tolerated when expressed constitutively. Despite the advantages of conditional expression systems, only a limited number have been described for yeast (e.g., see Kramer et al., 1984; Fujita et al., 1990; Poletti et al., 1992; Louvion et al., 1993; Mumberg et al., 1994; Mascorro-Gallardo et al., 1996; Ronicke et al., 1997; Belli et al., 1998).

The most widely used conditional promoter is derived from the yeast *GAL1*gene (reviewed in Johnston, 1987). The *GAL1* promoter (*GAL1*p) has several features that surpass other available promoters for conditional expression of genes. For example, *GAL1*p activity is easy to control by growing the yeast on different inexpensive sugars; galactose in the media induces and glucose represses transcription of *GAL1*p-linked transgenes (Yocum et al., 1984). *GAL1*p also allows relatively high levels of expression, and the levels of expression can be varied to some extent by changing the ratios of galactose and glucose in the media (St John and Davis,

1981; West et al., 1984). Finally, the activity of *GAL1*p has a very broad dynamic range; e.g., the activity of a fused gene can vary over 4 orders of magnitude between the fully repressed and fully induced states. While it would be useful to have other conditional expression systems, for example, to allow independent control of multiple genes in the same yeast, most of the other systems that have been described fall short in one or more of the properties that make *GAL*1p ideal. Other systems, for example, require expensive or toxic reagents to control expression, or display limited ranges of expression. Indeed, a common problem with other conditional promoters is high background expression even under repressing conditions, particularly where high-copy vectors are used to maximize the induced levels (e.g., Mumberg et al., 1994). This not only limits the dynamic range of expression levels, but also prevents studies with proteins that are detrimental to yeast.

One of the most widely used techniques requiring expression of proteins in yeast is the two-hybrid system, a simple genetic assay for protein-protein interactions (Fields and Song, 1989). In this assay the two proteins to be tested are expressed as hybrids, one fused to a transcription activation domain (AD) and one to a DNA-binding domain (DBD). If the two proteins interact with each other they will activate a reporter gene containing binding sites for the DBD. The successful use of this assay to characterize many individual proteins has inspired efforts to attempt comprehensive screens to examine protein interactions on a genome-wide scale (Evangelista et al., 1996; McCraith et al., 2000; Uetz et al., 2000; Walhout et al., 2000; Ito et al., 2001). These large-scale screens use a mating approach in which the DBD and the AD expression plasmids are initially introduced into two different haploid yeast strains (Bendixen et al., 1994; Finley and Brent, 1994). Interaction assays are then conducted by mating the two haploid strains and measuring reporter activity in the resulting diploid cells. For comprehensive screens, large libraries of haploid strains must be collected, amplified and stored prior to mating. Because many proteins can be detrimental to yeast viability, it would be of value to use expression constructs that remain inactive until the two-hybrid assay is to be performed. Although *GAL*1p has been used to express the AD fusion in one two-hybrid system (Gyuris et al., 1993), which has been adapted for large-scale screens (Finley and Brent, 1996; Buckholz et al., 1999), thus far only constitutive yeast promoters have been used to express DBD fusions in large-scale two-hybrid screens.

We reasoned that the mating assay used for large-scale two-hybrid assays could be exploited to generate a system for regulating the expression of DBD fusions. Here we describe such a system using the yeast *MAL62* promoter (*MAL62*p). *MAL62*p is induced by maltose and repressed by glucose (Ni and Needleman, 1990; Levine et al., 1992). We show that we can increase the dynamic range of this system by maintaining expression constructs in a *mal*$^-$ yeast strain, where *MAL62*p activity is effectively eliminated. To express the protein, the *mal*$^-$ strain can be mated with a *MAL*$^+$ strain. We show that this is a useful approach for expressing DBD-fused proteins in the yeast two-hybrid

system, and that it can be used along side *GAL1*p to express AD fusions. We also show that this approach can allow the introduction and maintenance of genes that are toxic to yeast when constitutively expressed.

2. Materials and Methods

2.1 Strains and Media

Haploid *S. cerevisiae* yeast strains RFY231 (MAT**a** *his3*, *trp1*Δ::*hisG*, *ura3*, 3LexAop-*Leu2*::*leu2* *MAL*$^+$) and RFY206 (MAT**a** *trp1*Δ::*hisG* *his3*Δ200 *leu2-3 lys2*Δ201 *ura3-52 mal*$^-$) have been described (Finley and Brent, 1994; Kolonin and Finley, 1998). Yeast YPD (yeast extract plus peptone and glucose) and minimal media lacking the indicated nutrients were prepared as described (Golemis et al., 1998-2001); -his lacks histidine, -ura lacks uracil, and -trp lacks tryptophan. Sugars added to the media included either 2% glucose (Glu), 2% maltose (Mal), or 2% galactose (Gal). 0.2% raffinose (for interaction assays) or 2% raffinose (for transcription assays) was added to the Mal, Gal and Mal/Gal media to enhance growth. Indicator plates contained 160μg/ml XGal.

