Production of Two *S. cerevisiae* Strains Designed to Enhance Utilization of the Yeast Two Hybrid System

by

Kevin Campbell Graham

Submitted to the Faculty of Graduate Studies in Partial Fulfilment of the Requirements of

Degree of Master of Science

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PRODUCTION OF TWO S. *cerevisiae* STRAINS DESIGNED TO ENHANCE UTILIZATION OF THE YEAST TWO HYBRID SYSTEM

BY

KEVIN CAMPBELL GRAHAM

A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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And finally to my family. Mom, Dad and Cheryl, your love and support have kept me going. If you are as proud of me as I am of you, then that is all the reward I need.

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Abbreviations

3-AT = 3-amino-1,2,4-triazole
APS = ammonium persulfate
BEB = band elution buffer (50 mM Tris•Cl pH 7.6, 0.2 M NaCl, 1 mM EDTA, 0.1% SDS)
βME = ß-mercaptoethanol
bp = base pair(s)
BSA = bovine serum albumin
CDS = coding sequence
ddH_2O = double distilled water
DMF = N-N-dimethylformamide
DTT = dithiothreitol
EDTA = ethylene diamintetraacetic acid
EtBr = ethidium bromide
FOA = 5-fluoro-orotic acid
LB = Luria Bertani
LiAc = lithium acetate
MCS = multiple cloning site
ONPG = ortho-nitrophenyl ß-D-galactopyranoside
PEG = polyethylene glycol
SC = synthetic complete
SDS = sodium dodecyl sulfate
SSC (20X) = 3 M NaCl, 0.3 M Sodium citrate ($Na_3C_6H_5O_7•2H_2O$), pH 7.0
TAE (1X) = 0.04 M Tris-acetate, 0.001 M EDTA
TBE (1X) = 0.09 M Tris-borate, 0.001 M EDTA
TE = 10 mM Tris-HCl pH 8.0; 1 mM EDTA
TEMED = *N',N',N',N'*-tetramethylethylene diamine
TGE = 50 mM glucose, 25 mM Tris•Cl (pH 8.0), 10 mM EDTA
UV = ultraviolet irradiation
Xgal = 5-bromo-4-chloro-3-indoyl-ß-D-galactopyranoside

ABSTRACT

The two-hybrid system is a molecular genetic test in *Saccharomyces cerevisiae* that can identify and study protein:protein interactions *in vivo*. This system can be used to identify the motifs that are involved in the interaction of two proteins. Alternatively, the system can be used to identify novel binding partners of a protein of interest. Currently, all yeast strains used for the two-hybrid system have numerous features which prevent full utilization of the system. This thesis describes the construction of two new and improved yeast strains for use in the two-hybrid system. The yeast genes *URA3*, *LYS2* and *LEU2* were manipulated to form three disruption cassettes. These cassettes were used to disrupt the *URA3* and *LYS2* genes and stably integrate *GAL1-lacZ* and *GAL1-HIS3* reporter genes. Both reporter gene constructs have low levels of basal activity allowing for the detection of weak protein:protein interactions. The strain KGY37 can be used with $Gal4_{BD}$ fusions and can accommodate three independently replicating plasmids to enable the study of phosphorylation-mediated two-hybrid interactions. The second yeast strain, KGY94, provides a nutritional reporter gene (*HIS3*) for use with $lexA_{BD}$ fusions. These new strains will allow easier identification of previously uncharacterized two-hybrid protein:protein interactions.

1. INTRODUCTION

The yeast *Saccharomyces cerevisiae* has been used for decades as a biochemical and genetic tool for the study of many eukaryotic cellular processes. The ability of this species to grow in both the haploid and diploid states has greatly facilitated the use of genetic approaches to the analysis of biological processes. This is because the haploid state phenotype directly reflects the genotype (Lacroute, 1975). The mating of different haploid strains to form stable diploids permits the interactions of different mutations to be tested, allowing the determination of complementation groups (Herskowitz, 1988). Other advantages of *S. cerevisiae* include a short doubling time (100 min), unicellular morphology, non-pathogenicity and the ability to grow both in liquid and on solid medium. All of these features, combined with recent advances in yeast molecular biology, and the complete sequencing of the yeast genome, have allowed the genetics of this organism to progress further than perhaps any other eukaryotic organism and have contributed greatly to its use as a model system (Sherman, 1991; Oliver *et al.*, 1992; Johnston *et al.*, 1994; Kaiser *et al.*, 1994; Galibert *et al.*, 1996).

The usefulness of yeast has expanded dramatically with the advent of recombinant DNA technology and the development of transformation protocols (Beggs, 1978; Hinnen *et al.*, 1978; Ito *et al.* 1983), resulting in a "new age of yeast genetics" (Struhl, 1983). The transfer of DNA between strains using transformation has allowed the isolation of genes by the complementation of mutant alleles with cloned fragments of genomic DNA. This capability has led to an increased understanding of many mammalian cellular processes by the study of mammalian genes in conjunction with their yeast homologues (Sherman, 1991). One significant resource to emerge as a result of these advances is the two-hybrid system, an *in vivo* genetic assay for protein:protein interactions (Fields and Song, 1989).

1.1 Markers and Transformation

The manipulation of yeast as a molecular biological tool revolves around the use of nutritional genetic markers as selectable genes on plasmid vectors, and the introduction of these vectors by transformation into various yeast strains (Beggs, 1978; Hinnen *et al.*, 1978). The production of independently replicating vectors for yeast required some selection for their maintenance in the yeast cell. Auxotrophic mutations which interrupt biosynthetic pathways and require the addition of a specific nutrient to media to support growth are utilized for this purpose. When a plasmid containing a working copy of a mutated gene (a selectable marker) is transformed into the yeast strain it complements the mutated gene in the biosynthetic pathway and permits growth on medium lacking the specific nutrient. For example, the *ura3-52* allele (Rose and Winston, 1984), a non-reverting Ty element insertion in the coding sequence of the *URA3* gene (Rose *et al.*, 1984), is a commonly utilized auxotrophic mutation. Loss of the *URA3* gene product interrupts the uracil biosynthesis pathway and necessitates either the addition of uracil to growth medium, or the presence of a plasmid harboring a functional *URA3* gene.

The original methods used to introduce DNA into yeast cells by transformation involved enzymatic digestion of the yeast cell wall and treatment of the resulting spheroplast with polyethylene glycol (PEG) to permit DNA uptake, followed by regeneration of the cell wall on special growth medium (Hinnen *et al.*, 1978; Beggs, 1978). This method was laborious and gave variable results when different strains were used. Currently it is used for the transformation of YACs (yeast artificial chromosomes) and large pieces of DNA which are not easily transformed by other methods (Burke *et al.*, 1987; Gietz and Schiestl, 1995). An alternative method was developed by Ito *et al.* (1983) who used lithium acetate (LiAc) and PEG to stimulate DNA uptake without the need for cell wall digestion. This protocol was easier and did not require special regeneration medium, however it resulted in a decrease in overall transformation efficiency (Ito *et al.*, 1983). Schiestl and Gietz (1989) improved the efficiency of LiAc/PEG transformation by

including single stranded carrier DNA (ssDNA) in the transformation reaction. Further improvements of the method which further increase transformation efficiency have been published (Gietz *et al.*, 1992; Schiestl *et al.*, 1993; Gietz and Woods, 1994; Gietz *et al.*, 1995, Gietz and Schiestl, 1995). Yeast can also be transformed by electroporation, however this technique is limited in the amount of DNA which can efficiently be transformed (Becker and Guarente, 1991; Manivasikam and Schiestl, 1993). With the development of efficient methods of transformation new plasmid vectors were being developed to aid in molecular analysis.

1.2 Yeast - *E. coli* Shuttle Vectors

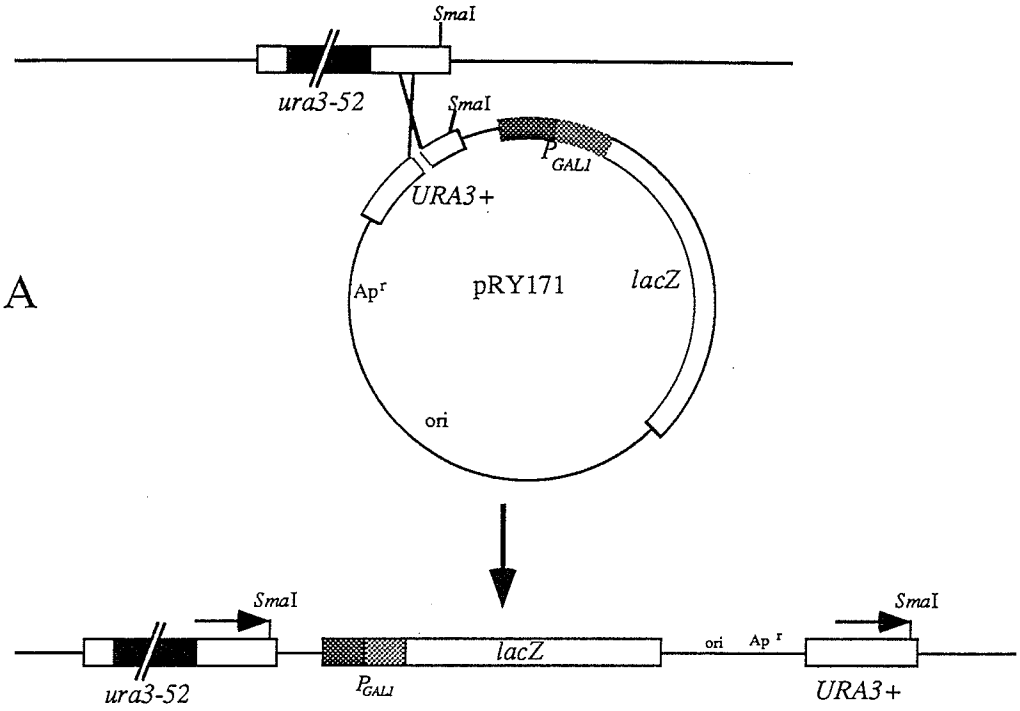
The vectors used for transformation into yeast have several important features. In addition to a yeast DNA replication origin and a selectable marker for maintenance in yeast, they also have a bacterial origin of replication and an antibiotic resistance gene for selection and maintenance in *E. coli*. In the absence of a yeast origin of replication, plasmids can only be maintained by integration into the genomic DNA by recombination into a locus corresponding to yeast DNA in the plasmid (Hinnen *et al.*, 1978; Hicks *et al.*, 1978; Struhl *et al.*, 1979; Scherer and Davis, 1979; Orr-Weaver *et al.*, 1981). This event is infrequent with a circular DNA molecule, but if the plasmid is first linearized with a restriction enzyme which cuts in the yeast DNA sequences, the high efficiency of homologous recombination in *S. cerevisiae* results in an increase in the frequency of integration. The free DNA ends initiate recombination and result in a higher efficiency of integration (Orr-Weaver *et al.*, 1981; 1983). If the plasmid contains a contiguous gene which is cleaved the recombination event produces a direct repeat of the gene separated by the plasmid DNA as depicted in Figure 1A. This type of site specific integration, which produces a duplication of the target locus, is unstable. The direct repeat reverts at a frequency of 1% in 15 generations or

Figure 1 Integration of transformed DNA fragments.

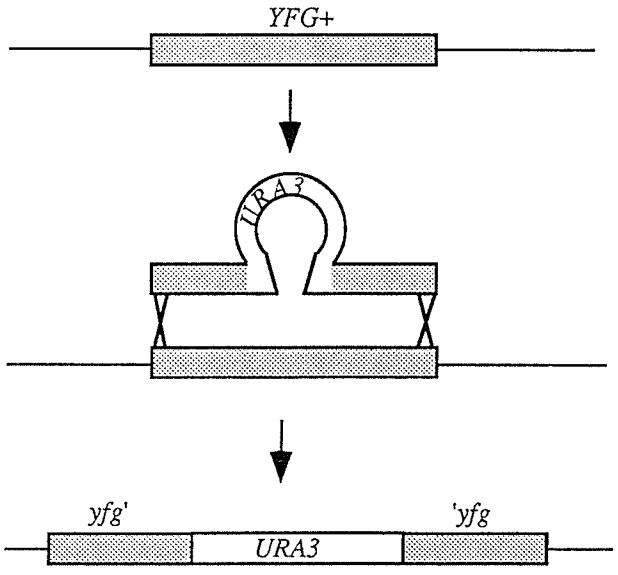
A. Integration of a circular plasmid produces direct repeats of the target locus. Digestion within the *URA3* gene targets integration to the homologous region within *ura3-52*. This site specific integration produces a direct repeat (indicated by arrows) consisting of the *ura3-52* and *URA3* genes. The resulting structure produces a Ura+ strain but is unstable and can is released from the genome by another recombination between the homologous regions.

B. Integration of a non-contiguous gene. Your favorite gene (*YFG*) which is disrupted by a working copy of the *URA3* gene digested free of its plasmid and transformed into a Ura- yeast strain. Double recombination between the free ends results in replacement of the genomic *YFG* and with the disrupted copy which is detected by a Ura+ phenotype. (After Rothstein, 1983).

A


SmaI
ura3-52
SmaI
P GAL1
URA3+
Ap r
pRY171
lacZ
ori
SmaI
SmaI
ura3-52
lacZ
ori
Ap r
P GAL1
URA3+

B


YFG+
URA3
yfg'
'yfg
URA3

approximately 1 in a 1000 cells per generation (Struhl *et al.*, 1979). If the plasmid contains homologous DNA fragments that are non-contiguous, such as a gene disrupted by another selectable marker, the recombination event is similar to a double cross over which results in replacement of the genomic DNA with the transformed fragment (Figure 1B).

The ability to integrate various DNA fragments and maintain them as heritable elements during DNA replication gives a tremendous degree of flexibility for genome manipulation and strain construction. Selective genome manipulation can be accomplished by *in vitro* mutagenesis of a specific gene, followed by direct introduction of the mutant allele. These methods circumvent the traditional methods of mutation such as exposure to ultraviolet irradiation (UV) and chemical treatment (Struhl, 1983).

1.3 *LacZ* Fusion Reporter Systems

The bacterial gene *lacZ*, derived from *E. coli*, has been used as a reporter to study gene expression in yeast and other systems. The *lacZ* gene product, ß-galactosidase, can be easily detected by assaying for cleavage of the chromogenic substrates Xgal (5-bromo-4-chloro-3-indoyl-ß-D-galactopyranoside) and ONPG (ortho-nitrophenyl ß-D-galactopyranoside) on either solid medium or by liquid assay (Miller, 1972). Studies with promoters fused to *lacZ* have allowed characterization of promoter functions in diverse species such as lambda phage (Ptashne *et al.*, 1980), *E. coli* (Berman and Beckwith, 1979), yeast (Guarente and Ptashne, 1981), *Drosophila* (Hama *et al.*, 1990) and mammalian systems (Geller and Freese, 1990).

Yeast researchers have taken advantage of the development of yeast vectors and transformation methods to move the *lacZ* reporter system into the yeast cell to study of eukaryotic genes in a cellular environment more reflective of the *in vivo* situation. Initial work in this area involved the study of expression from the *CYC1* promoter (Guarente and Ptashne, 1981). Plasmids containing a *CYC1-lacZ* gene fusion were found to express ß-galactosidase under conditions conducive to expression from the *CYC1* promoter. Deletion

of the DNA upstream of *CYC1* allowed the identification of specific upstream activating sequences (UAS_C) which controlled *CYC1* gene expression. Other studies have demonstrated similar control regions with the *URA3* gene promoter region fused to *lacZ* (Rose *et al.*, 1981). *In vitro* replacement of UAS_C with DNA from the intergenic control region of the *GAL1* and *GAL10* genes (St. John and Davis, 1981; St. John *et al.*, 1981) conferred galactose-inducibility upon the *CYC1-lacZ* reporter, and allowed characterization of the *GAL10* promoter region (Guarente *et al.*, 1982). Integrated and self-replicating vectors containing *lacZ* gene fusion constructs were used to further characterize the *GAL1-10* divergent promoters by identifying an upstream activating region containing Gal4p binding sites (see below) (West *et al.*, 1984; Yocum *et al.*, 1984).

1.4 Gal4 Protein and UAS_G

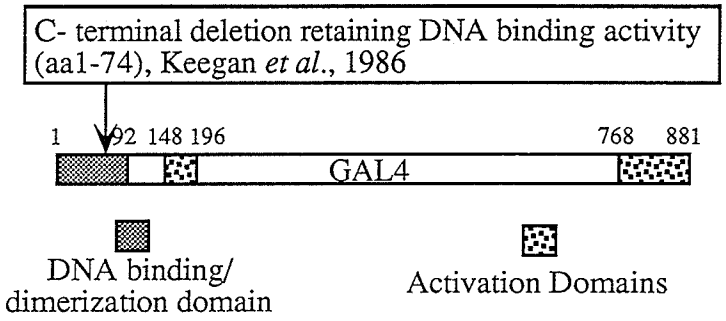
The *GAL4* gene encodes a transcriptional activator which regulates the expression of the *GAL1*, *GAL10*, *GAL7*, and *GAL2* genes in response to the sugar galactose. The 881 amino acid Gal4 protein (Gal4p), (see Figure 2A), contains an N-terminal DNA binding domain (aa 1-74), a self-dimerization domain (aa 74-94), and two transcriptional activation domains, one located behind the DNA binding domain (aa 148-196) and one at the C-terminal end of the protein (aa 768-881) (Johnston and Carlson, 1992; Marmorstein *et al.*, 1992; Lohr *et al.*, 1995). In the absence of galactose, Gal4p is inhibited by the binding of Gal80p to the C-terminal transcriptional activation domain of Gal4p. Galactose interacts with the Gal3 protein, which can then bind to and alter the structure of the Gal4p/Gal80p complex, releasing Gal4p. Gal4p then binds to a 17bp upstream activating sequence (UAS_G) located in the promoter region of the several genes it induces. This 17bp sequence features a two fold rotational symmetry to which Gal4p binds as a dimer (Johnston and Carlson, 1992; Marmorstein *et al.*, 1992). Four of these binding sites are located between the divergently transcribed *GAL1* and *GAL10* genes (see Figure 2B)

Figure 2 The transcriptional activator Gal4p.