2.2 Plasmids

pHZ5 was constructed by first amplifying a DNA fragment containing the *MAL61-62* divergent promoter (Ni and Needleman, 1990; Levine et al., 1992) region from a genomic clone, Yip5-MAL61-62 (kindly provided by Richard Needleman, Wayne State University School of Medicine, Detroit, MI), using a 5' primer, derived from within the *MAL61* coding region, x130 (5'CACGCCATTCTCGATCTCATCTAAG3'), and a 3' primer, x131 (5'GAGAGTTAATTAACCGGT**CAT**TTATGTAATTTAGTTAGC3'), which introduces an *Age*I site (underlined) downstream of the *MAL62* ATG (bold). The amplified product was digested with *Age*I and *Pac*I, which cuts at a natural site 3 bp upstream of the *MAL61* ATG, and subcloned into the PacI-AgeI sites of pPacAge-NLex to create pHZ5 (Fig. 1). pPacAge-NLex had been created by replacing the *ADH1* promoter of pEG202 (Golemis et al., 1998-2001) with a *Pac*I and *Age*1 site, and replacing the LexA coding region with LexA-NLS. pHZ5-NRT and pHZ5-RT were constructed by introducing the appropriate sequences (see Fig. 1) into pHZ5 by homologous recombination in yeast (Orr-Weaver and Szostak, 1983). Recombinant plasmids were extracted from yeast, purified through bacteria, and sequenced. The plasmid for expressing LexA from *GAL1*p, pGILDA (Golemis et al., 1998-2001), was obtained from D. Shayowitz and K. Kaiser (MIT, Cambridge, MA). The 2μm HIS3 plasmid used as a control in Fig. 2, pRFHM0, was constructed by deleting the *Sph*I fragment containing *lexA* sequences from pEG202. Plasmids used in the two-hybrid assay (Fig. 4) for expressing the *ADH1*p-driven LexA-DmCdk1 and -DmCdk2, pRFHM12 and pRFHM13, respectively, and for *GAL1*p-driven expression of an AD fused to *Drosophila* Cks1, cyclin D, Dacapo, cyclin J, and cyclin E were described previously (Finley and Brent, 1994; Finley et al., 1996). Plasmids for *MAL62*p-driven expression of LexA fused to DmCdk1 or DmCdk2 were constructed as follows. *Bam*HI-*Sal*I fragments containing the DmCdk1 and DmCdk2 coding regions were isolated from the corresponding PCR-amplified regions of pRFHM12 and pRFHM13, respectively, and then subcloned into

pHZ5 digested with *Bam*HI and *Sal*I. Plasmids for expression of LexA fused to *Drosophila* Ftz, Cks1, and G6, were constructed by recombination in yeast as part of a project to construct a *Drosophila* protein interaction map (C.A.Stanyon, L.Giot, J.Zhong, H.Zhang, J.Knight, M.McKenna, and R.L.Finley Jr., unpublished). The open reading frames from the ATG to the stop codon were amplified by PCR using gene-specific primers that also contained common 5' and 3' Recombination Tags (5RT and 3RT, Fig. 1) sequences homologous with sequences flanking the cloning site of pHZ5-NRT and pHZ5-RT. After co-transformation of yeast with the vector and PCR product, recombinant plasmids were purified and verified by sequencing prior to introduction into RFY206 and RFY231 (Fig. 3). Destination vectors (pHZ5attR and pNLexAattR) for use with the Gateway™ *in vitro* recombination cloning system (Invitrogen, Carlsbad, CA) were constructed by inserting the attR1-ccdB-Cm^R-attR2 cassette into pHZ5 and pNLex-NLS, which is identical to pHZ5 except that the LexA-NLS fusion proteins are expressed from *ADH1*p. The Drosophila cyclin A (DmcycA) ORF was subcloned from a sequence-verified Gateway™ entry clone into the two destination vectors using the LR recombination reaction to create pHZ5attB-DmCycA and pNLexattB-DmCycA. The sequence of the attB site in these plasmids on the coding strand is: 5'GTGACAAGTTTGTACAAAAAAG CAGGCTTA**ATG**3', from the last codon of the NLS (underlined) to the ATG from DmCycA (bold). To construct pHZ5attB-DmCycAΔSstII and pNLexattB-DmCycAΔSstII, in which the cyclin A ORF contains a frame shift, pHZ5attB-DmCycA and pNLexattB-DmCycA were linearized at a unique SstII site in DmCycA, the 3' overhangs were removed with Klenow, and the blunt ends were re-ligated. The resulting plasmids contain a frame shift at codon 46 of DmCycA, resulting in a truncated protein (DmCycA*), which is no longer toxic to yeast. Inserts of expression clones were verified by sequencing.

2.3 Immunobotting

Haploid or RFY206/RFY231 diploid yeast strains containing the LexA plasmids were grown on -his minimal media containing 2% of each of the indicated sugar: glucose, maltose, or galactose (Glu, Gal, Mal). Gal and Mal media also contained 2% raffinose. Diploid yeast also contained a *TRP1* AD vector and were grown on -his-trp minimal media. All cultures were grown to mid-log phase (OD600 ~1.0), harvested and resuspended in 50μl of SDS sample buffer per 1ml culture, and frozen at –80ºC before boiling and loading to SDS-10%-polyacylamide gel (PAGE), as previously described (Golemis et al., 1998-2001). Similar results were obtained by growing the cultures to saturation in minimal glucose media, then washing away the glucose and incubating for 24 hours in the new sugar. The amount of protein in each extract was quantified by analyzing digital images of Coomassie-stained PAGE gels with NIH Image. Subsequently, equal amounts of protein were loaded to each lane of a new PAGE gel and the proteins were transferred to Hybond-P PVDF membranes (Amersham Pharmacia, Piscataway, NJ). Immunoblotting was performed with rabbit polyclonal anti-LexA antisera and detected with an HRP-conjugated goat-

anti-rabbit secondary antibody (Biorad, Hercules, CA) using the ECL detection kit (Amersham Pharmacia, Piscataway, NJ). To verify equal loading, the membranes were stripped and re-probed with an antibody to yeast replication factor RFA (kindly provided by George Brush, Karmanos Cancer Institute, Detroit, MI).

2.4 Yeast transcription and two-hybrid assays.

All yeast contained the sensitive *lacZ* reporter plasmid pSH18-34 (provided by Steve Hanes, Axelrod Institute, Albany, NY), which contains *URA3*, 2μm, and six LexA binding sites (operators) upstream of *lacZ* (Golemis et al., 1998-2001). Haploid strains containing the indicated *LexA* plasmids were streaked to -his-ura minimal media containing 2% glucose and XGal. Diploid strains also contained a *TRP1* AD plasmid, pRF4-5o (unpublished) derived from pJG4-5 (Gyuris et al., 1993), and were selected on –his-ura-trp media containing 2% glucose plus XGal. Yeast two-hybrid interaction mating assays were performed as previously described (Finley and Brent, 1994). Briefly, horizontally streaked RFY206 strains containing pSH18-34 and the indicated *HIS3* LexA plasmids were mated with vertically streaked RFY231 strains containing the indicated *TRP1* AD plasmids on YPD media. After 24 hours growth at 30ºC, in which time diploids form at the intersections of the two strains, the YPD plate was replicated to -ura-his-trp Glu plates to select for diploids, then replica-plated to -ura-his-trp XGal indicator plates containing the indicated sugars (Fig. 4).

2.5 Transformation efficiency assay

Plasmids, pNLexattR, pHZ5attR, pNLexattB-DmCycA, pHZ5attB-DmCycA, pNLexattB-DmCycAΔSstII, and pHZ5attB-DmCycAΔSstII were prepared using Qiagen Tip-100 columns. Yeast strain RFY206 was transformed with 500ng of each plasmid using the LiOAc method (Golemis et al., 1998-2001). After the heat shock step, yeast were resuspended in 50μl -his Glu medium and dilutions were plated onto –his Glu plates. Colonies were counted after 4 days incubation at 30ºC.

3. Results and Discussion

3.1 Vectors for expressing LexA fused proteins from the MAL62 promoter

To test the *MAL62* promoter for use in conditional expression of proteins in yeast we constructed vectors for expression of LexA and LexA fusions. pHZ5 contains the *MAL61-62* intergenic region oriented with *MAL62*p driving expression of LexA fused to a nuclear localization signal (NLS) (Fig. 1). We chose *MAL62*p because it was previously shown to express a heterologous gene, *lacZ*, at a higher level than the *MAL61* promoter under both inducing (2% maltose) and non-inducing conditions (2% galactose) (Levine et al., 1992). The high level of expression possible in galactose would allow *MAL62*p to be used alongside *GAL1*p, for example, to enable conditional expression of two different proteins in the same strain (see below). We also constructed versions of pHZ5 with and without the NLS (pHZ5-NRT and pHZ5-RT). These plasmids also contain common recombination tag sequences (5RT and 3RT, Fig. 1) upstream and downstream of the multiple cloning site (MCS), to allow open reading frames (ORFs) to be inserted using recombination in yeast (Orr-Weaver and Szostak, 1983). To insert an ORF into one of these vectors, the ORF can be amplified with flanking 5'RT and 3'RT sequences, then used to co-transform yeast along with the vector linearized at the MCS; the yeast gap repair machinery inserts the ORF into the vector. Correct insertions can be achieved with over 85% efficiency using these 20bp recombination tags (data not shown). The ability to insert ORFs containing common tags into multiple vectors is particularly useful for characterizing large sets of ORFs (e.g., Hudson et al., 1997). We have also constructed versions of pHZ5 that have recombination tags compatible with the Gateway™ *in vitro* recombination cloning system (see Materials and Methods).

3.2 Strain-dependent expression of proteins using the MAL62 promoter

We used immunoblotting to analyze the levels of LexA expressed from *MAL62*p in a *MAL*$^{+/-}$ diploid yeast strain grown under different conditions (Fig. 2A). Our results show that the steady-state levels of LexA expressed from *MAL62*p are similar in yeast grown in maltose, or in maltose plus galactose, and only slightly less in galactose alone (Fig. 2A). This result is consistent with the fairly high level of background activation previously observed for the native *MAL62* gene under non-repressing and non-inducing conditions (Levine et al., 1992). The level of LexA expression from *MAL62*p is comparable with the level produced by the *ADH1* promoter (*ADH1*p) (Fig. 2A), which has been used to constitutively express LexA fusions in the yeast two-hybrid system (Gyuris et al., 1993; Vojtek et al., 1993). As expected, under repressing conditions (2% glucose), the steady state level of LexA is reduced relative to the induced or non-induced levels. Nevertheless, a substantial amount of LexA accumulates even in the cells grown in glucose. By comparison, LexA expressed from *GAL1*p is undetectable in yeast grown in glucose, and the ratio of induced to repressed levels is high (Fig. 2A).

The lack of full repression of *MAL62*p could compromise its usefulness for studies requiring conditional expression, where often the

primary goal is to minimize protein expression until it is needed for a particular assay. Thus, to further reduce the repressed activity of *MAL62*p we introduced the vector into a *mal*$^-$ yeast strain, RFY206; *mal*$^-$ strains often lack the transcription factor encoded by *MAL63*, or the maltose transporter encoded by *MAL61*, both of which are required for activation of *MAL62*p (Federoff et al., 1983). As shown in Fig. 2B, the steady state level of LexA expression from *MAL62*p is barely detected in RFY206 grown in either glucose or maltose (with raffinose added to support growth). Thus, expression from MAL62p can be minimized by maintaining the plasmid in a *mal*$^-$ haploid yeast strain. Expression can be induced, however, by mating the *mal*$^-$ strain with a *MAL*$^+$ strain (Fig. 2A,B). This provides an approach to increase the dynamic range over which expression can be regulated (compare Fig. 2B, lanes 2 and 6). To test the utility of regulating *MAL62*p by mating we performed three functional assays: expression of transcription factors fused to LexA, expression of DNA-binding domain (DBD) fusions in the yeast two-hybrid system, and expression of a toxic protein.