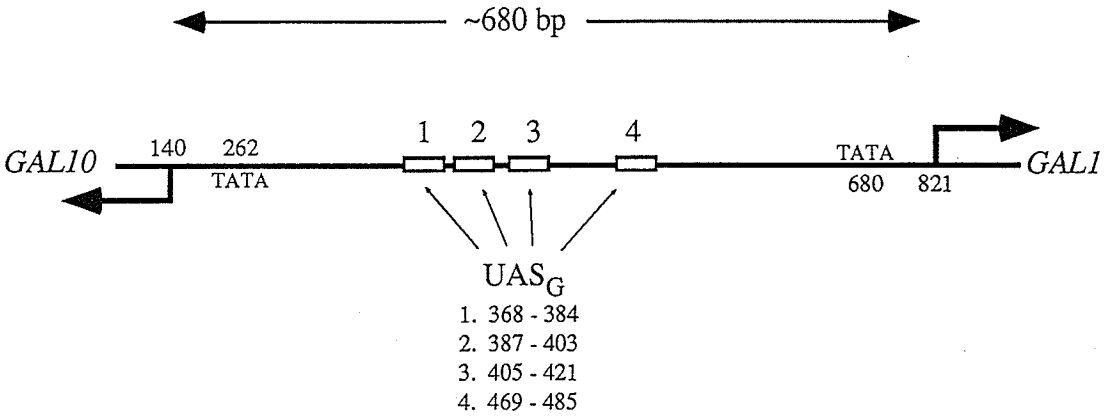
A. Diagram showing the relative positions of the Gal4 protein DNA binding, dimerization, and transcriptional activation domains. The arrow indicates the size of the largest C-terminal deletion made by Keegan *et al.* (1986) that still retained Gal4 DNA binding activity.

B. The *GAL1-10* intergenic control region. The open boxes indicate the positions of the four dyad symmetric Gal4p binding sites (UAS_G). The positions of the transcriptional start sites, the TATA boxes and the ranges of the four UAS_G sequences are indicated in base pairs according to the sequence numbering of Yocum *et al.*, (1984).

A


C- terminal deletion retaining DNA binding activity
(aa1-74), Keegan *et al.*, 1986
1
92
148
196
768
881
GAL4
DNA binding/
dimerization domain
Activation Domains

B


~680 bp
GAL10
140
262
TATA
1
2
3
4
TATA
680
821
GAL1
UAS_G
1. 368 - 384
2. 387 - 403
3. 405 - 421
4. 469 - 485

(Giniger *et al.*, 1985). Deletion studies of this region determined that a single copy of the UAS_G sequence does not bind Gal4p efficiently, but that two or more of the sites work synergistically to activate transcription (Giniger and Ptashne, 1988). A synthetic 17bp sequence (the 17mer - 5'- CGGAAGACTCTCCTCCG-3') designed from a consensus sequence of 5 different UAS_G sequences results in a high degree of galactose-activated transcription. Two or more of these sites work cooperatively to activate transcription of a *lacZ* reporter gene fused to the *GAL1* gene (Giniger *et al.*, 1985; Giniger and Ptashne, 1988).

The Gal4p transcription factor has been found to be modular in nature, a feature it shares with another yeast activator (Gcn4p) and other eukaryotic transcription factors (Brent and Ptashne, 1985; Hope and Struhl, 1986; Keegan *et al.*, 1986; Ma and Ptashne, 1987a). The DNA binding and transcription activating domains of Gal4p can be physically separated from each other and still maintain their respective functions. These features of Gal4p are illustrated in Figure 3. Brent and Ptashne (1985) showed that while full length lexA protein bound to a specific operator sequence (*lexAop*) inserted between the UAS_G and TATA box of a *GAL1-lacZ reporter* promoter it did not activate transcription (Figure 3A-i). In fact, the bacterial repressor decreased the level of basal transcription from the *GAL1-lacZ* reporter. However, a hybrid protein comprised of the lexA binding domain fused to the Gal4p activation domain ($lexA_{BD}$-$Gal4p_{AD}$) was able to activate transcription when bound to the *lexA* operator sites (Figure 3A-ii) (Brent and Ptashne, 1985). This conversion of a transcriptional repressor to an activator was the first example of a hybrid protein being able to activate transcription. Following this, serial deletion of the C-terminal end of the Gal4 protein combined with S1 nuclease protection assays narrowed down the Gal4p DNA binding domain to amino acids 1-74; these alone retained the binding ability in the absence of the remainder of the protein (see above) (Keegan *et al.*, 1986). The position of the DNA binding domain was confirmed and the position of the activation domain determined by C-terminal and internal deletion analysis of Gal4p. Ma and Ptashne (1987a)

Figure 3 Gal4 hybrid fusions which activate transcription.

A. Demonstration of the activation of *lacZ* transcription by lexA-GAL4 when a *lexAop* sequence is placed upstream of a reporter gene.

i. LexA protein binds to *lexAop* but does not activate transcription due to lack of an activation domain.

ii. Fusion of the activating domains of Gal4 protein to the lexA binding domain produces a hybrid activator that activates transcription when bound to *lexAop* (Adapted from Brent and Ptashne, 1985.)

B.i. Gal4 protein binds to UAS_G and activates transcription of a reporter gene. Multiple Gal4 proteins are indicated to represent the four UAS_G sites upstream of *GAL1*.

ii. The DNA binding domain ($Gal4_{BD}$) binds to UAS_G but does not activate transcription due to lack of an activation domain.

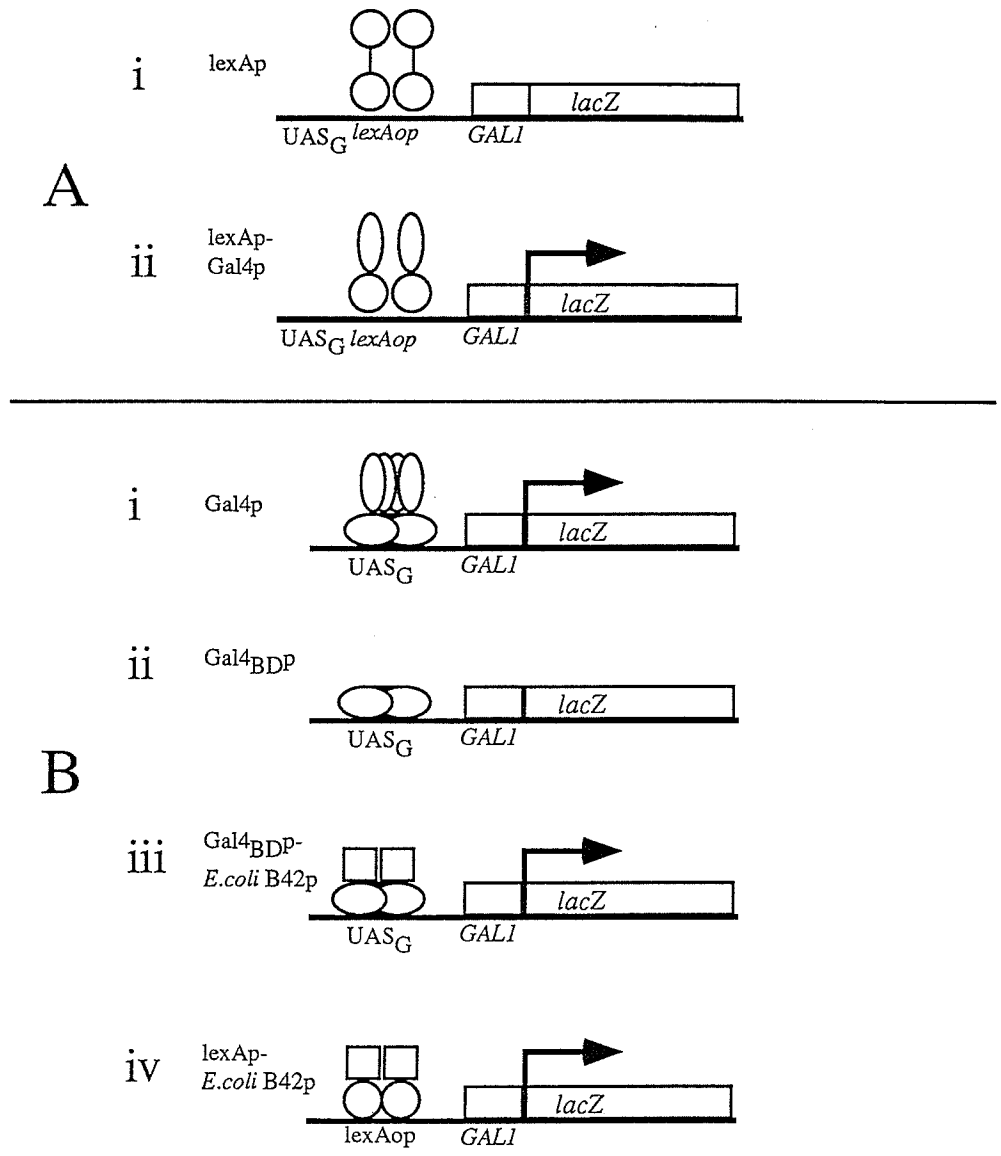
iii. The fusion of $Gal4_{BD}$ and an *E.coli* transcriptional activator (B42) replaces Gal4 function.

iv. A lexA-B42 fusion also activates transcription if UAS_G is replaced with a lexAop sequence (Adapted from Ma and Ptashne, 1987b)

C. Conversion of a transcriptional repressor to an activator.

i. Gal80 binds to Gal4 to inhibit transcription in the absence of glucose.

ii. Insertion of the B42 domain into Gal80 overrides its inhibitory power and results in transcription. (Adapted from Ma and Ptashne, 1988).


A
i
lexAp
lacZ
UAS$_G$ lexAop
GAL1
ii
lexAp-
Gal4p
lacZ
UAS$_G$ lexAop
GAL1
B
i
Gal4p
lacZ
UAS$_G$
GAL1
ii
Gal4$_{BD}$p
lacZ
UAS$_G$
GAL1
iii
Gal4$_{BD}$p-
E.coli B42p
lacZ
UAS$_G$
GAL1
iv
lexAp-
E.coli B42p
lacZ
lexAop
GAL1
C
i
Gal80p
Gal4p
lacZ
UAS$_G$
GAL1
ii
Gal80p-B42p
Gal4p
lacZ
UAS$_G$
GAL1

determined that there were two acidic domains which could activate transcription. These activation domains are located just behind the DNA binding domain (aa 148-196) and at the C-terminal end of the protein (aa 768-881) (Ma and Ptashne, 1987a). Work with the DNA binding and dimerization domains of Gal4 (aa 1-147) fused to random *E. coli* DNA fragments identified several acidic peptides (e.g. B42) which were able to re-establish the activation of transcription from the *GAL1* promoter (Figure 3B-iii) (Ma and Ptashne, 1987b). When incorporated into a lexA hybrid protein, the B42 peptide was able to induce transcription when transformed into a yeast strain containing a *lexAop-GAL1-lacZ* reporter gene (Figure 3B-iv). As described earlier, Gal80p acts as a transcriptional repressor by binding to the C-terminal activation domain of Gal4p and interfering with activation (Figure 3C-i). When the DNA fragment coding for the B42 acidic peptide was cloned into the *GAL80* gene, the resulting protein fusion converted Gal80p from a repressor of Gal4p to a transcriptional activator (Figure 3C-ii) (Ma and Ptashne, 1988). The discovery of the modularity of the Gal4 protein and the work with hybrid proteins constructed with the different domains was fundamental to the development of the two-hybrid system (Fields and Song, 1989), a molecular genetic method for the study of protein:protein interactions.

1.5 The Two-hybrid System

With the discovery of the modular nature of transcription factors, and the ability of hybrid fusions to activate transcription of a reporter gene, Fields and Song (1989) put forth the brilliant proposal that interacting proteins, when fused to the Gal4p binding ($Gal4p_{BD}$) and activating ($Gal4p_{AD}$) domains, could restore the function of the two separated domains of Gal4p and be identified through the activation of a reporter gene. The resulting "two-hybrid system" is an *in vivo* molecular genetic assay which allows the identification of interacting proteins. It can be used to study known interactions of two proteins or to identify unknown interacting partners of YFP (your favorite protein) by screening a library of genomic or cDNA clones (Fields and Song, 1989; Chien *et al.*, 1991).

The operation of the two-hybrid system is illustrated in Figure 4. Normal activity of Gal4p in the presence of galactose results in activation of genes with UAS_G binding sites; this is detected by a *GAL1-lacZ* reporter construct (Figure 4A). A fusion between $Gal4p_{BD}$ and a selected protein (X) can bind to the UAS_G sequence upstream of the *GAL1-lacZ* reporter fusion, but is unable to activate transcription due to the lack of an activation domain (Figure 4B). A corresponding fusion between $Gal4p_{AD}$ and a protein that interacts with protein X (Y) is unable to bind to UAS_G and therefore can not activate transcription (Figure 4C). Simultaneous production of both hybrid proteins, $Gal4p_{BD}$/Xp and $Gal4p_{AD}$/Yp allows the X and Y proteins to interact and results in the correct placement of $Gal4p_{BD}$ and $Gal4p_{AD}$, initiating transcription of a reporter gene such as *lacZ* (Figure 4D). Fields and Song (1989) showed that fusions of the *GAL4* sequences coding for the DNA binding and transcription activating domains with the yeast genes *SNF1* and *SNF4* respectively, which code for a known pair of interacting proteins, resulted in activation of the *GAL1-lacZ* reporter only when both plasmids were present within the yeast strain.

Fields and co-workers suggested that new protein interactions could be identified by screening a library of random genomic yeast DNA fragments fused to the $GAL4_{AD}$ with a known protein of interest fused to $GAL4_{BD}$ (Chien *et al.*, 1991). A gene fragment coding for YFP is cloned in frame behind the $GAL4_{BD}$ in a plasmid which constitutively produces the fusion protein in yeast. A library of random genomic fragments or cDNA is cloned into three different $GAL4_{AD}$ plasmids, which produce fusions in the three possible reading frames. The $GAL4_{BD}$ plasmid and a mixture of the $GAL4_{AD}$ libraries are transformed into a reporter strain which is plated onto synthetic minimal growth medium. Positive interactions are identified by selection for the two plasmid markers and, in some yeast strains, a nutritional reporter gene such as *HIS3*. The positives are confirmed by a second screen by assaying for the production of the *GAL1-lacZ* reporter gene. The clones which pass the second test are retained for further study. Since each fusion protein is produced

Figure 4 The yeast two-hybrid system

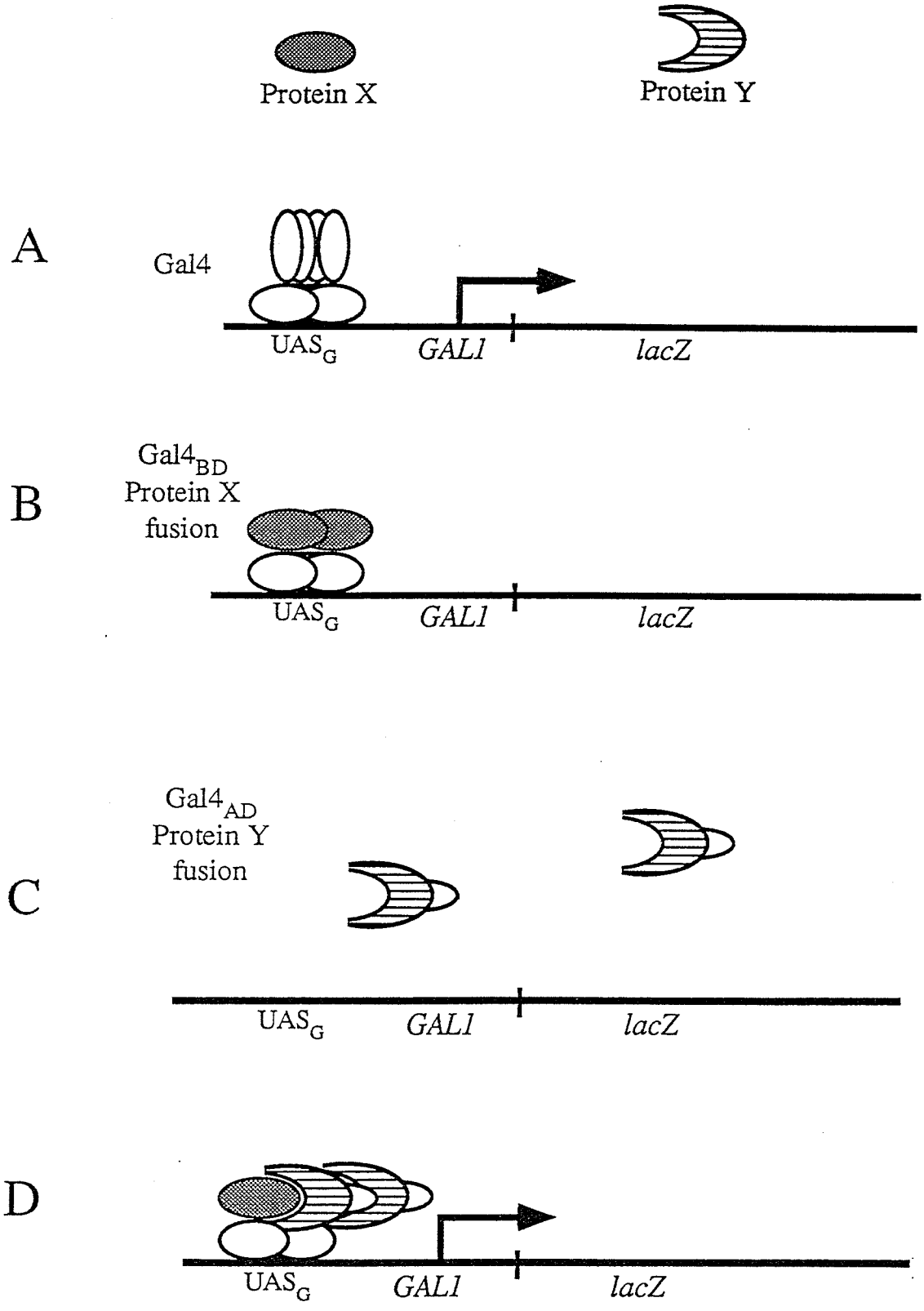
Protein X and Protein Y are a pair of interacting proteins.

A. In the presence of galactose Gal4 binds to UASG and activates transcription or downstream genes.

B. The $Gal4_{BD}$-Protein X fusion protein still binds to UASG but does not activate transcription.

C. The $Gal4_{AD}$-Protein Y fusion protein does not activate transcription as it cannot bind to UAS_G.

D. The interaction of Protein X with Protein Y reconstitutes the activity of Gal4 and activates transcription, which is detected by *lacZ* activity.