3.3 Regulating expression of MAL62p by mating haploid yeast strains

We expressed LexA fused to two different transcription activators and measured their ability to activate a sensitive *lacZ* reporter gene containing upstream LexA operators. We used *Drosophila* Cks1 and ftz, which were previously shown to activate a high and moderate level of transcription, respectively, when fused to a DNA-binding domain such as LexA (Fitzpatrick and Ingles, 1989; Finley and Brent, 1994). We compared *MAL62*p with *ADH1*p, which has been used extensively to express proteins in yeast. As shown in Fig. 3, expression of LexA-activators from *ADH1*p leads to high expression of the *lacZ* reporter in both the *MAL*$^+$ (RFY231) and *mal*$^-$ (RFY206) haploid strains, and in the *MAL*$^{+/-}$ diploid that results from mating RFY231 and RFY206. In contrast, LexA-activators expressed from *MAL62*p result in detectable expression of *lacZ* only in the *MAL*$^+$ strain, and not in the *mal*$^-$ strain (Fig. 3). When the *mal*$^-$ strain was mated with the *MAL*$^+$ strain, the *MAL62*p expressed sufficient LexA-fused activator to activate the *lacZ* reporter in the diploid cells, and the *lacZ* levels in the diploid cells were similar for *ADH1*p and *MAL62*p. Combined with our immunoblotting results (Fig. 2B), these results show that a *mal*$^-$ haploid strain can be used to minimize the background level of a gene expressed from the *MAL62*p, and that expression can be induced in diploids generated by mating the *mal*$^-$ strain with a *Mal*$^+$ strain.

3.4 Use of MAL62p in two-hybrid interaction mating assays

Next, we compared *MAL62*p and *ADH1*p in two-hybrid interaction-mating assays. Fig. 4 shows the results of interaction mating assays with previously characterized *Drosophila* cell cycle regulators. The DBD-fused proteins were LexA fusions to *Drosophila* Cdk1 or Cdk2 (DmCdk1 and DmCdk2). These DBD proteins were expressed from *ADH1*p or *MAL62*p in the *mal*$^-$ yeast strain RFY206. This strain was mated with the *MAL*$^+$ strain, RFY231, expressing various proteins fused to an activation domain (AD), from *GAL1*p. The AD-fused proteins included strong, moderate, and weak

Cdk-interacting proteins, as well as two Cdk-interacting proteins that inhibit yeast growth (*Drosophila* cyclins EI and EII; Fig. 4, lanes 5 and 6). Figure 4 shows that *MAL62*p works approximately as well as *ADH1*p for expressing DBD fusions in the two-hybrid system. For example, both moderate and weak interactions were detected when LexA-Cdk proteins were expressed from *MAL62*p.

The interaction assays in Fig. 4 also suggest that using *MAL62*p to express DBD proteins and *GAL1*p to express AD proteins can broaden the range of detectable interactions. The interactions detected on galactose or maltose plus galactose were the same, as might be expected from our immunoblotting results showing that *MAL62*p and *GAL1*p express similar levels of LexA or AD, respectively, under these conditions (Fig 2A). On maltose, however, some new interactions are detected while others become less obvious. Interactions with AD-fused Dap, for example, were best detected on media containing galactose (Fig 4, column 3). Interactions with the AD-fused cyclin J (Fig. 4, column 4), on the other hand, were best detected on maltose medium, where we expect the protein levels of the AD fusions to be lower (e.g., see Fig. 2A). One possible explanation for this counterintuitive observation is that the interaction of LexA fusions with cyclin J multimers may inhibit DNA binding. Interestingly, although expression of the AD from *GAL1*p in maltose cannot be detected by immunoblotting (Fig. 2A), sufficient AD fusion protein is present to allow activation of the sensitive two-hybrid reporter. This is consistent with previous results showing that a very strong interaction can be detected with a *GAL1*p-driven AD protein even in glucose, where expression is significantly repressed, and should be even lower than in maltose (e.g., (Finley and Brent, 1994)).

Another type of interaction that can be variably detected by changing the expression levels of the AD and DBD is an interaction involving an AD-fused protein that inhibits yeast growth. *Drosophila* cyclins EI and EII, for example, are inhibitory when expressed as fusions to an AD (Fig. 4, columns 5 and 6; data not shown). As shown in Fig. 4, the high-level expression of the cyclin E proteins induced in media containing galactose results in minimal yeast growth. This inhibition is enhanced in the presence of Cdk2, the natural kinase partner for cyclin E. The lower level of cyclin E expression in maltose, however, allows growth and detection of interactions, for example, with DmCdk2. Thus, we can vary the expression of the AD from very high (Gal), to low (Mal), while maintaining high expression of he DBD. Combined, these results suggest that different protein interactions can be detected by varying the expression levels of the AD and DBD. The ability to vary both AD and DBD independently (e.g., in maltose or galactose) should increase the number of interactions that can be detected.

3.5 Regulated expression of toxic proteins using MAL62p

One goal of using a conditional expression system is to enable transient expression of proteins that may be detrimental to yeast viability. Proteins that are particularly toxic will inhibit growth, as is the case with *Drosophila* cyclin E (e.g., see Fig. 4). Attempts to

express such proteins from a constitutive promoter generally fail at the step of trying to introduce the expression construct into yeast. For less toxic proteins, it may be possible to obtain yeast transformed with a constitutively expressing construct, but the protein will eventually be down-regulated over the course of several generations due to selection of yeast expressing lower levels the protein. With a conditional expression system, however, it becomes possible to introduce the expression construct and grow the yeast in the absence of the toxic protein, as has been demonstrated with *GAL1*p (Barnes and Rine, 1985; Burke et al., 1989). The protein can then be transiently expressed in order to perform particular assays, such as two-hybrid interaction assays (see Fig. 4).

To test whether *MAL62*p can be used to express a toxic protein in yeast, we subcloned the ORF for a very toxic protein, *Drosophila* cyclin A (DmCycA), into the Gateway expression vectors, pHZ5attR and pNLexattR (see Materials and Methods), which express LexA fusions from *MAL62*p or *ADH1*p, respectively. As shown in Fig. 5A, the presence of the DmCycA ORF in the *ADH1*p vector dramatically reduced transformation efficiency relative to the parent vector. Indeed, only a few transformant colonies were obtained with the construct that constitutively expressed LexA-DmcycA. Introduction of a frame shift into the cyclin A ORF restored the ability of the plasmid to transform yeast (Fig. 5A, DmCycA*), indicating that the loss of transformation efficiency was due to expression of the toxic cyclin A protein. In contrast, insertion of the cyclin A ORF into the *MAL62*p vector reduced the transformation efficiency by only 2-3-fold; allowing hundreds of transformant colonies to be obtained (Fig. 5A). These results show that *MAL62*p is sufficiently inactive in the *mal*$^-$ strain grown on glucose to allow the introduction of a gene whose expression severely inhibits yeast growth. Moreover, transient expression of the toxic DmCycA protein using *MAL62*p enabled detection of protein-protein interactions in a yeast two-hybrid interaction-mating assay (Fig 5B).

3.5 Conclusions

We set out to develop a yeast conditional expression system that would share some of the useful properties of *GAL1*p. The system that we have described utilizes *MAL62*p, which allows simple control of heterologous gene expression using inexpensive sugars and requires no exotic inducer or repressor. Use of a *mal*$^-$ yeast to minimize background activity, and mating with a *MAL*$^+$ strain to induce high level of expression, allows a broad dynamic range of expression levels, similar to *GAL1*p. Finally, differences in the inducers of *GAL1*p and *MAL62*p activity allow independent control over the expression levels of two genes in the same yeast cells.

The ratio of induced to repressed expression levels can be maximized by mating *mal*$^-$ and *MAL*$^+$ yeast strains. A similar mating scheme could be used with other promoter systems, including promoters that are not normally amenable to regulated expression. This approach may be useful, for example, in combination with a promoter that expresses at a higher level than *GAL1*p

or *MAL62*p for high-level expression of heterologous proteins. A strain lacking a specific transcription activator for the promoter could easily be constructed and used in conjunction with a strain that constitutively expresses the transcription factor. This approach is analogous to the UAS-Gal4 system in *Drosophila*, in which one strain contains a transgene with upstream Gal4-binding sites (UAS) and the other strain expresses the Gal4 transcription factor under control of various promoters (Brand and Perrimon, 1993). Using a mating scheme to control promoter activity avoids the need for exogenous inducers or repressors, which may in some cases be expensive or interfere with particular yeast assays.

The mating scheme and MAL62p provide a method for tightly regulating expression of DBD fusion proteins in the yeast two-hybrid system. This may be particularly useful for comprehensive yeast two-hybrid screens. These screens require that large libraries of yeast strains be constructed that contain expression constructs for a diverse set of ORFs. The libraries, which are often maintained in an array format, must be amplified a number of times and stored for extended periods before the actual two-hybrid assays are performed. Conditional expression of both the AD and DBD proteins using *GAL*1p and *MAL62*p can allow assays with proteins that are normally toxic to yeast. In addition, our two-hybrid assays indicate that the ability to alter the relative levels of the AD and DBD proteins can be exploited to detect different sets of interactions. This capability may be of great benefit to large-scale yeast two-hybrid studies as it would enable testing for interactions at various expression levels without the need to subclone each into multiple vectors.

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Figures

Fig. 1. Vectors for expression of LexA fusions from *MAL62*p.
pHZ5 contains the *MAL62* promoter and first codon fused to codons 2-202 of *lexA*, followed by a sequence encoding a 8 amino acid nuclear localization signal (NLS) and a multiple cloning site (MCS). All of these vectors share the same backbone, which includes the pMB1 origin of replication, AmpR gene, yeast *HIS3* and 2 μm origin of replication, and the *ADH1* transcription terminator following the MCS. All vectors have the same LexA sequence (Common 5' Sequence) followed by different sequences preceding the ADH terminator. pHZ5-NRT contains a recombination tag (5RT) between the sequences encoding the NLS and MCS. pHZ5-RT contains the same 5RT but lacks the NLS. 3RT is a sequence that can be used as a 3' recombination tag along with 5RT to insert open reading frames by recombination in yeast (see text). Sites in the MCS are unique unless underlined.

Fig. 2. Expression of LexA in MAL^+, mal^-, and $MAL^{+/-}$ strains using MAL62p.
Immunoblots containing protein extracts from a $MAL^{+/-}$ diploid (A), or MAL^+ or mal^- haploid yeast strains (B), were probed with anti-LexA antibodies or anti-HA to recognize AD-HA. Equal amounts of total protein were loaded to each lane based on image analysis of Coomassie-stained gels. Asterisks indicate cross-reacting yeast proteins that are not LexA (i.e., they are present in extracts lacking LexA). LexA was expressed from the indicated promoters on 2μm plasmids pHZ5 (*MAL62*p), pNLex (*ADH1*p), or pGILDA (*GAL1*p). The AD-HA fusion protein was expressed from *GAL1*p in the 2μm AD vector, pJG4-5. Lanes labeled C contain extracts of yeast grown in Gal/Mal but lacking the LexA or AD-HA coding sequences.