Protein X
Protein Y
A
Gal4
UAS$_G$
GAL1
lacZ
B
Gal4$_{BD}$
Protein X
fusion
UAS$_G$
GAL1
lacZ
C
Gal4$_{AD}$
Protein Y
fusion
UAS$_G$
GAL1
lacZ
D
UAS$_G$
GAL1
lacZ

from a different plasmid, the protein coding sequence carried by the $GAL4_{AD}$ plasmid can be isolated from the reporter yeast strain.

The protein:protein interactions that have been identified using the two-hybrid system are too numerous to list here. Some prominent examples are shown in Table 1. The two-hybrid system has also been used to verify and study interacting partners identified by *in vitro* methods. Examples of protein:protein interactions analyzed in this fashion are listed in Table 2.

Increased use of the two-hybrid system has led to numerous modifications and improvements of the system. Paetkau *et al.* (1994) found that some "false positives" were the result of complete *GAL4* gene sequences present in the genomic library used to prepare the $GAL4_{AD}$/gene *Y* plasmid library. They circumvented this problem by deleting *GAL4* from the genomes of the yeast strains used to prepare the libraries. Bartel et al. (1993) have devised a "mating assay" to identify "self-activating" $GAL4_{AD}$/library plasmids. Alternate DNA binding domains such as lexA can be used with reporter constructs containing a *lexAop* sequence (Zervos *et al.*, 1993). Transcriptional activation domains such as herpes virus VP16 and the acidic bacterial peptide B42 have been successfully used in place of $Gal4p_{AD}$ (Vojtek *et al.*, 1993; Zervos *et al.*, 1993). New vectors that incorporate epitope tagging of the fusion proteins assist in the confirmation of interactions with *in vitro* studies (Durfee *et al.*, 1993; Harper *et al.*, 1994; Yavuzer and Goding, 1995). The addition of a reporter construct that uses the biosynthetic markers *HIS3* (Durfee *et al.* 1993) and *LEU2* (Gyuris *et al.*, 1993) allows for the direct selection of cells that contain interacting proteins by their ability to grow on selective yeast medium. While many *HIS3* reporters require the addition of an antimetabolite (3-AT) to decrease basal expression, a strain that does not require any 3-AT has been developed (Feilotter *et al.*, 1994).

The "regular" version of the two-hybrid system cannot be used to identify protein:protein interactions that are dependent upon tyrosine phosphorylation, such as those found in mammalian signal transduction pathways. A strategy for producing such a system

Table 1 Examples of protein:protein interactions discovered with the two-hybrid system.

BD Fusion	AD Fusion	Reference
Rap1	Rif1	Hardy *et al.*, 1992
Snf1*	Sip1*	Yang *et al.*, 1992
Mxi1	Max	Zervos *et al.*, 1993
Rb	protein phosphatase type 1 (PP-1α2)	Durfee *et al.*, 1993
Cdk2	Cip1	Harper *et al.*, 1993
Rad7*	Sir3*	Paetkau *et al.*, 1994
Fas/APO-1	FADD RIP	Chinnaiyan *et al.*, 1995 Stanger *et al.*, 1995
PI-3 kinase	insulin receptor IGF-1 receptor	Lamothe *et al.*, 1995
APC	EB1	Su *et al.*, 1995
Huntington	HIP-1	Kalchman *et al.*, 1996

BD = Binding Domain AD = Activation Domain
* Indicates yeast proteins. All others are human proteins isolated from AD:cDNA libraries.

Table 2 Examples of protein interactions studied with the two-hybrid system.

BD Fusion	AD Fusion	Reference
Ras	Raf	Van Aelst *et al.*, 1993; Vojtek *et al.*, 1993
Rad1*	Rad10*	Bardwell *et al.*, 1993
activation of NF-κB	Raf1	Li and Sedivy, 1993
p53	SV40 large T antigen	Li and Fields, 1993
Rad7*	Sir3*	Paetkau *et al.*, 1994
CKII α and α' CKII ß	CKII ß CKII ß	Gietz *et al.*, 1995 Kusk *et al.*, 1995 Boldyreff *et al.*, 1996
p85 subunit of PI 3-kinase	p110 subunit of PI 3-kinase	Holt *et al.*, 1994
Irs-1	Irk	O'Neill *et al.*, 1994
dystrophin	syntrophin	Castelló *et al.*, 1996
Rad51	Rad52	Shen *et al.*, 1996

BD = Binding Domain AD = Activation Domain
* Indicates yeast proteins. All others are human proteins.

would introduce a third plasmid containing a constitutive promoter driving the expression of the required kinase. The most convenient two-hybrid system yeast strains contain 2 or 3 reporter gene constructs, which eliminates a number of possible selectable markers that could be used for selection of a third plasmid containing a kinase. One auxotrophic marker, usually the *TRP1* gene, is needed to maintain the binding domain plasmid; another marker, usually *LEU2,* is used to maintain the activating domain plasmid. In addition, the majority of two-hybrid strains use the *URA3* gene to maintain an integrated *lacZ* reporter construct. The result is that no useful markers are left to maintain a third plasmid.

To develop a two-hybrid system dependent upon tyrosine phosphorylation we decided to use a three plasmid system. The *URA3* gene was chosen as the selective marker for the plasmid that would carry the gene coding for a tyrosine kinase. The use of *URA3* would allow for the simple testing of phosphorylation-dependent protein interactions by eviction of the *URA3*/kinase plasmid by replica plating onto FOA medium. FOA is converted to a toxic intermediate compound by the *URA3* gene product. Yeast cells containing this protein are therefore killed, while cells which lose the *URA3* plasmid survive (Boeke et al., 1984). As seen in Figure 5, transformants containing the $GAL4_{BD}$ (*TRP1*) and $GAl4_{AD}$ (*LEU2*) plasmids and the *URA3*/kinase plasmid would be assayed for production of the *GAL1-HIS3* reporter integrated into the genome of the host yeast strain (left plate). After His+ positives are identified, they are replicated onto FOA medium (middle plate) which induces loss of the *URA3* based kinase plasmid. The colonies are retested for induction of the reporter gene in the absence of the kinase (right plate). Positives which are found to be kinase dependent can then be isolated for further study. This simple secondary screen would allow for the testing of large numbers of initial positives.

To achieve this end the modification of a two-hybrid yeast strain was required to allow for selective maintenance of three plasmids. The *URA3* gene needed to be retrieved from its role maintaining the integrated *GAL1-lacZ* reporter system. The strategy used was

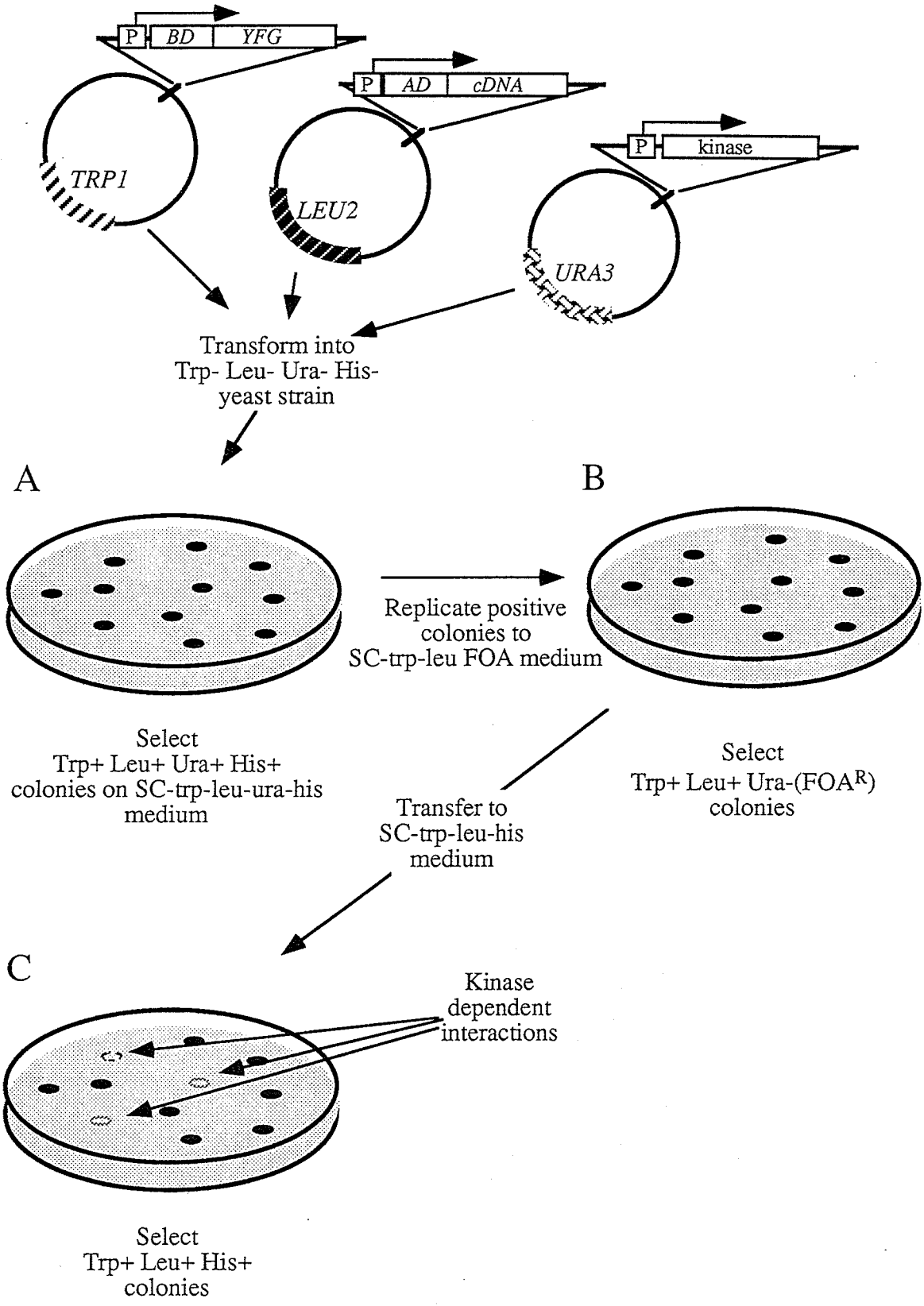
Figure 5 **Screening strategy to identify kinase dependent protein:protein interactions.**

To identify kinase dependent protein:protein interactions a activation domain library (*LEU2*) is screened with YFG in a binding domain plasmid (*TRP1*) and a kinase produced constitutively from a plasmid (*URA3*).

A. Initial positives are isolated by growth on SC-Trp-Leu-Ura-His media which selects for activation of the reporter gene in the presence of the three plasmids.

B. Colonies are replica plated to SC-Trp-Leu FOA media to retain the binding domain and activating domain plasmids and select for the loss of the *URA3* based kinase plasmid.

C. FOA^R colonies are then replica plated to SC-Trp-Leu-His media. Kinase dependent interactions are identified by the lack of activation of the *HIS3* reporter gene after loss of the kinase plasmid.


P
BD
YFG
P
AD
cDNA
P
kinase
TRP1
LEU2
URA3
Transform into
Trp- Leu- Ura- His-
yeast strain
A
B
Replicate positive
colonies to
SC-trp-leu FOA medium
Select
Trp+ Leu+ Ura+ His+
colonies on SC-trp-leu-ura-his
medium
Select
Trp+ Leu+ Ura-(FOA^R)
colonies
Transfer to
SC-trp-leu-his
medium
C
Kinase
dependent
interactions
Select
Trp+ Leu+ His+
colonies

to integrate the *GAL1-lacZ* at the *URA3* gene to disrupt its function, thereby giving a stable reporter gene construct combined with a uracil minus yeast strain.

This thesis describes the production and testing of two new two-hybrid yeast strains, KGY37 and KGY94. KGY37 (*MATa ade2 gal4 gal80 his3-Δ200 trp1-Δ901 leu2::pUC18 ura3::GAL1-lacZ lys2::*$UAS_{G\ 17mers(x3)}$*GAL1-HIS3)* contains two stably integrated reporter genes (*lacZ* and *HIS3*) which are controlled by Gal4p and exhibit low levels of basal activity. Two additional auxotrophic markers (*URA3* and *LYS2*) have been made available by disruption of their genomic loci with the reporter constructs. Recovered markers permit the transformation of additional plasmids to introduce modifying proteins such as a tyrosine kinase. These features of KGY37 will allow the identification of a new class of two-hybrid interactions which are dependent upon modifying proteins not normally present in yeast. KGY94 (*MATa ade2 gal4 gal80 his3-Δ200 trp1-Δ901 leu2-3,112 ura3-52 URA3::GAL1-lacZ lys2::lexAop* $_{(x3)}$*GAL1-HIS3*) contains *lacZ* and *HIS3* reporter genes which are activated from lexA binding sites. This strain provides the opportunity to use lexA fusions combined with a *HIS3* reporter gene to easily screen large mammalian cDNA libraries by nutritional selection for positives. The use of the *HIS3* gene circumvents the laborious replica plating needed to test for *lacZ* activation. Both strains have proven to be useful additions to the family of yeast strains used for two-hybrid research.

2. MATERIALS AND METHODS

2.1 Bacterial strains

The *E. coli* strain DH5α (Gibco BRL) strain was used for the manipulation, amplification and storage of all plasmids. The genotype of this strain is F⁻ f80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *pho*A *hsd*R17(r_K^-, m_K^+) *sup*E44 *thi*-1 *gyr*A96 *rel*A1. All bacterial stocks were maintained at -80°C in 20% glycerol.

2.2 Yeast strains

Yeast strains used in this project are listed in Table 3 below.

Table 3 Yeast strains

Strain	Genotype	Source	Reference
CTY10-5D	*MATa ade2 gal4 gal80 his3-Δ200 leu2-3,112 trp1-Δ901 ura3-52 URA3::lexAopGAL1-lacZ*	S. Fields	Bartel *et al.*, 1993a
DGY63	*MATa ade2 gal4 gal80 his3-Δ200 leu2-3,113 trp1-Δ901 ura3-52*	R.D. Gietz	
DGY63::171	*MATa ade2 gal4 gal80 his3-Δ200 leu2-3,113 trp1-Δ901 ura3-52 URA3::GAL1-lacZ*	R.D. Gietz	
DGY74	*MATa ade2 gal4 gal80 his3-Δ200 leu2-3,113 trp1-Δ901 URA3+*	R.D. Gietz	
DBY31	*MATa ade2 gal4 gal80 his3-Δ200 trp1-Δ901 URA3+ $LEU2_{EKM}$*[1]	R.D. Gietz	
YPH2	*MATα ade2-101 (ochre) ura3-52, lys2-801 (amber), GAL+*	R. Schiestl	Sikorski and Hieter, 1989

[1] $LEU2_{EKM}$ (*LEU2* *Eco*RI *Kpn*I mutated) is an allele of the yeast *LEU2* gene which has had the wild type *Kpn*I and *Eco*RI sites removed by *in vitro* mutagenesis without affecting the action of the gene product (Gietz and Sugino, 1988).

Yeast strains were stored at -80°C in 20% glycerol, and were recovered by streaking onto solid YPAD or selective medium and incubating at 30°C for 2 to 3 days prior to use.

2.3 Plasmids

Plasmids not produced during this project are listed in Table 4 below.

Table 4 Plasmids

Plasmid		Genes	Source	Reference
pAS1	*	*TRP1*, Ap^R, $GAL4_{BD}$	S. Elledge	Durfee *et al.*, 1993
pBluescript SK+		Ap^R, f1(+) origin	R.D. Gietz	Stratagene
pBM2389	*	*TRP1*, Ap^R, *ARS1CEN4*, *GAL1-HIS3*	M. Johnston	Liu *et al.*, 1993
pDG82		Ap^R, *hisG:URA3:hisG*	R.D. Gietz	Alani *et al.*, 1987
pDG108		Ap^R, *LYS2*	R.D. Gietz	
pDG317		Ap^R, *LEU2*	R.D. Gietz	Gietz and Sugino, 1988
pDG649	*	*TRP1*, Ap^R, $lexA_{BD}$, *RAD7*	R.D. Gietz	Paetkau *et al.*, 1994
pDG663		*LEU2*, Ap^R, $GAL4_{AD}$, *RAD10*	R.D. Gietz	
pDG862	*	*TRP1*, Ap^R, $GAL4_{BD}$, *RAD7*	R.D. Gietz	
pDG98		Ap^R, *URA3*	R.D. Gietz	
pDP3		Ap^R, *URA3*	R.D. Gietz	
pGBD-KKQ1[1]	*	*TRP1*, Ap^R, $GAL4_{BD}$, *KKQ1*	P. James	
pJR3	*	*LEU2*, Ap^R, $GAL4_{AD}$, *SIR3*	R.D. Gietz	Paetkau *et al.*, 1994
pRY171	*	*URA3*, Ap^R, *GAL1-lacZ*	R.D. Gietz	Yocum *et al.*, 1984
YCplac22	*	*TRP1*, Ap^R, *ARS1*, *CEN4*	R.D. Gietz	Gietz and Sugino, 1988
YCplac33	*	*URA3*, Ap^R, *ARS1*, *CEN4*	R.D. Gietz	Gietz and Sugino, 1988
YEp13	*	*LEU2*, Ap^R,	R.D. Gietz	Broach *et al.*, 1979

[1] pGBD-KKQ1 contains a gene fusion with the $GAL4_{BD}$ which produces a fusion protein that self activates expression of a Gal4p controlled reporter construct (P. James, personal communication).
* Shuttle vectors capable of replication in either bacteria or yeast hosts are indicated by an asterisk.

All plasmids were stored in TE buffer at -20°C.

2.4 Media

Media were prepared in 600 ml volumes, with all components being autoclaved on a liquid cycle for 20 min., at a temperature of 121°C and a pressure of 15 psi (pounds per square inch), unless otherwise indicated.

2.4.1 Bacterial media

All bacterial media were prepared as described by Sambrook *et al.* (1989).