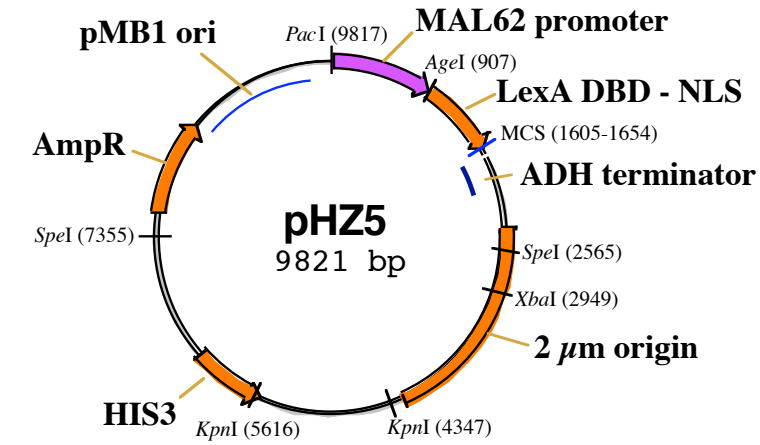
Fig. 3. Regulated expression of transcription factors from *MAL62*p by mating. The ability of LexA-fused transcription factors to activate a sensitive LexA-operator-*lacZ* reporter was assayed on glucose XGal indicator plates. LexA fused to *Drosophila* proteins (ftz or Cks1) that activate transcription in yeast, either with or without a nuclear localization signal (NLS), were expressed from *ADH1*p or *MAL62*p in three different yeast strains: a MAL^+ haploid yeast strain (RFY231), a mal^- haploid strain (RFY206) or the diploid exconjugant from these two strains ($MAL^{+/-}$). Yeast with the indicated expression constructs were streaked to XGal indicator plates containing glucose and grown for 2 days (RFY231 and diploids) or 4 days (RFY206); note that RFY206 is a slow-growing strain. The three columns of diploids were generated in independent matings. MAL6p-G6 encodes a LexA fusion that does not activate transcription.

Fig. 4. Yeast two-hybrid assays with regulated expression of both AD and DBD fusion proteins. Interaction mating assays between different LexA DBD strains (rows) and AD strains (columns). The DBD strains expressing LexA (vector), or LexA fused to *Drosophila* Cdk1 or Cdk2 either from the constitutive *ADH1*p or from *MAL62*p, were mated with AD strains containing the AD vector with no insert (v), or the vector encoding AD fused to various *Drosophila* cell cycle regulators; Cks1 (1), cyclin D (2), Dacapo (3), cyclin J (4), cyclin EI (5), and cyclin EII (6). All AD fusions are expressed from *GAL1*p. After the DBD and AD strains were mated, they were replica-plated to

media that selects for diploid growth and that contained XGal and 2% maltose plus 2% galactose (Mal/Gal), 2% galactose (Gal), or 2% maltose (Mal). The blue color resulting from activation of the *lacZ* reporter gene indicates an interaction between the DBD and AD. Lack of growth for the cyclin E strains in the presence of galactose is due to the toxicity of high-level expression of AD-cyclin E proteins. On Mal media the *GAL1*p is not induced (e.g., see Fig. 2) and the lower level of AD-cyclin E protein is tolerated.

Fig. 5. Expression of a toxic protein using MAL62p. (A) Yeast strain RFY206 was transformed with plasmids expressing LexA alone (Vector), LexA fused to DmCycA (DmCycA), or LexA fused to a truncated cyclin A (DmCycA*). The average number of transformants for each plasmid from nine independent transformations is shown. Error bars indicate the standard deviation. Fewer than 4 transformant colonies/500ng plasmid were obtained for each transformation with the plasmid expressing LexA-DmCycA from the constitutive *ADH1*p. (B) Detection of interactions with Dmcyclin A expressed from *MAL62*p. Interaction mating assay performed as described in Fig. 4 and Materials and Methods. Two separate RFY206 transformants containing pHZ5attB-DmCycA, or a vector expressing LexA fused to *Drosophila* cyclin D (*ADH1*p-DmCycD) were mated with four different RFY231-derived strains that expressed AD fused to DmCdk2 (column 1), DmCdk1 (column 2), or DmDiM1 (columns 3 and 4). Growth on the –leucine plates indicates that the *LEU2* reporter is active. Cyclin D interacts with DmCdk2 and DiM1, whereas DmCycA interacts strongly with DiM1.

Fig. 1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PacI (9817)
MAL62 promoter
AgeI (907)
LexA DBD - NLS
MCS (1605-1654)
ADH terminator
AmpR
pHZ5
9821 bp
SpeI (7355)
SpeI (2565)
XbaI (2949)
2 μm origin
HIS3
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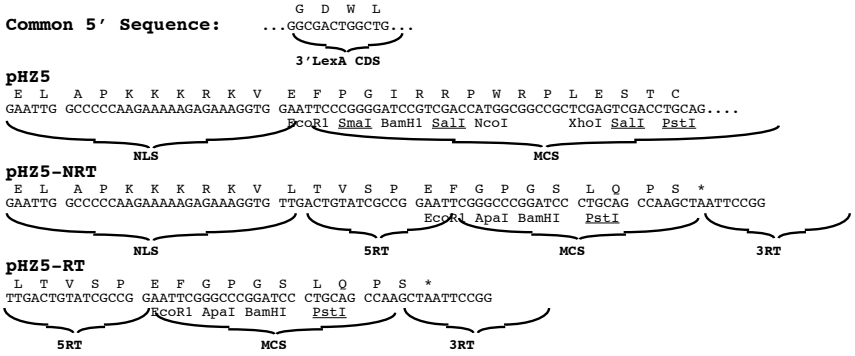
Common 5' Sequence:
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3'LexA CDS
pHZ5
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GAATTG GCCCCCAAGAAAAAGAGAAAGGTG GAATTCCCGGGGATCCGTCGACCATGGCGGCCGCTCGAGTCGACCTGCAG....
EcoR1 SmaI BamH1 SalI NcoI XhoI SalI PstI
NLS
MCS
pHZ5-NRT
E L A P K K K R K V L T V S P E F G P G S L Q P S *
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EcoR1 ApaI BamHI PstI
NLS
5RT
MCS
3RT
pHZ5-RT
L T V S P E F G P G S L Q P S *
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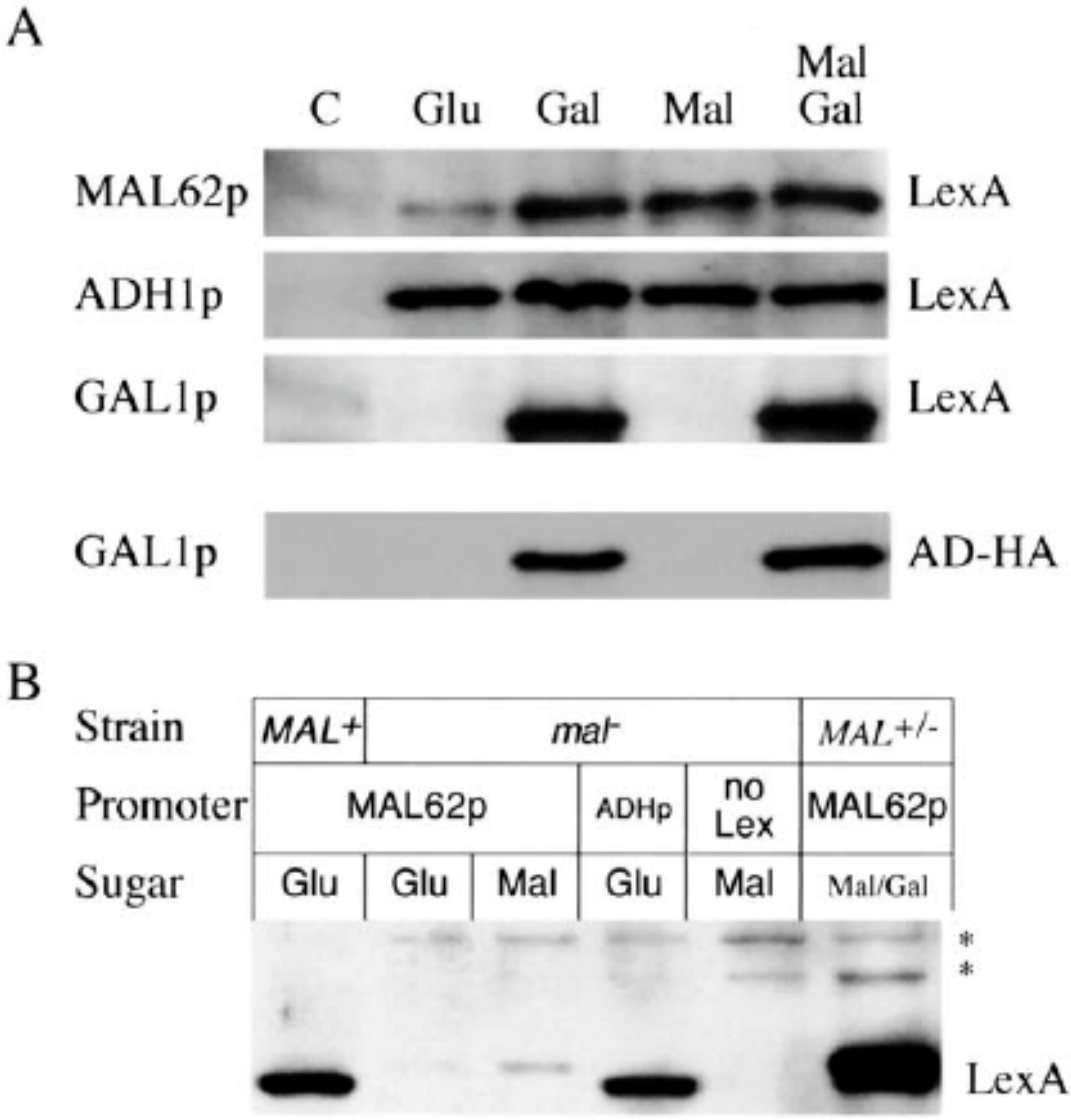
5RT
MCS
3RT

Figure 2.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Gal
Mal
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LexA
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LexA
GAL1p
LexA
GAL1p
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Strain
MAL+
mal-
MAL+/-
Promoter
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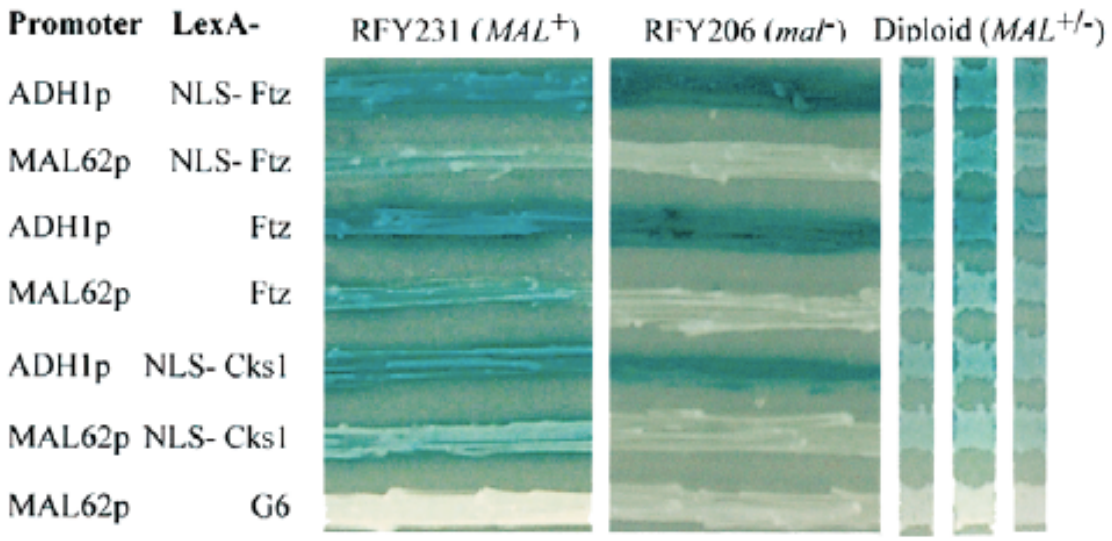
Glu
Mal
Glu
Mal
Mal/Gal
*
*
LexA

Figure 3.