2.4.1.1 LB (Luria-Bertani)

Difco Bacto-yeast extract	3 g
Difco Bacto-tryptone	6 g
NaCl	6 g
Difco Bacto agar	10 g

The ingredients were dissolved in 600 ml of double distilled water (ddH_2O), titrated to pH 7.0 with 10 N NaOH and added to a 1 litre flask containing 10 grams of Bacto-agar. The medium was autoclaved, cooled to 55°C and supplemented with the antibiotic carbenicillin (final concentration = 50 μg/ml) if desired. After mixing to distribute the molten agar, the medium was poured into plastic petri dishes in 30 ml aliquots, allowed to harden, and dried for 4 to 5 days covered at room temperature. Liquid medium was produced by omitting the Bacto-agar and autoclaving in glass bottles with loosened caps. The chosen antibiotic supplement for liquid LB medium was ampicillin, added to a final concentration of 50 μg/ml. This less stringent and cheaper antibiotic was sufficient for selection during growth in liquid media.

Xgal Plates

For plates requiring Xgal for blue/white color selection of transformants a solution of 25 mg/ml in dimethyl formamide (DMF) was aseptically spread onto LB + carbenicillin plates to a final concentration of 50 μg/ml.

2.4.1.2 SOC

Bacto-yeast extract	12 g
Bacto-tryptone	3 g
NaCl	0.36 g
KCl	0.108 g (or 600μl of 2.5 M KCl)
Dextrose	1.2 g

The above ingredients were dissolved in 600 ml of ddH_2O, titrated to pH 7.0 with 10 N NaOH and autoclaved in glass bottles with loosened caps. Before use, a 2 molar

solution of magnesium salts (1 M $MgCl_2$:1 M $MgSO_4$ (filter sterilized)) was added to a final concentration of 0.5%.

2.4.2 Yeast media

2.4.2.1 YPAD (Sherman, 1991)

Bacto-yeast extract	6 g
Bacto-peptone	12 g
Dextrose	12 g
Adenine hemisulfate	48 mg (Solid medium) 24 mg (Liquid medium)

Adenine hemisulfate reduces the growth advantage of revertants at the *ade2* locus (Sherman, 1991; Kaiser *et al.*, 1994)..

The above media constituents (minus the adenine hemisulfate) were dissolved in 600 ml of ddH_2O. Liquid medium was autoclaved in glass bottles with loosened caps, while solid medium was produced by combining the liquid phase with 10 grams of Bacto-agar and the appropriate amount of adenine in a 1 litre flask, autoclaving and pouring into plastic petri dishes.

2.4.2.2 Synthetic Complete (SC) Dropout medium

(after Sherman *et al.*, 1979; Sherman, 1991; and Kaiser *et al.*, 1994)

Bacto-yeast nitrogen base w/o $(NH_4)_2SO_4$ or amino acids	1 g
Ammonium sulfate	3 g
Drop out mix	350 mg
Dextrose	12 g
Bacto-agar	10 g

All components listed above except the Bacto-agar were dissolved in 600 ml of ddH_2O and titrated with 10 N NaOH to pH 5.6-5.8. The solution was then combined with the agar in a 1 litre flask and sterilized by autoclaving. Liquid medium was prepared in screw cap bottles.

2.4.2.2.1 Drop out mix for synthetic medium

	Amount	Final Concentration
Adenine sulfate	4 g	46 mg/l
Arginine HCl	2 g	23 mg/l
Aspartic Acid	2 g	23 mg/l
Glutamic Acid HCl	2 g	23 mg/l
*Histidine HCl	2 g	23 mg/l
Homoserine	6 g	70 mg/l
Isoleucine	2 g	23 mg/l
*Leucine	2 g	23 mg/l
*Lysine HCl	2 g	23 mg/l
Methionine	2 g	23 mg/l
myo-Inositol	2 g	23 mg/l
p-aminobenzoic acid (PABA)	0.2 g	2 mg/l
Phenylalanine	3 g	35 mg/l
Serine	2 g	23 mg/l
Threonine	2 g	23 mg/l
*Tryptophan	2 g	23 mg/l
Tyrosine	2 g	23 mg/l
*Uracil	2 g	23 mg/l
Valine	9 g	105 mg/l

Complete mix contains all of the components listed above. Synthetic complete minus (SC -) drop out medium was produced by omitting one or more of the supplements indicated by an asterisk. SC galactose medium was prepared by substituting filter sterilized galactose for the dextrose. Twelve grams of galactose were dissolved in 100 ml of sterile ddH_2O and filter sterilized. The medium was prepared as described for SC medium with 100 ml less water to accommodate the filter sterilized galactose which was added after the medium had cooled to 55°C.

2.4.2.3 3-Aminotriazole medium

To increase the selection pressure for histidine prototrophy the chemical 3-aminotriazole (3-AT) was added to media at various concentrations (Kishore and Shah, 1988).

2.4.2.4 SC Xgal medium (Chien *et al.*, 1991)

Bacto-yeast nitrogen base w/o $(NH_4)_2SO_4$ or amino acids	1 g
Ammonium sulfate	3 g
Drop out mix	0.35 g
Sucrose	12 g
Bacto-agar	10 g
Water to	565 ml

Xgal medium was prepared as SC medium with 35 ml less water, cooled to 50°C, supplemented with 35 ml of sterile 1.0 M $NaH_2PO_4 \cdot H_2O$ sodium phosphate buffer (pH 7.0) and 1 ml of Xgal solution (60 mg/ml in N-N-dimethylformamide) and poured into plates (Chien *et al.*, 1991).

2.4.2.5 FOA medium (Sikorski and Boeke, 1991)

Bacto-yeast nitrogen base w/o $(NH_4)_2SO_4$ or amino acids	1 g
Ammonium sulfate	3 g
FOA	600 mg
Complete mix	400 mg
Dextrose	12 g

Ten grams of Bacto-agar was combined with 300 ml of ddH_2O, autoclaved for 20 minutes at 121°C and cooled to 60°C. The components listed above were dissolved in another 300 ml of ddH_2O, heated to 60°C, filter sterilized, combined with the molten agar and poured into plates.

2.5 Electroporation of E. coli DH5α

Plasmid DNA and ligation products were transformed into the electrocompetent *E. coli* strain DH5α (Gibco BRL) by electroporation as described by Dower *et al.* (1988).

2.5.1 Preparation of electrocompetent bacteria

A 50 ml starter culture of liquid LB medium was inoculated with a single colony of DH5α from solid LB medium. The starter culture was grown overnight with shaking at

37°C and diluted 1/100 into two litres of prewarmed LB medium. This culture was grown with good aeration for two hours and 15 minutes to an OD_{600} between 0.5 and 1.0. Cells were collected by an 8670 *g* centrifugation at 4°C in a Beckman J2-21 centrifuge using a JA-14 rotor. The cells were resuspended in two litres of ice cold sterile ddH_2O, pelleted and resuspended with one litre of sterile ddH_2O. After a second centrifugation the cell pellet was resuspended in a total volume of 50 ml sterile ddH_2O in an ice cold 50 ml centrifuge tube. The cells were pelleted and resuspended in 20 ml of ice cold 10% glycerol (filter sterilized), pelleted a final time and resuspended in 2 ml of ice cold 10% glycerol. The cells were dispensed into 0.5 ml microfuge tubes in 50 µl aliquots, flash frozen in liquid nitrogen and stored at -80°C (Dower *et al.*, 1988).

2.5.2 Electroporation of electrocompetent DH5α

A 50 µl aliquot of electrocompetent cells was thawed on ice, and mixed with 0.04 to 1 µg of plasmid DNA by carefully pipetting up and down. The cell/DNA mixture was then transferred to an ice cold electroporation cuvette (BioRad) with a 0.1 cm aperture and subjected to an electric pulse, generated by a BioRad Gene Pulser™ set to 1.25 kV with a 25 µF capacitor connected to a pulse controller set to 400 ohms (Miller, 1988; Dower *et al.*, 1988). The average pulse was 7 to 8 milliseconds with a 25 µF capacitor. The electroporated bacteria were immediately resuspended in 1 ml of room temperature SOC medium and incubated at 37°C for 30 min. Various volumes of this culture were spread onto solid LB + carbenicillin plates, which contained Xgal if blue/white color selection was required, and incubated at 37°C overnight.

2.6 Concentration of DNA by ethanol precipitation

Solutions of DNA were concentrated by absolute ethanol precipitation using sodium acetate (Sambrook *et al.*, 1989). A one-tenth volume of sodium acetate (3.0 M, pH 6.0) were added to the DNA solution followed by 2.5 volumes of ice cold absolute ethanol and

these were mixed together using a vortex. The mixture was then incubated for one to 16 hours at -20°C. The nucleic acids were collected by centrifugation for 5 minutes at 14 000 *g* at 4°C. After removal of the supernatant the pellet was carefully washed with 100µl of room temperature 70% ethanol and left to air dry. Pellets were resuspended in 50 µl of TE (10 mM Tris•Cl pH 8.0, 1 mM EDTA) unless otherwise indicated.

2.7 Phenol extraction

Restriction enzymes were removed from DNA preparations by phenol/chloroform extraction according to the method of Sambrook *et al.* (1989). Phenol was vacuum distilled to remove impurities and then saturated with TE buffer (pH 9.0). At the time of use the TE saturated phenol was mixed with 1 volume of chloroform and the lowest solvent phase was used for extraction. One volume of the phenol:chloroform mixture was added to DNA solutions, mixed on a vortex apparatus, and the phases separated by a one minute centrifugation at 14 000 *g* at room temperature. The aqueous phase was removed to a fresh tube and the DNA precipitated with ethanol.

2.8 Isolation of plasmid DNA from bacteria

Plasmid DNA was isolated from *E. coli* by the alkaline lysis miniprep method described by Birnboim and Doly (1979). Single colonies were picked from LB + carbenicillin plates into 2 ml of liquid LB containing 50 µg/ml ampicillin and grown overnight at 37°C with good aeration provided by shaking the culture at 200 rpm. The following day 1.5 ml of the saturated culture was decanted into a 1.7 ml microfuge tube and the cells were pelleted by a 30 second spin at 14 000 *g* in a table top microcentrifuge. After discarding the medium, cell pellets were resuspended in 100 µl of ice cold TGE (25 mM Tris•Cl (pH 8.0), 50 mM glucose, 10 mM EDTA) and lysed by the addition of 200 µl lysis buffer (0.2 N NaOH, 1% SDS) followed by gentle mixing. Cellular proteins and the large *E. coli* chromosomes were precipitated out of solution by the addition of 150 µl of ice

cold 5 M potassium acetate pH 4.8, followed by vigorous mixing of the solutions and a 5 minute 14 000 *g* centrifugation at 4°C to pellet debris. The supernatant was transferred to a fresh tube, extracted with one volume of phenol:chloroform and the phases separated by centrifugation for 1 minute at 14,000 *g*. The upper aqueous layer was removed to a fresh tube and the nucleic acids were precipitated with 1 ml of ice cold absolute ethanol. Nucleic acids were collected by centrifugation for 5 minutes at 14 000 *g*, washed with 100µl of 70% ethanol and air dried before being resuspended in 50µl of TE buffer.

2.9 Restriction endonuclease digestion of DNA

Restriction endonuclease digestion of DNA was done as per manufacturer's directions or as described by Sambrook *et al.* (1989). Plasmid DNA digestions for identification of specific clones were performed as follows; 2 µl of miniprep plasmid DNA, containing approximately 200 ng of plasmid, was dispensed into a 0.5 ml microfuge tube to which was added 2.5 µl of 10X reaction buffer (appropriate to the enzyme being used), RNase A (final concentration 40 µg/ml), 0.5 µl of each restriction enzyme (equivalent to 2 to 10 units) and sterile ddH_2O to a final volume of 25 µl. Reactions were incubated at the designated incubation temperature for 1 to 16 hours. The digested DNA was ethanol precipitated, resuspended in 20µl of TE, and analyzed by agarose gel electrophoresis. The banding pattern of all restriction enzyme digests were photographed.

Plasmid digestions to be used for DNA fragment purification and vector preparation for cloning were scaled up so that 10-20 µg of DNA (or an entire miniprep) were treated in a final volume of 500 µl. These reactions also contained 1X strength reaction buffer, RNase A (final concentration 80µg/ml), and up to 100 units of restriction enzyme. Enzymes were either heat inactivated at 65°C for 20 minutes or removed by phenol:chloroform extraction, after which DNA was concentrated by ethanol precipitation.

2.10 DNA agarose gel electrophoresis

DNA fragments prepared by digestion with restriction endonucleases were separated by electrophoresis in agarose gels made in TAE (0.04 M Tris-acetate, 0.001 M EDTA) supplemented with ethidium bromide (Sharp *et al.*, 1973; Sambrook *et al.*, 1989). Powdered electrophoresis grade agarose (Gibco BRL) was dissolved at the appropriate concentration (ranging from 0.7% w/v to 2.5%) in TAE by boiling. Ethidium bromide (EtBr) was added to a final concentration of 0.5 μg/ml after cooling to 55°C and the agarose was poured into horizontal gel molds. EtBr intercalates within the DNA helix and fluoresces when exposed to UV light, allowing the direct visualization of as little as 1 ng of DNA (Sambrook *et al.*, 1989). Tracking dye (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 50% (v/v) glycerol) was diluted into samples to a final concentration of 10% (v/v). The samples were then dispensed into individual gel wells and the DNA fragments separated by electrophoresis at a voltage ranging from 2 to 12 volts/cm. Migration of the DNA bands was monitored by visualization under ultraviolet light (λ = 254 nm) produced by a hand held transilluminator (UVP Inc.). DNA band patterns on gels were photographed on a Spectroline (TR-254) transilluminator, using Polaroid type 667 film and a Polaroid MP-4 land camera (f8, 10 sec exposure) fitted with a Kodak #9 Wratten filter.

2.11 Purification of DNA

2.11.1 DNA fragment isolation from agarose gels

The DNA purification method of Girvitz *et al.* (1980) was utilized to isolate DNA fragments. Ten to twenty micrograms of plasmid DNA were digested to completion with a restriction endonuclease. Following ethanol precipitation, the DNA pellet was resuspended in 100 μl of TE containing 10% (v/v) tracking dye. The sample was loaded into two adjacent wells (2 mm x 5 mm) on a 0.7% (w/v) agarose gel containing ethidium bromide and the fragments were electrophoresed at 5 volts/cm. The gel was examined with 360 nm

UV light to minimize damage (Sambrook *et al.*, 1989). When the fragments were fully separated a clean scalpel blade was used to make a vertical cut in the gel just downstream of the desired fragment, perpendicular to the path of the DNA. A piece of 3MM Whatman filter paper (approximately 1 cm by 2 cm) was used as a template to cut a piece of dialysis tubing the same size. The 3MM:dialysis membrane sandwich was soaked in TAE buffer and then inserted with clean forceps into the incision in the gel with the dialysis membrane facing the anode of the gel box. Larger DNA fragments were excised from the gel before continuation of electrophoresis to decrease the possibility of contamination.

During electrophoresis an "elution chamber" was constructed. An 18 gauge needle was used to punch a hole in the bottom of a 0.5 ml microfuge tube. The tube was placed into a 1.5 ml tube and the caps were cut off of both tubes. When the DNA had completely migrated onto the 3MM: dialysis membrane the sandwich was removed with forceps and placed in the 0.5 ml tube. Care was taken not to lose the liquid absorbed to the 3MM filter paper as this would result in loss of DNA. One hundred and one µl of Band Elution Buffer (50 mM Tris•Cl pH 7.6, 0.2 M NaCl, 1 mM EDTA, 0.1% SDS) was added to the paper and membrane which was then spun for 10 sec at 350 *g* in a microcentrifuge. The eluant was transferred to a fresh 1.5 ml microfuge tube and the wash repeated twice, with a final spin of 30 sec at 11000 *g* to collect the buffer remaining in the filter paper. The DNA containing solution was then phenol:chloroform extracted and the DNA ethanol precipitated and dissolved in 20 µl of TE.

2.11.2 Polyethylene glycol (PEG) purification of DNA

High molecular weight plasmid DNA was purified from short fragments of DNA or oligoribonucleotides produced by RNase A digestion by PEG exclusion (Sambrook *et al.*, 1989). A two-third volume of 20% PEG8000 in 2.5 M NaCl was thoroughly mixed with the plasmid DNA and the mixture was incubated in an ice water bath for 1 hour. The precipitated large molecular weight DNA was pelleted by centrifugation at 14000 *g* at 4°C

for 10 minutes. This treatment leaves the small DNA and/or RNA fragments in solution in the PEG mixture. The supernatant was removed and the DNA pellet was carefully washed with ice cold 70% ethanol, allowed to air dry and dissolved in 50 µl of TE. The resulting purified linear or circular plasmid DNA was used for ligation and DNA sequencing purposes.

2.12 Ligation

2.12.1 Dephosphorylation of linearized plasmid DNA

For ligation reactions, purified preparations of linear plasmid DNA were treated with calf intestinal alkaline phosphatase (CIP) (Boehringer Mannheim). This removes the terminal 5' phosphate groups from DNA and prevents self-ligation (Sambrook *et al.*, 1989). Up to 20 µg of linear DNA in 50 µl TE was treated with 2 units of CIP enzyme in the presence of 1X Dephosphorylation Buffer (0.5 M Tris-HCl, 1 mm EDTA, pH 8.5). After a 30-45 minute incubation at 37°C the enzyme was removed by phenol:chloroform extraction, and the dephosphorylated DNA was ethanol precipitated and dissolved in 50µl of TE.

2.12.2 Ligation of DNA

Ligations were done as described in Sambrook *et al.* (1989) with some modifications. An insert to vector molar ratio of two fold or greater was used to enhance the degree to which products of mixed population ligations favored dimeric concatemers (Sambrook *et al.*, 1989).

All vector DNA sequences were dephosphorylated by treatment with CIP to decrease the fraction of self ligated vector in the ligation products except in cases where intramolecular ligation was desired. Samples of the vector and insert DNA to be ligated were analyzed on an agarose minigel with a DNA size standard (1 kb ladder, Gibco BRL) of known concentration. Amounts of the vector and insert were estimated by comparing

the fluorescence to a band of known DNA content in the 1 kb ladder. All ligations were prepared with 200 ng of vector DNA.

2.12.2.1 Sticky end ligations

Two hundred nanograms of vector DNA were ligated with an approximately two molar excess of insert DNA fragment. The DNA samples were combined with 2 µl of 10X ligation buffer (0.66 M Tris•Cl pH 7.5, 50 mM $MgCl_2$, 50 mM DTT, 10 mM ATP), 0.625 Weiss units of T4 DNA Ligase (Boehringer Mannheim) and ddH_2O to a final volume of 20 µl. The reaction was incubated overnight at 12°C, ethanol precipitated and dissolved in 20 µl of TE. Two µl of the ligation (approximately 40 ng of DNA) were transformed into electrocompetent DH5α as described above. The transformants were selected by plating onto LB + carbenicillin (20µg/ml) plates, supplemented with Xgal (final concentration 58 mg/ml) if blue/white color selection was used.