Promoter
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RFY231 (MAL+)
RFY206 (mal-)
Diploid (MAL+/-)
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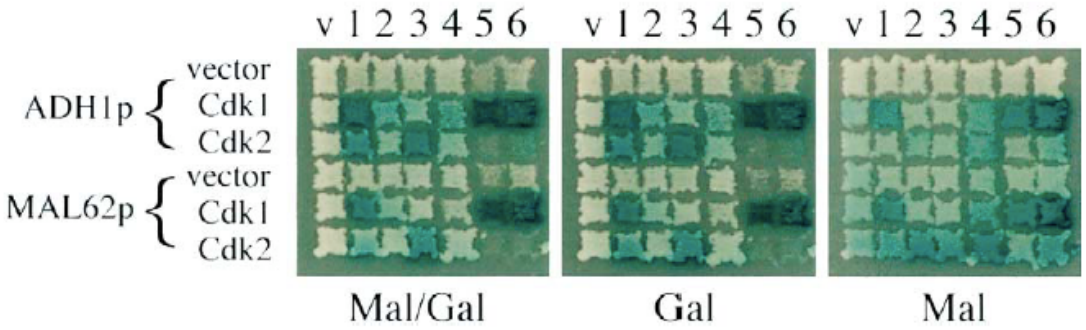
MAL62p NLS- Ftz
ADH1p Ftz
MAL62p Ftz
ADH1p NLS- Cks1
MAL62p NLS- Cks1
MAL62p G6

Figure 4.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v 1 2 3 4 5 6
v 1 2 3 4 5 6
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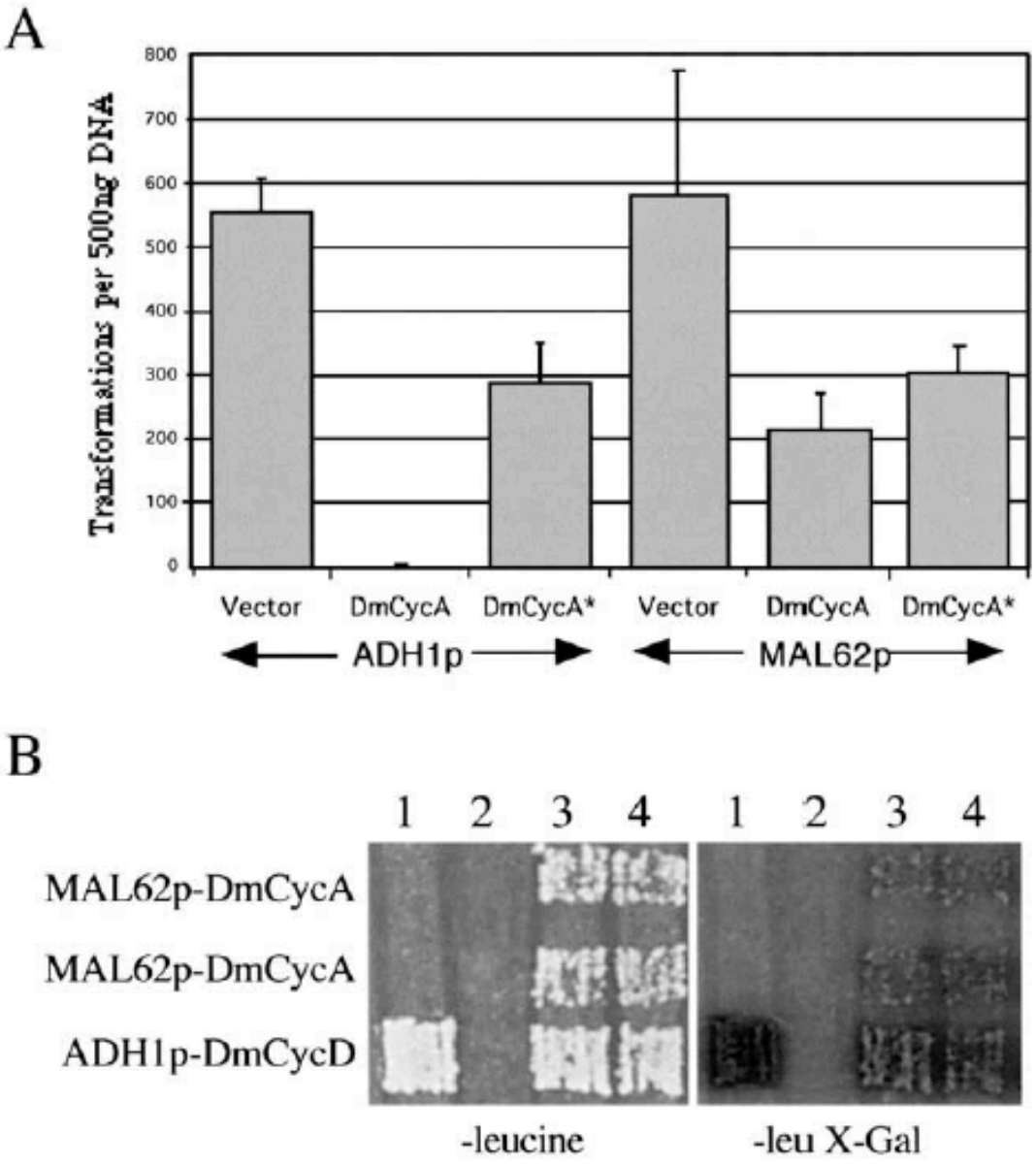
vector
Cdk1
Cdk2
MAL62p
vector
Cdk1
Cdk2
Mal/Gal
Gal
Mal

Figure 5.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Transformations per 500ng DNA
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600
500
400
300
200
100
0
Vector
DmCycA
DmCycA*
Vector
DmCycA
DmCycA*
ADH1p
MAL62p
B
1 2 3 4
1 2 3 4
MAL62p-DmCycA
MAL62p-DmCycA
ADH1p-DmCycD
-leucine
-leu X-Gal