2.12.2.2 Blunt end ligations

Blunt end ligations requiring the filling of 3' recessed ends using Klenow polymerase were performed as follows. To maximize the blunt end ligation efficiency 200 ng of vector DNA were mixed with an approximately 3 molar excess of insert DNA fragment as estimated from photographs of minigels. The reaction was brought to 50 mM Tris•Cl (pH 7.2), 10 mM $MgCl_2$, and 100 µM each of dA, dC, dG, dTTP in a final volume of 20 µl. Five units of Klenow polymerase (*E. coli* DNA polymerase I large fragment, New England Biolabs) was added and the reaction incubated for 20 minutes at room temperature to allow the 3' recessed ends to be filled. The reaction was then supplemented with ATP and DTT to final concentrations of 1 mM and 5 mM respectively. T4 DNA Ligase (Boehringer) (5 units) was added and the reaction incubated overnight at 12°C. The ligated DNA was precipitated with ethanol, dissolved in TE and electroporated into *E. coli*.

2.13 Annealing of complementary oligonucleotides

Complementary oligonucleotides were annealed by mixing 10 µg of each in 50 mM NaCl in a 0.5 ml microfuge tube. Tubes were heated to 95°C for 5 min., then moved to a heating block set 10-15°C below the melting temperature (T_m) for each oligonucleotide pair as determined by the methods of Rychlik and Rhoads (1989) using the Dataminder computer program (version 1.0). The tubes were incubated for 1 hour at the appropriate temperature and then slowly cooled by removing the block from its heat element and letting the water cool to room temperature. Annealed oligonucleotides were stored at -20°C until used.

2.14 Phosphorylation of oligonucleotide fragments

In the event that purchased oligonucleotides had not been phosphorylated the annealed oligonucleotide pairs were 5' phosphorylated prior to ligation by treatment with T4 polynucleotide kinase (Gibco BRL) according to the manufacturer's directions.

2.15 Sequencing of plasmid DNA

Plasmid DNA was sequenced using the dideoxy method (Sanger *et al.*, 1977; modified by Hattori and Sakaki 1986) utilizing a Sequenase® kit V 2.0 (United States Biochemicals). Approximately 10 µg of plasmid DNA prepared by the alkaline lysis miniprep method (Sambrook *et al.*, 1989) was digested with RNase A (40µg/ml) for 30 minutes at 42°C, and precipitated with PEG as described above. The purified plasmid DNA was dissolved in 20 µl of TE and denatured by adding 18 µl of the solution to 2 µl of 2 N NaOH and incubating at room temperature for 5 minutes. The DNA was precipitated by adding 8 µl of 5 M ammonium acetate and 100 µl of 100% ethanol, followed by a 1 hour incubation at -20°C (Hattori and Sakaki, 1986). After centrifugation the DNA pellet was washed with 100 µl of 70% ethanol, air dried, and dissolved in 7µl of sterile water. To anneal the oligonucleotide sequencing primer to the denatured template, one microlitre

of a stock solution (6 ng/µl) followed by 2 µl of 5X Sequenase® reaction buffer (200 mM Tris•HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl) were added to the denatured template DNA and the mixture was heated to 65°C for 5 minutes in a heating block. The block was removed from the heater and allowed to equilibrate to room temperature (30-45 min). The tubes were centrifuged briefly to collect the solution and the sequencing reactions were prepared by the addition of 2 µl of diluted Labelling mix (1.5 µM dGTP, 1.5 µM dCTP, 1.5 µM dTTP), 1 µl of DTT (0.1 M), 0.5 µl of ^{35}S-dATP (1270 Ci/mmol, NEN Dupont), and 2 µl of diluted Sequenase® enzyme (6.5 µl Sequenase® dilution buffer, 1 µl Sequenase®, 0.5µl Pyrophosphatase). Reactions were incubated at room temperature for 2.5 minutes and then dispensed in 3.5 µl aliquots into each of four termination tubes which had been prewarmed to 37°C and contained 2.5 µl of the appropriate termination mix. Termination mixes were comprised of the four deoxynucleotide triphosphates (dATP, dCTP, dGTP and dTTP @ 80µM each), one dideoxynucleotide triphosphate (ddATP, ddCTP, ddGTP or ddTTP @ 8µM) and 50 mM NaCl. After exactly 5 minutes the reactions were stopped by the addition of 4 µl of Stop solution (95% formamide, 20 mM EDTA, 0.05% Bromophenol blue, 0.05% Xylene cyanol FF), and stored on ice or at -20°C for future use.

2.16 Separation of sequencing products by denaturing polyacrylamide electrophoresis

Sequencing reaction products were separated on a vertical sequencing apparatus (BioRad) containing a 6% polyacrylamide urea gel (Sambrook *et al.*, 1989). Gels were prepared by combining 25.2 grams of ultraPURE urea (Gibco BRL) with 8 ml ddH_2O, 12 ml 5X TBE (0.45 M Tris-borate, 0.005 M EDTA) and 9 ml premixed 40% (w/v) (19:1 acrylamide:bis) acrylamide mixture (BioRad). The mixture was dissolved by heating with stirring on a combination hot plate and brought to a final volume of 60 ml with ddH_2O. The solution was vacuum filtered through a buchner funnel fitted with Whatman filter paper

(No 2) and degassed under vacuum. Ten ml of this gel solution was mixed with 67 µl 25% ammonium persulfate (APS) and 50 µl TEMED and used as a plug to seal the bottom of the BioRad SequiGen gel form assembled in accordance with the manufacturer's directions. The remaining 50 ml of gel solution was combined with 63 µl of 25% APS and 50 µl of TEMED, mixed briefly and quickly injected using a 60cc syringe between the glass plates of the gel form. A 24 well sharkstooth comb was placed tooth side up between the glass plates to form a flat interface in the top of the polyacrylamide gel. When the acrylamide had polymerized the gel form was placed into the lower buffer chamber and the upper and lower chambers were filled with one litre of 1X TBE. The sharkstooth comb was removed and the gel prewarmed by electrophoresing the gel under constant power at 55 watts. When the gel temperature reached 50°C (as indicated by a temperature indicator affixed to the front glass plate of the apparatus) the sharkstooth comb was inserted, teeth down, to form sample wells on the interface. The wells were rinsed out with 1X TBE to remove acrylamide fragments and urea just prior to loading. Samples were heated to 85-90°C for 5 minutes, then loaded into gel lanes. The gel was run at constant power (55 W) to maintain the gel temperature at 50°C until the bromophenol blue indicator dye had migrated to the bottom edge of the gel (approximately 90 minutes). The gel was removed from the glass plate sandwich, laid on to 3MM Whatman paper, covered with plastic wrap and dried under vacuum at 80°C for 1 hour using a Savant gel drying system. The dried gel was then exposed to Kodak X-Omat AR or Dupont Reflections™ Xray film at room temperature for at least 12 hours and developed using a model 14-XL automated Xray film processor (AFP Imaging, Elmsford, N.Y.).

2.17 DNA isolation from yeast

DNA was isolated from yeast cultures using either the glass bead method of Hoffman and Winston (1987) or Zymolyase based on the methods of Cryer *et al.* (1976).

2.17.1 Hoffman and Winston glass bead prep

The method of Hoffman and Winston (1987) was used to isolate plasmid DNA from yeast. This method also yielded sufficient genomic DNA to perform southern blot analysis. Cells from a single yeast colony were inoculated into 2 ml of the appropriate medium, grown overnight at 30°C with shaking and decanted into a 1.7 ml microfuge tube. Cells were pelleted by a 30 sec. centrifugation at 11 000 *g*, the medium was removed by aspiration and the cells washed in 500 µl of sterile ddH_2O. The cells were resuspended in 200 µl yeast cracking buffer (2%(v/v) Triton X-100, 1%(w/v) SDS, 100 mM NaCl, 10 mM Tris•Cl pH 8.0, 1 mM EDTA) and approximately 200 µl of acid washed glass beads (425-600 microns, Sigma G-8772) was added to each tube. Two hundred microlitres of phenol:chloroform was added and the cell suspension was mixed on a vortex apparatus (Canlab) for three times for 30 seconds with a 30 second incubation on ice between each round of mixing. The tubes were centrifuged for 2 min. at 14 000 *g* at room temperature to pellet the cellular debris and separate the phases. The supernatant was removed to a fresh microfuge tube and DNA was ethanol precipitated and dissolved in 50 µl TE.

2.17.2 Spheroplast method

To isolate genomic DNA from yeast for use in Southern blot analysis a gentler extraction method involving enzymatic cell wall digestion was employed (Cryer *et al.*, 1976). The cells from a 5 ml culture grown in YPAD or selective medium for 1 to 2 days were collected in a 1.7 ml microfuge tube, washed with 1 ml of sterile ddH_2O, and resuspended in 400 µl of SCE (1 M sorbitol, 0.1 M sodium citrate, 0.05 M EDTA) containing 3.2 µl of ß-mercaptoethanol and 0.4 mg of yeast lytic enzyme (70,000 units/g, ICN). Tubes were incubated in a 37°C water bath until spheroplasts were formed (about 2 hours). The formation of spheroplasts was verified by adding 20% SDS to induce lysis in an aliquot of cells while observing them under a microscope. The cells were lysed by the addition of 400 µl of lysis buffer (2% SDS, 50 mM Tris, 10 mM EDTA) and gentle

mixing. Following the addition of 200 µl of 5 M NaCl, the solution was mixed well and the tubes were placed on ice for 1 hour. The nucleic acids were pelleted by centrifugation at 14 000 *g* for 10 minutes. The supernatant was discarded and the pellet dissolved in 400 µl TE with a sterile toothpick to mix the solution. The suspension was extracted with one volume of phenol:chloroform and the nucleic acids precipitated with 800 µl of ice cold 95% ethanol. The DNA pellet was washed with 70% ethanol, air dried and dissolved in 100 µl of TE.

2.18 High efficiency yeast transformation

The high efficiency lithium acetate/single stranded DNA/PEG (LiAc/ssDNA/PEG) transformation protocol of Gietz and Woods (1994) was used for all yeast transformations. A 10 ml starter culture of either YPAD or the appropriate synthetic complete (SC) dropout medium was inoculated from solid medium and grown overnight at 30°C with shaking in a 150 mm glass test tube. The titre of the culture was determined by using a Bright Line® hemacytometer (Reichert, Buffalo, N.Y.). Fifty millilitres of prewarmed YPAD medium was inoculated from the starter culture to a concentration of 5 x 10^6 cells per ml. Cells were grown at 30°C for 3-5 hours or until 2 doublings had occurred, to bring the cell concentration to 2 x 10^7 cells/ml. The log phase culture was pelleted by a 5 min. centrifugation at 3380 *g* in an IEC Centra MP4 table top centrifuge. The cells were washed with 20 ml of sterile water, pelleted, resuspended in 1 ml of sterile water and transferred to a 1.5 ml microfuge tube. The cells were pelleted a third time and resuspended in 100 mM lithium acetate (LiAc) to a final concentration of 2 x 10^9 cells/ml and incubated at 30°C for 15 minutes.

A 50 µl aliquot of cells was pipetted into a 1.5 ml microfuge tube for each transformation. The cells were pelleted by a 10 second spin and the LiAc removed. Transformation mix was added to the yeast pellet in the following order: 240µl of 50% (w/v) PEG 3350, 36 µl of 1 M LiAc, 25 µl of boiled single stranded carrier DNA (salmon

testes DNA @ 2 mg/ml), 100 ng - 5 μg of plasmid DNA and sterile ddH_2O to a final volume of 350 μl. The pellet was resuspended in the transformation mixture by vortexing, and the resulting mixture equilibrated at 30°C for 30 minutes. The yeast were then subjected to a heat shock at 42°C for 20 min. and collected by a 15 sec centrifugation at 8 000 *g*. The transformation mix was removed with a micropipette and the cells were resuspended in 1.0 ml of sterile ddH_2O. Samples were plated onto appropriate medium to select for transformants and incubated for 2 to 5 days at 30°C until colonies grew to be readily visible.

2.19 Yeast colony replica plating

The standard method of replica plating with velveteen squares (Lederberg and Lederberg, 1952) was utilized to transfer yeast colonies from one type of medium onto another. A sterile 10 cm^2 piece of velvet was affixed to a circular wood support by means of a metal collar. The source plate was gently pressed onto the velvet to leave an impression of the colonies on the material. Successive replicas (up to 5) were then made from the imprint by pressing fresh plates onto the velvet in turn. This protocol provided a time efficient method for transferring and testing the same set of colonies for several auxotrophic markers at once. After use the velveteen squares were washed in tap water, rinsed, dried flat, de-linted and sterilized by autoclaving.

2.20 Yeast colony filter lift analysis

Activation of *lacZ* reporter gene constructs was detected by filter lift analysis (Breeden and Nasmyth, 1985). A sterile Whatman filter disc (75 mm) was placed onto a plate containing yeast colonies to be tested. The orientation of the disc on the plate was marked by asymmetrically placed punctures with an 18 gauge needle. Care was taken to ensure that all of the colonies made contact with the filter. The filter was lifted slowly from the plate and submerged colony side up in a bath of liquid nitrogen for 10 sec. The filter

was allowed to thaw at room temperature for 5 minutes and then frozen twice more to ensure adequate lysis of the cells. After the final thawing the filter was placed on a second filter paper disc in a sterile petri dish containing 1.25 ml of Z buffer (60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$) supplemented with 3.3 µl of ß-ME and 21 µl of Xgal (25 mg/ml in DMF) (Miller, 1972). The dishes were placed in plastic bags to retard evaporation and incubated at 37°C until blue color appeared. Colonies with *lacZ* reporter genes activated by strong positives turned blue in 20 min.

2.21 ß-Galactosidase quantitation by ONPG assay

ß-galactosidase expression induced by two-hybrid positives was quantified by liquid ONPG cleavage assays (Miller, 1972). Transformants from SC minus dropout plates were used to inoculate liquid dropout medium and cultures were grown overnight with good aeration at 30°C. The OD_{600} of the saturated culture was measured and 1.0 ml of the culture was then dispensed into a 1.7 ml microfuge tube. The cells were pelleted by a 1 min. centrifugation at 14 000 *g*, the medium was removed and the cells washed with 1 ml of Z buffer (60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$). The cells were pelleted again, the Z buffer removed and the pellet resuspended in 300 µl of Z buffer to concentrate the cells. 100 µl of the suspension was transferred to a 1.7 ml microfuge tube with a hole punctured in the lid with an 18 gauge needle. The tubes were immersed in liquid nitrogen for 15 seconds and then placed in a 42°C water bath to freeze/thaw the cells. The freeze/thaw was repeated once to ensure adequate lysis. The cells were then mixed with 700 µl of Z buffer containing ß-ME (39 mM) followed by 160 µl of a 4 mg/ml solution of ortho-nitrophenyl ß-D-galactopyranoside (ONPG). The tubes were incubated at 30°C until a yellow color developed, at which time the reaction was stopped by the addition of 400 µl of 1 M Na_2CO_3 and the elapsed time recorded. The cellular debris was pelleted by a 3 min centrifugation at 14 000 *g* and the

OD_{420} of the supernatant determined. β-galactosidase activity (Miller units) was calculated by the formula $1000 * OD_{420} / (t * v * OD_{600})$ where t is the reaction time and v is the volume (in this case 0.33 ml) of cells used in the assay. Measurements were determined from two independent transformants and the results averaged.

2.22 Southern blotting

2.22.1 Digestion, separation and transfer of DNA

Genomic DNA was digested with the appropriate restriction endonuclease. Samples and DNA size standards (1 kb ladder, Gibco BRL) were separated by agarose gel electrophoresis on a 14 cm 0.75% agarose gel at 2 V/cm for 12-16 hours. The gel was stained in 1X TAE buffer containing EtBr at a concentration of 5 μg/ml and photographed. The DNA was transferred to Zetaprobe® membrane (BioRad) by alkaline transfer (Reed and Mann, 1985) for 3-4 hours with 0.4 N NaOH using the TurboBlotter™ apparatus (Schleicher and Schuell) assembled according to manufacturer's directions. After transfer, the membrane was rinsed in 2X SSC (0.3 M NaCl, 30 mM Sodium citrate, pH 7.0) and allowed to air dry. The membrane was stored between sheets of filter paper in a sealed plastic bag at room temperature until probed.

2.22.2 Klenow labelling of DNA

DNA fragments used as probes were isolated by the method of Girvitz *et al.* (1980) and labelled by the random priming method of Feinberg and Vogelstein (1983; 1984). The fragment DNA to be labelled (300 ng) was diluted to a total volume of 100 μl of TE in a microfuge tube. The tube was placed in a boiling water bath for 5 minutes, then 33 microlitres (100 ng) of the denatured DNA was mixed with 2 μl BSA (10 mg/ml), 10 μl oligonucleotide labelling buffer (OLB) (see Table 5 below) 5 μl ^{32}P-dCTP (3000 Ci/mmol) (NEN Dupont) and 2 μl (10 units) of Klenow polymerase (New England Biolabs). After a

two hour incubation at room temperature, the reaction was terminated by the addition of one volume (50 µl) of Oligonucleotide Labelling Buffer stop solution (0.2% SDS in TE and bromophenol blue (0.1 mg/ml)).

Table 5 Oligonucleotide Labelling Buffer (dCTP label)

Solution O	Solution A	Solution B	Solution C
1.25 M Tris•Cl 0.125 M $MgCl_2$ pH 8.0	1 ml Solution O + 18 µl βME, 5 µl dATP 5 µl dTTP 5 µl dGTP	2 M Hepes pH 6.6 (Titrated with 4 M NaOH)	Hexadeoxyribonucleotides in TE @ 90 OD units/ml

The OLB mix for dCTP label is comprised of Solutions A, B and C mixed in a 100:250:150 ratio. Stock solutions of deoxynucleotide triphophates (dNTPs) used in solution A were 100 mM in 3 mM Tris•Cl pH 7, 0.2 mM EDTA (Feinberg and Vogelstein, 1984).

Unincorporated label was removed by centrifugation of the reaction mixture through a Biospin 6 chromatography column (BioRad) using an IEC model CL clinical centrifuge at maximum rpm. The radioactive eluate containing the labelled probe was quantified by aliquoting 1 µl to 1 ml of water and analyzed in a Beckman scintillation counter (Model LS1800). Ten million cpm of a probe with a specific activity ranging from 1×10^8 to 1×10^9 cpm per microgram (usually 10 - 20 µl) were used for every 100 cm^2 of membrane.

2.22.3 Membrane hybridization

Hybridization was carried out in glass tubes in a Robbins Scientific hybridization oven at 65°C. Membranes were prehybridized for 1 to 3 hours at 65°C with 5 ml of Westneat hybridization buffer (7% SDS, 1 mM EDTA pH 8, 0.263 M Na_2HPO_4 pH 7.2, 1% BSA) containing 250 mg of single stranded salmon testes carrier DNA (Westneat *et al.*, 1988). The prepared probe was carefully added into the hybridization buffer in the tube without touching the blot. (Direct application of the probe to the membrane can result in

obliteration of a clean signal due to high background caused by nonspecific hybridization at the area of application.) The tube was returned to the oven at 65°C for 12-16 hours.

After hybridization the membrane was washed to remove unbound probe. Two 15 minutes washes at room temperature in wash solution 1 (2X SSC, 0.2% SDS) were followed by two 15 minutes washes at 65°C in wash solution 2 (0.1X SSC, 0.1% SDS) (Sambrook *et al.*, 1989). After washing the membrane was wrapped in plastic film and exposed to Kodak XOmat AR or DuPont Reflections™ Xray film between two DuPont Cronex® Lightning Plus intensifying screens at -80°C for one to seven hours. The film was developed using a model 14-XL automated Xray film processor (AFP Imaging, Elmsford, N.Y.).

3. RESULTS AND DISCUSSION

3.1 Experimental rationale

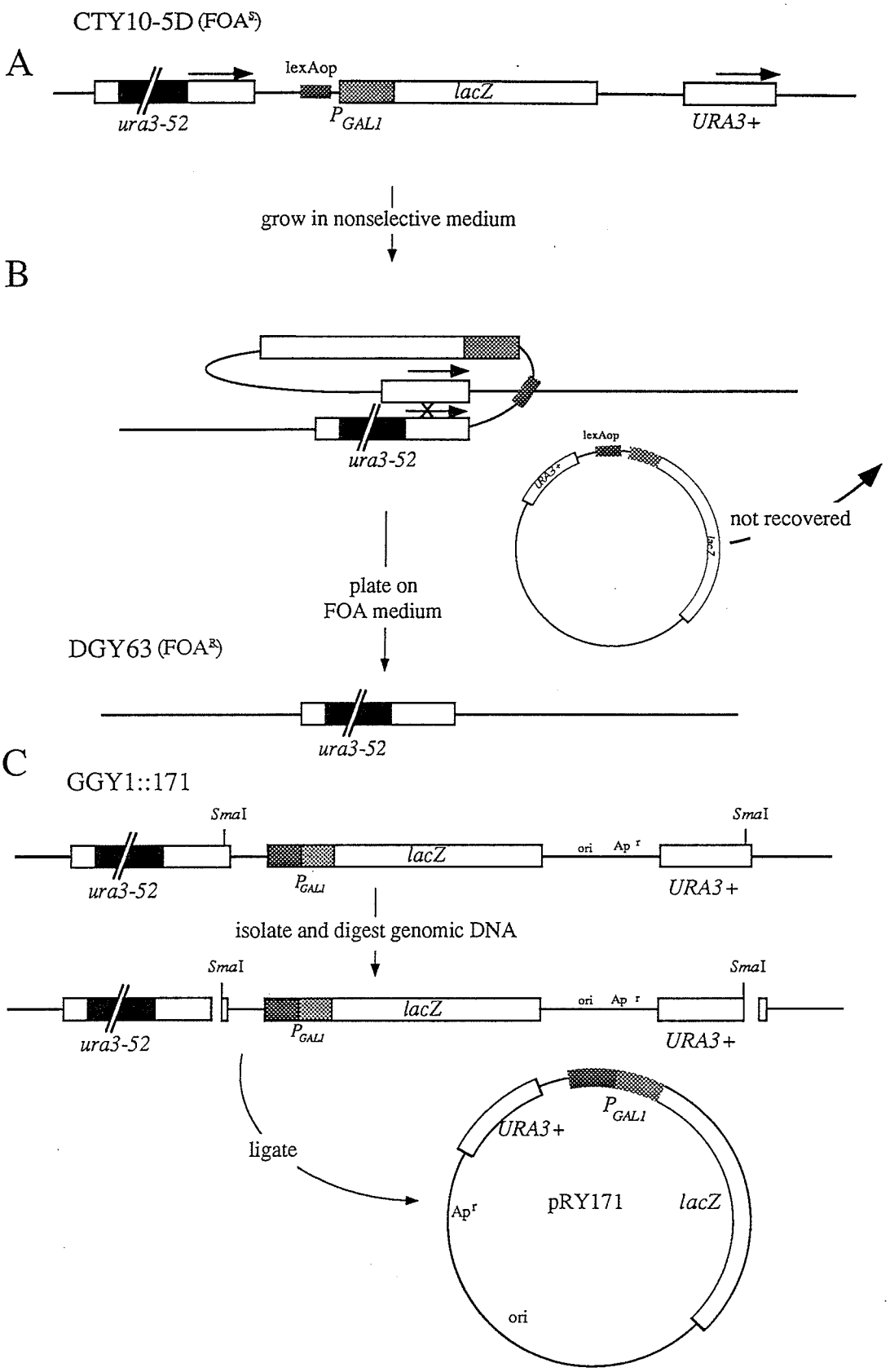
Most of the yeast strains utilized in the two-hybrid system have the *lacZ* reporter construct integrated at the *URA3* locus (Bartel *et al.*, 1993a; Bartel and Fields, 1995; Gietz *et al.*, 1996). This integration involves transforming a *ura3-52* strain with a linearized plasmid carrying the reporter gene and the selectable *URA3* gene. Integration involves two recombination events between homologous sequences in *ura3-52* and *URA3*. It results in a construct like the one shown in Figure 6A. Note that the *ura3-52* and *URA3* sequences form non-tandem repeats on either side of the reporter gene (Struhl *et al.*, 1979; Orr-Weaver *et al.*, 1981). This integrant is prototrophic for uracil and prevents the experimenter from using *URA3* as the selectable marker on a plasmid. In addition, the non-tandem repeats recombine with a frequency of 1% per 15 generations resulting in the excision of the DNA between them, including the reporter gene (Figure 6B) (Orr-Weaver *et al.*, 1981; Schiestl *et al.*, 1988). In preparation for high efficiency transformation, an integral part of a two-hybrid screen, a yeast culture passes through 10 or more generations. Uracil prototrophic yeast strains such as CTY10-5D (Bartel *et al.*, 1993a; see Figure 2A) must be grown in SC-ura selective medium prior to transformation to prevent loss of the reporter construct within a large portion of the yeast cell population (Struhl *et al.*, 1979). We decided to modify an existing strain used for two-hybrid screening to allow use of the *URA3* gene as a selectable marker. To this end "inverted" cassettes of the *URA3*, *LYS2* and *LEU2* genes were produced. A working copy of the gene being inverted was digested at a unique restriction site near the middle of the gene's open reading frame (ORF). Another linearized plasmid (e.g. pUC18 cut with *Bam*HI) was ligated into the gene. The plasmid backbone from the original plasmid was then separated from the gene by digestion at the 5' and 3' ends of the gene fragment, and these ends were then ligated together to

Figure 6 **Nontandem repeats result in reporter construct instability and allow recovery of plasmid.**

A. Structure of the *lexAop GAL1-lacZ* reporter construct in CTY10-5D (Bartel *et al.*, 1993a).

B. Structure of possible recombination intermediates.
Lack of selection pressure allows loss of the intervening plasmid DNA sequences through homologous recombination between nontandem repeats.

C. The recovery of pRY171 after genomic integration.
Genomic DNA from the yeast strain GGY1::171 (Gill and Ptashne, 1987) was restricted with *Sma*I, ligated and electroporated into bacteria. Plasmid DNA isolated from carbenicillin resistant blue colonies from Xgal medium contained the plasmid pRY171 (Yocum *et al.*, 1984) as verified by restriction mapping.


CTY10-5D (FOA^S)
A
lexAop
lacZ
ura3-52
P GAL1
URA3+
grow in nonselective medium
B
ura3-52
lexAop
URA3+
lacZ
not recovered
plate on
FOA medium
DGY63 (FOA^R)
ura3-52
C
GGY1::171
SmaI
SmaI
lacZ
ori
Ap r
ura3-52
P GAL1
URA3+
isolate and digest genomic DNA
SmaI
SmaI
lacZ
ori
Ap r
ura3-52
P GAL1
URA3+
ligate
P GAL1
URA3+
Ap r
pRY171
lacZ
ori

from the inverted gene cassette. These new gene cassettes (*ura3-inv, lys2-inv, leu2-inv*) allow one step gene replacement by cutting the plasmid to free the 5' and 3' ends of the inverted gene. The cassettes themselves can be cloned into any plasmid to target the integration of that plasmid as a stable disruption of the "inverted" gene. By incorporating a reporter gene into the plasmid backbone, strains carrying these integrated plasmids have a chromosomally located reporter gene and remain auxotrophic for uracil, lysine and adenine (Ura-, Lys- and Leu-).

3.2 Two-hybrid Yeast strain construction

3.2.1 Strain modification

The first step in the construction of the new strain was the modification of the strain CTY10-5D to act as a host for reception of the integrative plasmids containing the *ura3-inv*, *lys2-inv* and *leu2-inv* cassettes (see below). This required removal of the *lexAop:GAL1-lacZ* reporter gene and replacement of the mutant alleles *ura3-52*, *leu2-3,112* with their wild type alleles *URA3* and *LEU2*. Previous to this project strain CTY10-5D was grown in YPAD and plated onto FOA medium to select for the loss of the *URA3* allele by homologous recombination between the *ura3-52* and *URA3* non-tandem repeats, as shown in Figure 6(B). The resulting strain was designated DGY63. The *ura3*-52 allele in strain DGY63 was replaced with the wild type *URA3*+ gene by transformation of the 5 kb *Bam*HI fragment isolated from plasmid pDP3 (see Figure 7A). The transformed yeast were plated onto SC-ura selective medium and incubated at 30°C for three days. Of the several hundred Ura+ colonies produced, four were tested for stability by growing overnight in liquid YPAD medium and then plating 10^7 cells onto FOA plates. Unstable integrants should exhibit the high frequency loss of the Ura+ phenotype, measured by the ability to survive on FOA medium. Of the 4 colonies, two were found to be stable (no FOA^S, Ura- cells in 10^7 cells). The replacement of *ura3-52* by *URA3* in one of them,

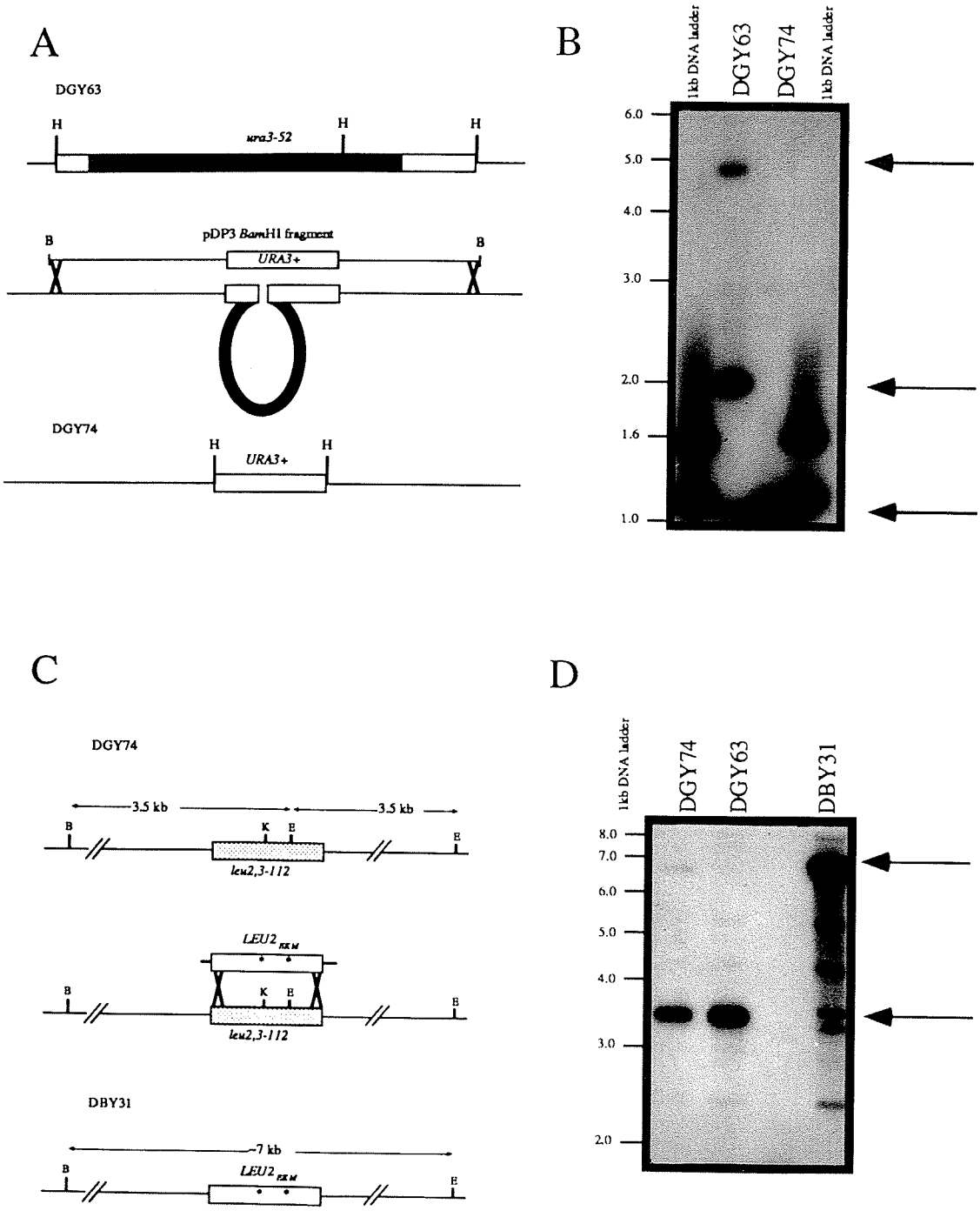
Figure 7 **Replacement of *ura3-52* and *leu2,3-112* alleles in CTY10-5D with the wild type alleles *URA3+* and $LEU2_{EKM}$.**

A. *URA3* Gene Replacement Schematic.
The *ura3-52* allele in strain DGY63 is replaced by homologous recombination. DGY63 was transformed with a 5 kb fragment from plasmid pDP3 containing the wild type *URA3* gene.

B. Verification of *ura3-52* Replacement.
Southern blot shows *Hin*dIII digested genomic DNA probed with the 1.1 kb *Hin*dIII *URA3* fragment. Replacement is indicated by the loss of the 5 kb and 2 kb fragments from *ura3-52* and the appearance of the 1.1 kb wild type fragment. The confirmed strain was designated DGY74.

C. *LEU2* Gene Replacement Schematic.
The $LEU2_{EKM}$ gene from plasmid pDG317 lacking the *Eco*RI and *Kpn*I restriction sites present in the wild type sequence (Gietz and Sugino, 1988) was transformed into the strain DGY74 and Leu+ transformants selected.

D. Southern Blot to Verify *LEU2* Structure.
Genomic DNA digested with *Bam*HI and *Eco*RI produces two 3.5 kb fragments from the *leu2-3,112* allele. The lack of the *Eco*RI site in $LEU2_{EKM}$ produces a 7 kb band. The confirmed strain was designated DBY31. Nonspecific bands are unexplained but considered inconsequential because they appeared in negative controls.


A
DGY63
H
ura3-52
H
H
pDP3 BamHI fragment
B
URA3+
B
DGY74
H
URA3+
H
B
1kb DNA ladder
DGY63
DGY74
1kb DNA ladder
6.0
5.0
4.0
3.0
2.0
1.6
1.0
C
DGY74
3.5 kb
3.5 kb
B
K
E
E
leu2,3-112
LEU2 NKM
B
K
E
E
leu2,3-112
DBY31
7 kb
B
LEU2 NKM
E
D
1kb DNA ladder
DGY74
DGY63
DBY31
8.0
7.0
6.0
5.0
4.0
3.0
2.0

designated DGY74, was verified by Southern blot analysis (Figure 7B). DGY74 was next converted to *LEU2* by replacement of the *leu2-3,112* mutant allele with the $LEU2_{EKM}$, allele. This allele was mutagenized *in vitro* by Gietz and Sugino (1988) to remove the *Eco*RI and *Kpn*I sites but still produces an active protein. The *Bam*HI/*Eco*RI fragment from plasmid pDG317 was transformed into DGY74 to produce the *LEU+* strain DBY31 (Figure 7C). The replacement of *leu2-3.112* by $LEU2_{EKM}$ was confirmed by Southern blot (Figure 7D).

3.2.2 Recovery of pRY171 from the yeast genome

The plasmid pRY171 contains a well characterized *GAL1-lacZ* fusion which when integrated functions as an efficient reporter construct for two-hybrid research (Yocum *et al.*, 1984; Fields and Song, 1989; Durfee *et al.*, 1993). This plasmid was recovered by Dr. Gietz from the yeast strain GGY1::171 (Gill and Ptashne, 1987) by the plasmid eviction method (Winston *et al.*, 1983). Briefly, genomic DNA was prepared by the spheroplast method as described in Materials and Methods. Ten micrograms of genomic DNA were digested with the restriction endonuclease *Sma*I, which was known to have unique sites within the repeated *URA3* sequences (Figure 6C). DNA was self ligated and electroporated into DH5a. All blue colonies on LB + carbenicillin Xgal medium that were tested contained plasmid DNA with the size and restriction map of pRY171 as implied (Yocum *et al.*, 1984; D. Gietz, personal communication).

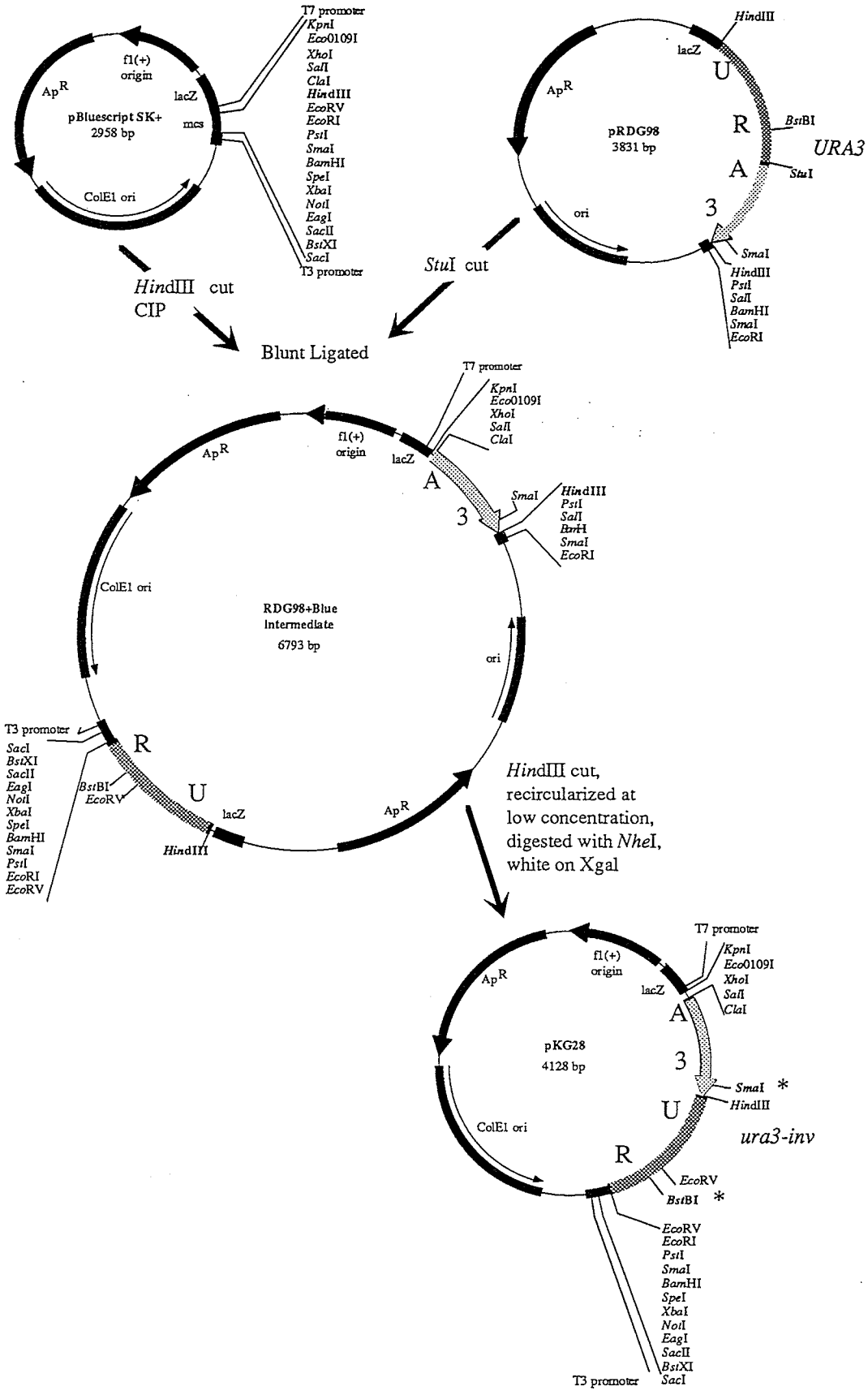
3.3 Construction of the inverted gene cassettes

3.3.1 Construction of *ura3-inv*

The *ura3-inv* gene was constructed by means of a two-step "cascade" ligation as depicted in Figure 8. The plasmid pRDG98 contains the *URA3* gene on a genomic 1.1 kb

Figure 8 **Construction of the *ura3-inv* cassette.**

pRDG98 was digested with *Stu*I and blunt end ligated into the *Hin*dIII site of pBluescript SK+ (Stratagene) producing the intermediate plasmid RDG98+Blue and destroying both sites. After deactivation of the ligase, the DNA was digested with *Hin*dIII to separate the pUC8 plasmid backbone of pRDG98 from the *URA3* gene. The *Hin*dIII enzyme was removed and the DNA sticky end ligated at low concentration to promote circularization of DNA molecules. Blunt end self ligation of *Hin*dIII cut pBluescript SK(+) or pUC8 could result in a viable vector with an *Nhe*I site, AAGCTAGCTT, so the final ligation was digested with *Nhe*I to linearize any such molecules before electroporation into bacteria. Plasmid DNA was isolated from carbenicillin resistant colonies which were white on Xgal medium. The vector that produced the expected restriction pattern with *Sma*I was designated pKG28.


pBluescript SK+
2958 bp
f1(+) origin
ApR
lacZ
mcs
ColE1 ori
T7 promoter
KpnI
Eco0109I
XhoI
SalI
ClaI
HindIII
EcoRV
EcoRI
PstI
SmaI
BamHI
SpeI
XbaI
NotI
EagI
SacII
BstXI
SacI
T3 promoter
pRDG98
3831 bp
URA3
BstBI
StuI
HindIII
PstI
SalI
BamHI
SmaI
EcoRI
HindIII cut
CIP
StuI cut
Blunt Ligated
RDG98+Blue
Intermediate
6793 bp
HindIII cut,
recircularized at
low concentration,
digested with NheI,
white on Xgal
pKG28
4128 bp
ura3-inv
*

*Hin*dIII fragment from YEp24 (Botstein *et al.*, 1979) cloned into pUC8. pRDG98 was digested with *Stu*I at base pair 435 of the *URA3* ORF (Rose *et al.*, 1984), which eventually results in a disruption at amino acid 146 of the 267 residue protein. The linearized vector was blunt end ligated to *Hin*dIII digested pBluescript SK(+) (Stratagene). The ligation was phenol extracted to remove the T4 ligase, ethanol precipitated and digested with *Hin*dIII, which digests the remaining *Hin*dIII sites in the *URA3* gene, releasing the pUC8 plasmid backbone of pRDG98. The *Hin*dIII was removed by phenol:chloroform extraction and the DNA was ligated at a low DNA concentration (~4 ng/µl) to favor intramolecular ligation (Sambrook *et al.*, 1989). The completed ligation was then digested with *Nhe*I to linearize any circularized pBluescript SK+ and pUC8 which may have formed by the ligation of Klenow filled *Hin*dIII ends. The ligation products were electroporated into *E. coli* and carbenicillin resistant colonies that were white on Xgal medium were verified as the anticipated product by restriction digestion. Plasmid pKG28 contained the inverted *URA3* gene structure (*ura3-inv*) shown in Figure 8 which could be removed from pBluescript SK(+) as a *Cla*I, *Eco*RI cassette.

3.3.2 Construction of the *ura3-inv::GAL1-lacZ* plasmids

To combine *ura3-inv* with the *GAL1-lacZ* reporter, plasmid pRY171 (Yocum *et al.*, 1984) was digested with *Bam*HI to separate the *GAL1-10* promoter fragment and the remaining vector DNA (Figure 9A). Both DNA fragments were purified as described in Materials and Methods. The *GAL1 Bam*HI fragment was retained for later use and the vector fragment was circularized upon itself, forming plasmid pKG145 (Figure 9B). pKG145 was cut with *Sma*I and *Hin*dIII to release the *URA3* gene, the larger vector band was purified and blunt-end ligated with the *Cla*I/*Eco*RI *ura3-inv* cassette from pKG28. Clones containing either orientation of the *ura3-inv* cassette were produced and designated pKG147 and pKG148 (Figure 9C). Both orientations of *ura3-inv* were isolated in the

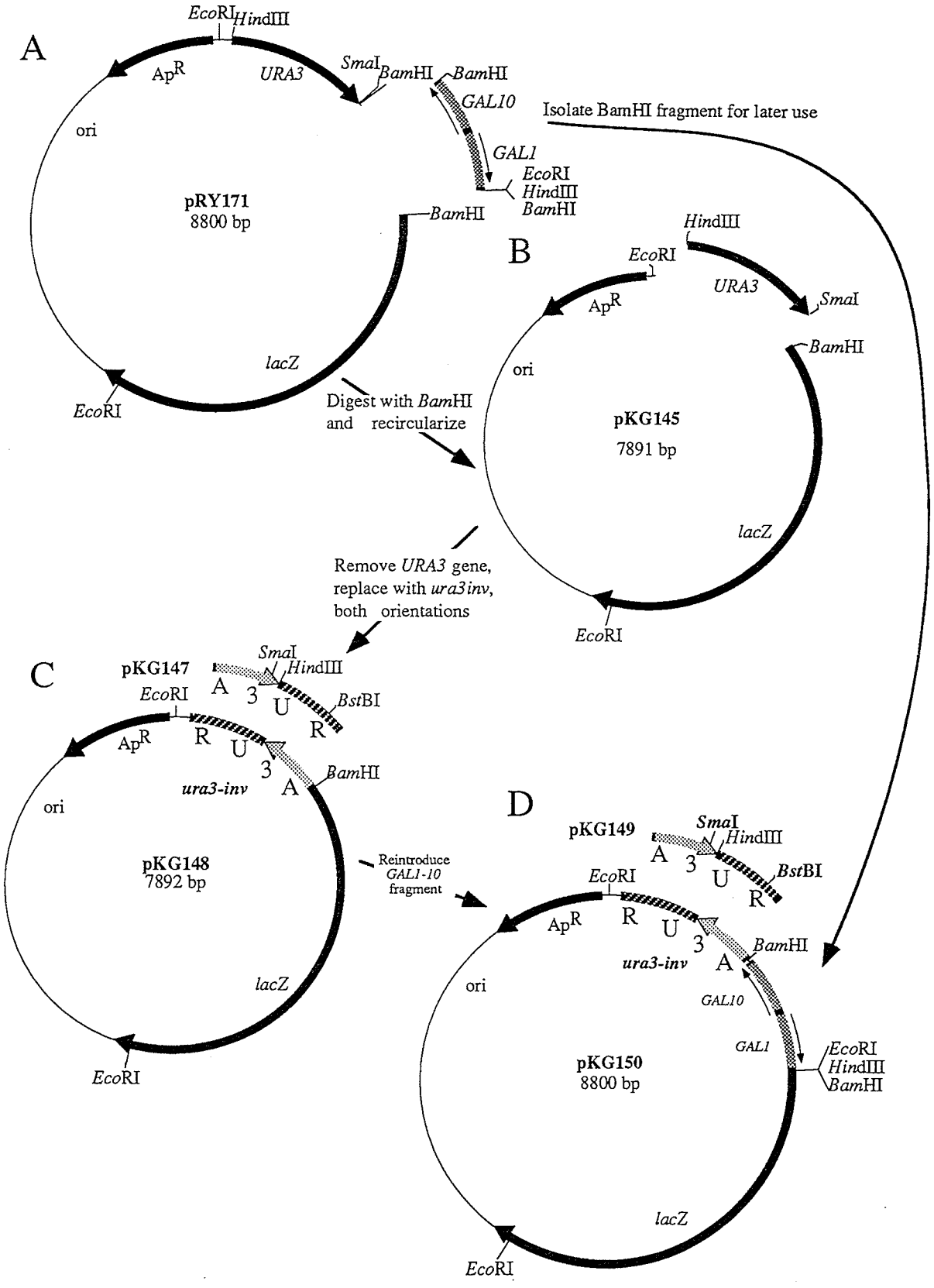
Figure 9 **Construction of the *ura3-inv::GAL1-lacZ* plasmids.**

A. Isolation of the *GAL1-10* promoter and ligation to form pKG145.
The vector pRY171 (Yocum *et al.*, 1984) was digested with *Bam*HI to isolate the *GAL1-10* promoter fragment from the rest of the plasmid. The promoter was retained for later use and the vector containing the *URA3* gene and *GAL1-lacZ* reporter religated to form plasmid pKG145.

B. Removal of the *URA3* gene.
pKG145 was digested with *Hin*dIII and *Sma*I to remove the *URA3* gene.

C. Introduction of *ura3-inv*.
The *ura3-inv Eco*RI/*Cla*I cassette from pKG28 was cloned into the *Hin*dIII and *Sma*I sites of pKG145. Both orientations of *ura3-inv* were isolated and designated pKG147 and pKG148 respectively.

D. Introduction of *GAL1-10*.
The *GAL1-10* promoter fragment was cloned into the *Bam*HI site of pKG147 and pKG148 to reproduce the original GAL1-lacZ fusion of pRY171. The plasmids were designated pKG149 and pKG150 respectively.


A
EcoRI
HindIII
ApR
URA3
SmaI
BamHI
BamHI
GAL10
GAL1
EcoRI
HindIII
BamHI
ori
pRY171
8800 bp
BamHI
lacZ
EcoRI
Isolate BamHI fragment for later use
Digest with BamHI
and recircularize
B
HindIII
EcoRI
URA3
SmaI
ApR
BamHI
ori
pKG145
7891 bp
lacZ
EcoRI
Remove URA3 gene,
replace with ura3inv,
both orientations
C
pKG147
SmaI
HindIII
EcoRI
A
3
U
BstBI
R
ApR
R
U
3
BamHI
ura3-inv
A
ori
pKG148
7892 bp
lacZ
EcoRI
Reintroduce
GAL1-10
fragment
D
pKG149
SmaI
HindIII
A
3
U
BstBI
EcoRI
R
ApR
R
U
3
BamHI
ura3-inv
A
ori
GAL10
GAL1
EcoRI
HindIII
BamHI
pKG150
8800 bp
lacZ
EcoRI

event that either orientation could potentially have been subject to activation by sequences flanking the *URA3* locus when integrated into the yeast genome. The *GAL1-10* promoter fragment previously isolated was reintroduced into the *Bam*HI site to restore the frame of the *GAL1-lacZ* fusion present in pRY171, forming plasmids pKG149 and pKG150 (Figure 9D). The presence of a *Hin*dIII site in the *GAL1-lacZ* fusion junction prevented linearization of the plasmid at the *Hin*dIII site in *ura3-inv*. The alternate unique restriction sites *Bst*BI and *Sma*I were identified. Cleavage with these enzymes produced a linear DNA molecule with homologous ends for integration at *URA3* (see Figure 9D).

3.3.3 Construction of *lys2-inv*

A similar cascade ligation approach was undertaken for the construction of the inverted *lys2* cassette (see Figure 10). A 3kb *Nco*I fragment containing the last 2/3 of the *LYS2* gene, coding for amino acids 514 - 1392, was isolated from plasmid pRDG108 and sticky end ligated to *Nco*I digested pJR5 (Paetkau et al, 1994). Plasmid pJR5 is a derivative of pACTII (gift of Dr. S. Elledge) containing a fragment of the yeast *SIR3* gene (Paetkau et al, 1994). It was utilized for this ligation because it is not cut by *Bam*HI, a feature which was essential for this construction. The resulting clone, pKG70, was digested with *Bam*HI, which cleaves at bp 3346 of the *LYS2* coding sequence, and ligated with *Bam*HI digested pUC18. The ligase was inactivated by phenol extraction and the DNA was digested with *Nco*I to dissociate the pKG70 backbone. After a subsequent phenol extraction, the DNA was ligated at low concentration to preferentially form circles, including the *lys2-inv*::pUC18 construct. The ligase was removed again by phenol:chloroform extraction and the DNA digested with *Bgl*II to linearize any recircularized pACTII based product. The ligation products were electroporated into DH5α and plated on LB plates containing carbenicillin and Xgal. Insert-containing white colonies were selected and the structure of the plasmid they contained analyzed by restriction enzyme digestion. One of these plasmids, pKG72 in Figure 10, was selected for further

Figure 10 Construction of the *lys2-inv* cassette.

The originating *LYS2* plasmid pKG70 was formed by ligating the *Nco*I fragment from the *LYS2* gene into the vector pJR5 as described in the text. Plasmid pKG70 was then digested with *Bam*HI and sticky end ligated into the *Bam*HI site of pUC18. After deactivation of the ligase, the ligation products, which contained the pKG70&pUC18 intermediate, were digested with *Nco*I to dissociate the pKG70 backbone from the concatomer. The *Nco*I enzyme was removed and the DNA sticky end ligated at low concentration to promote circularization of DNA molecules. The ligation was digested with *Bgl*II to linearize any recircularized pKG70 before electroporation into bacteria. Plasmid DNA was isolated from carbenicillin resistant colonies which were white on Xgal medium. The clone that produced the expected restriction pattern was designated pKG72.

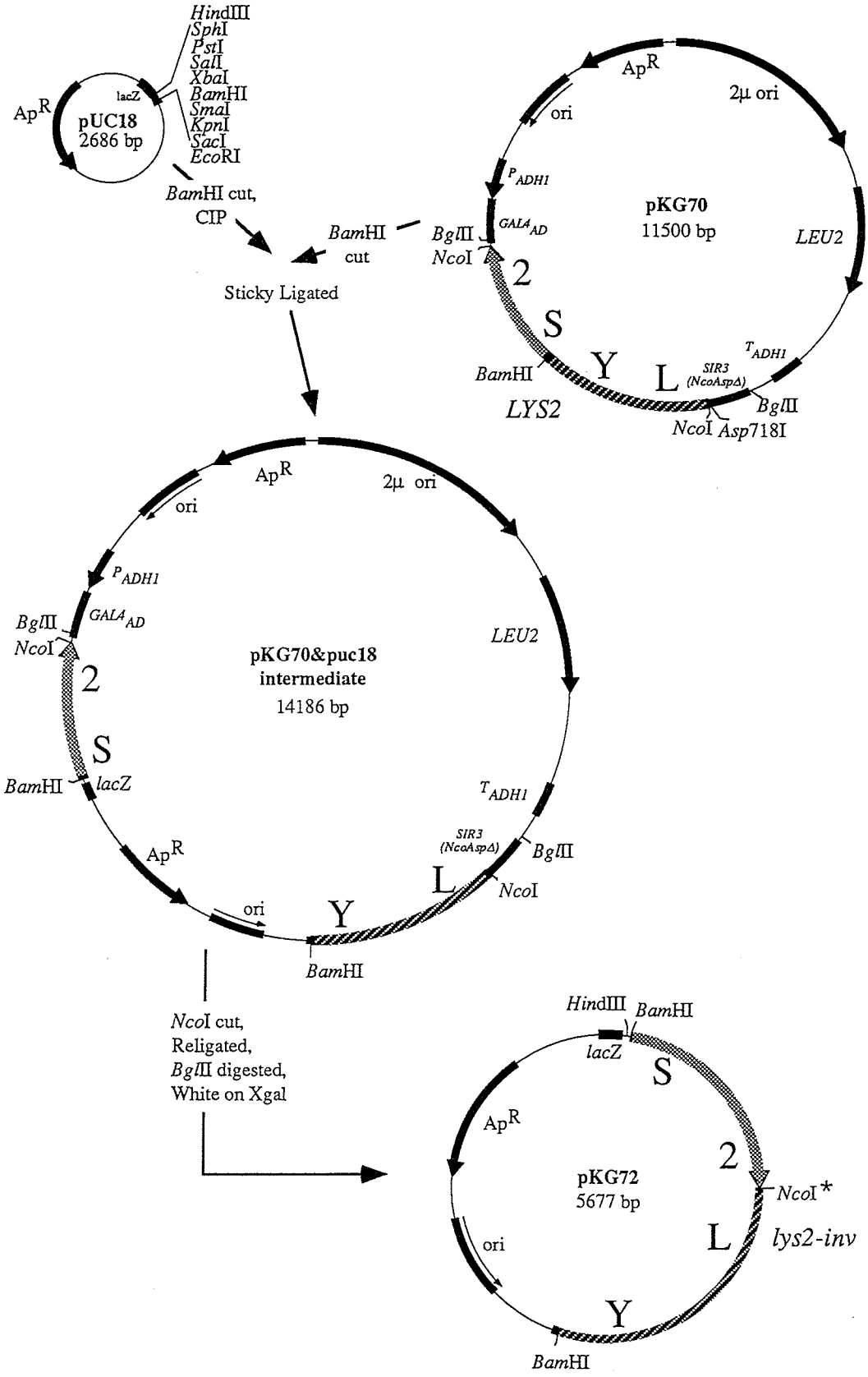
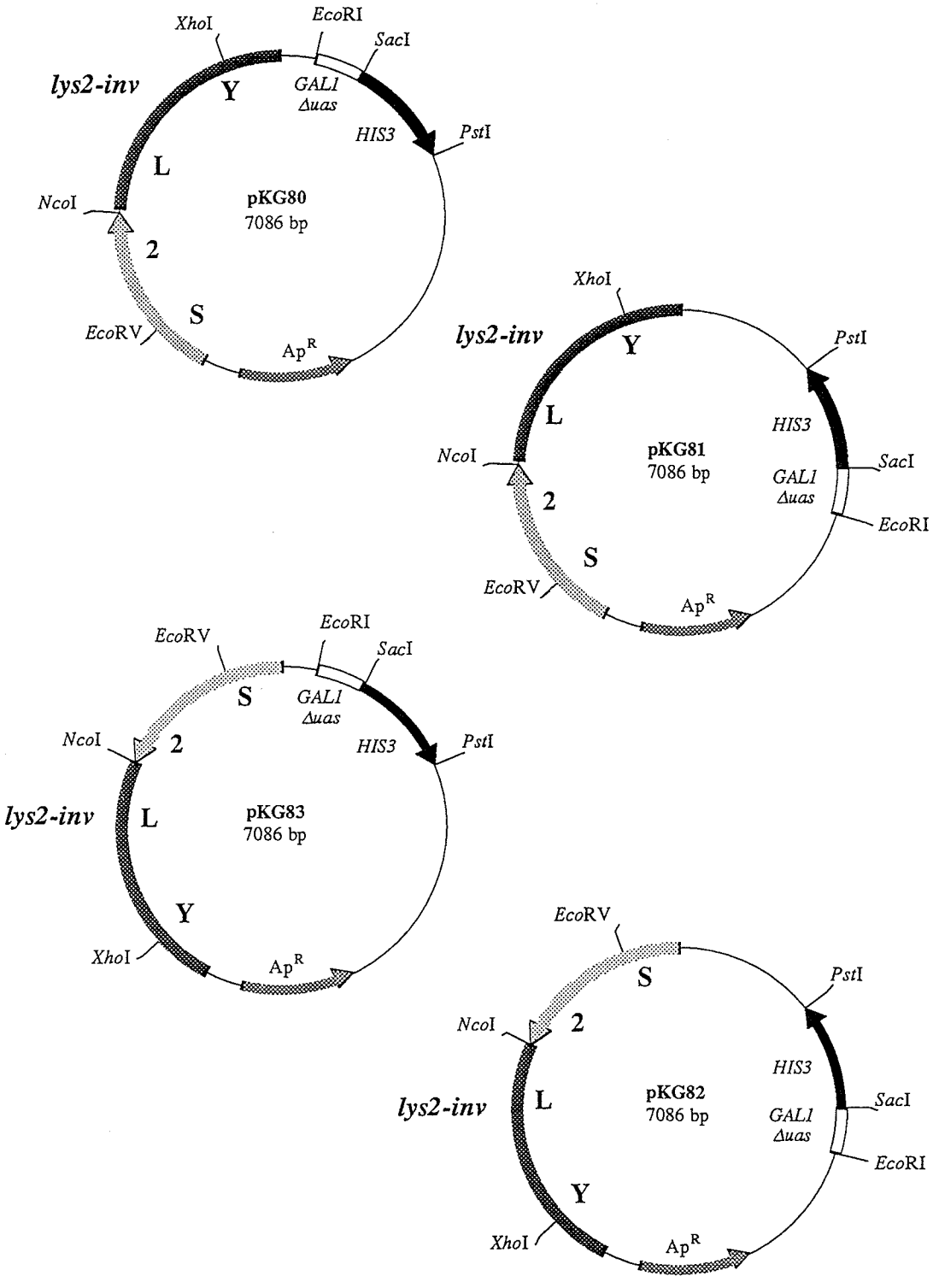

HindIII
SphI
PstI
SalI
XbaI
BamHI
SmaI
KpnI
SacI
EcoRI
ApR
lacZ
pUC18
2686 bp
BamHI cut,
CIP
BamHI
cut
Sticky Ligated
ApR
2µ ori
ori
PADH1
GALAAD
BglII
NcoI
2
S
Y
L
SIR3
(NcoAspΔ)
TADH1
BamHI
LYS2
NcoI
Asp718I
pKG70
11500 bp
LEU2
pKG70&puc18
intermediate
14186 bp
lacZ
NcoI cut,
Religated,
BglII digested,
White on Xgal
HindIII
BamHI
pKG72
5677 bp
NcoI*
lys2-inv

Figure 11 The *lys2-inv::GAL1-HIS3* reporter constructs.

The four plasmids produced from cloning of the *GAL1-HIS3* fragment from pBM2389 into the plasmids pKG74 and pKG75. An extra 800 bp of DNA was introduced 3' of the *PstI* site at the end of *HIS3*. This accounts for the shift in position of *GAL1-HIS3* between pKG80 and pKG81 (derived from pKG74) and between pKG82 and 83 (from pKG75).


XhoI
EcoRI
SacI
lys2-inv
Y
GAL1
Δuas
HIS3
PstI
L
pKG80
7086 bp
NcoI
2
S
EcoRV
ApR
XhoI
lys2-inv
Y
PstI
L
HIS3
pKG81
7086 bp
NcoI
SacI
GAL1
Δuas
2
EcoRI
S
EcoRV
ApR
EcoRV
EcoRI
SacI
S
GAL1
Δuas
NcoI
2
HIS3
PstI
lys2-inv
L
pKG83
7086 bp
Y
XhoI
ApR
EcoRV
S
PstI
NcoI
2
HIS3
lys2-inv
L
pKG82
7086 bp
SacI
GAL1
Δuas
EcoRI
Y
XhoI
ApR

work. The *lys2-inv* *Bam*HI fragment was isolated from plasmid pKG72 and cloned in both orientations into the *Eco*0109I site of pUC8, producing pKG74 and pKG75. These plasmids were digested with *Nar*I and *Sap*I to remove the *lacZ* gene alpha fragment and MCS which were replaced by the *Bam*HI/*Sal*I fragment from pBM2389 (Liu *et al.*, 1993; gift of Dr. Mark Johnston) containing a minimal *GAL1* promoter fused to the *Saccharomyces HIS3* gene (Figure 11). The minimal promoter in pBM2389 had been produced by deleting the *GAL4* upstream activating region and replacing it with unique *Bam*HI and *Eco*RI sites to facilitate introduction of any DNA sequence (Liu *et al.*, 1993). Both orientations of the *GAL1-HIS3* fragment were isolated for both ligations, again in anticipation that the expression from one particular orientation might be influenced by the site of integration. The resulting clones were designated pKG80, 81, 82 and 83 (see Figure 11). The *Bam*HI/*Sal*I fragment from pBM2389 contained 800 bp of DNA 3' of the *Pst*I site at the end of the *HIS3* gene. This extra DNA accounts for the shift in the position of the *GAL1-HIS3* reporter gene when inserted in opposite orientations (i.e. compare pKG81 and pKG81).

3.3.4 Introduction of UAS_G and *lexAop* binding sites

The absence of the natural upstream activating sequences in the *GAL1* promoter derived from pBM2389 necessitated the introduction of appropriate binding sites to allow transcription factor mediated activation of the *HIS3* reporter. We decided to take advantage of this situation and produce two sets of plasmids, one containing *lexA* binding sites and the other *GAL4* binding sites.

3.3.4.1 *LexAop*

Oligonucleotides containing the sequence for lexA binding, designed according to the *lexAop* binding sites found upstream of the *ColEI* gene (Ebina *et al.*, 1983), were kindly provided by Dr. Michael Hayden, Department of Medical Genetics at the University

of British Columbia. LexA oligonucleotide 1, 5'-AAT TCT GCT GTA TAT AAA ACC AGT GGT TAT ATG TAC AGT ACG-3' and oligonucleotide 2, 5'-AAT TCG TAC TGT ACA TAT AAC CAC TGG TTT TAT ATA CAG CAG-3' were annealed and phosphorylated as described in Materials and Methods. The inclusion of *Eco*RI sites at the 5' ends of the oligonucleotides facilitated cloning into the *Eco*RI site upstream of the *GAL1* promoter in plasmids pKG80, 81, 82 and 83. Two hundred nanograms of each plasmid, digested with *Eco*RI and treated with CIP enzyme to prevent self circularization, were ligated under sticky end conditions to an equimolar amount (42.5 femtomoles) of the phosphorylated LexA oligonucleotides. A unique *Bsr*GI restriction site was fortuitously introduced by the oligonucleotide; this allowed verification of ligation products by restriction analysis of miniprep DNA. The resulting vectors were designated pKG 92, 94, 96 and 98 respectively. Sequence analysis of pKG94 and pKG98 confirmed the insertion of three copies and one copy of the *lexAop* oligonucleotide respectively.

3.3.4.2 UAS_G

Oligonucleotides for UAS_G insertion were purchased 5'-phosphorylated from UCDNA Services at the University of Calgary. They were designed to incorporate the synthetic *GAL4* 17mer (CGG AAG ACT CTC CTC CG) which functions as a highly effective upstream activating sequence (UAS_G). The oligonucleotide permits the binding of Gal4 and results in galactose inducibility when placed upstream of a heterologous promoter (Giniger *et al.*, 1985). The reciprocal oligonucleotides, 5'-AAT TCG GAA GAC TCT CCT CCG-3' and 5'-AAT TCG GAG GAG AGT CTT CCG-3', were annealed as described in Materials and Methods. A small 20 bp fragment was produced, again with *Eco*RI complementary ends to facilitate sticky end ligation into the *Eco*RI site upstream of the minimal *GAL1* promoter. To simulate the natural arrangement of the UAS_G sequences in the *GAL1* promoter, the annealed fragments were ligated under standard conditions to form concatemers, which were fractionated at 4°C in a high resolution 3% Metaphor™

agarose gel (FMC). The 80 bp fragment corresponding to four copies of the oligonucleotide was purified by the method of Girvitz *et al.* (1983) and the concentration of the eluted DNA determined by spectroscopy. Two hundred nanograms of each of the four *lys2-inv::GAL1-HIS3* plasmids, (pKG 80, 81, 82 and 83) were ligated with equimolar amounts (42.5 femtomoles) of the UAS_G fragment. The 17mer sequence contains a *Bse*RI restriction site which was used to verify the ligation products by restriction analysis. The resulting four vectors were designated pKG101, 102, 103 and 104 respectively.

3.3.5 Construction of *leu2-inv*

The *leu2-inv* cassette was also produced with the cascade ligation method used in the construction of the *lys2-inv* and *ura3-inv* cassettes (Figure 12). The *Bgl*II fragment from YEp13 (Broach *et al.*, 1979) containing the *LEU2* gene was blunt end ligated into *Sal*I digested pUC9. This step reconstituted both *Bgl*II sites. The *LEU2*::pUC9 construct, pKG160, was digested with *Kpn*I, which cuts at bp 247 of the *LEU2* coding sequence (CDS) and eventually disrupts the 361 amino acid protein at residue 84. The *Kpn*I cut plasmid was sticky end ligated into *Kpn*I digested YEplac112 (Gietz and Sugino, 1988). After removal of the ligase by phenol extraction and ethanol precipitation of the DNA, the restriction endonuclease *Bgl*II was used to drop out the pUC9 backbone. The DNA was then ligated at low concentration to promote intramolecular ligation, and finally digested with *Nde*I to linearize recircularized pUC9. Positive *leu2-inv*::YEplac112 clones (pKG162) from antibiotic resistant colonies were identified by restriction enzyme digestion. The disruption construct pKG164, which was formed by cloning the *Kpn*I fragment containing the *leu2-inv* cassette into the *Kpn*I site of pUC18 (see Figure 12), was further manipulated to produce a second *leu2-inv* based construct.

Figure 12 Construction of the *leu2-inv* cassette.

The cascade ligation used to produce the *leu2-inv* cassette. Plasmids YEplac112 and pKG160 were digested with *Kpn*I and sticky end ligated to form the YEplac112&pKG160 intermediate. The intermediate was digested with *Bgl*II to dissociate the pUC9 backbone of pKG160, followed by ligation at low concentration to promote circularization of DNA molecules. The ligation was digested with *Nde*I to linearize any pUC9 DNA before electroporation into bacteria. Plasmid DNA was isolated from carbenicillin resistant colonies which were white on Xgal medium. The clone that produced the expected restriction pattern was designated pKG162. The disruption plasmid pKG164 was produced by cloning the *Kpn*I fragment from pKG162 containing *leu2-inv* into the *Kpn*I site of pUC18.

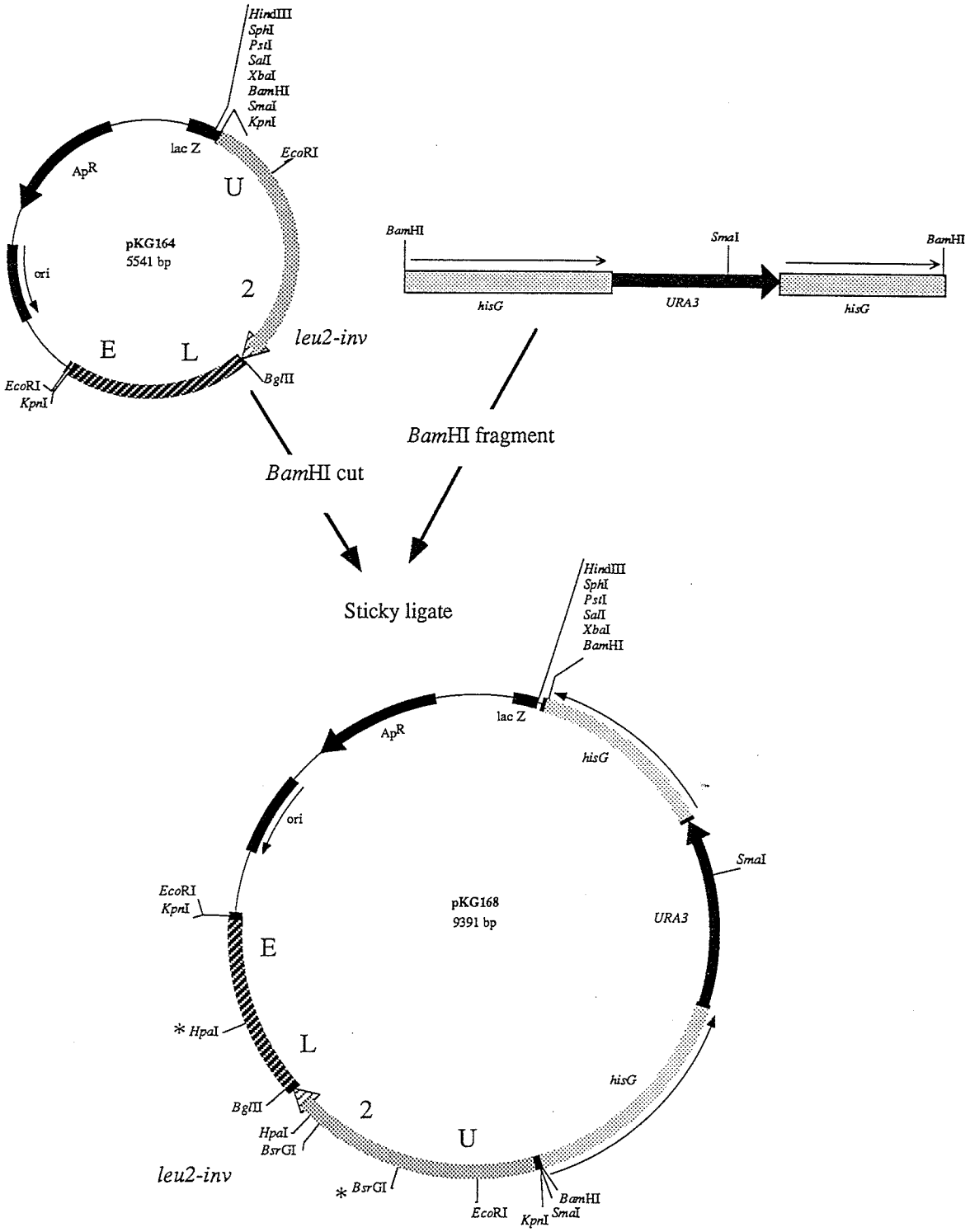
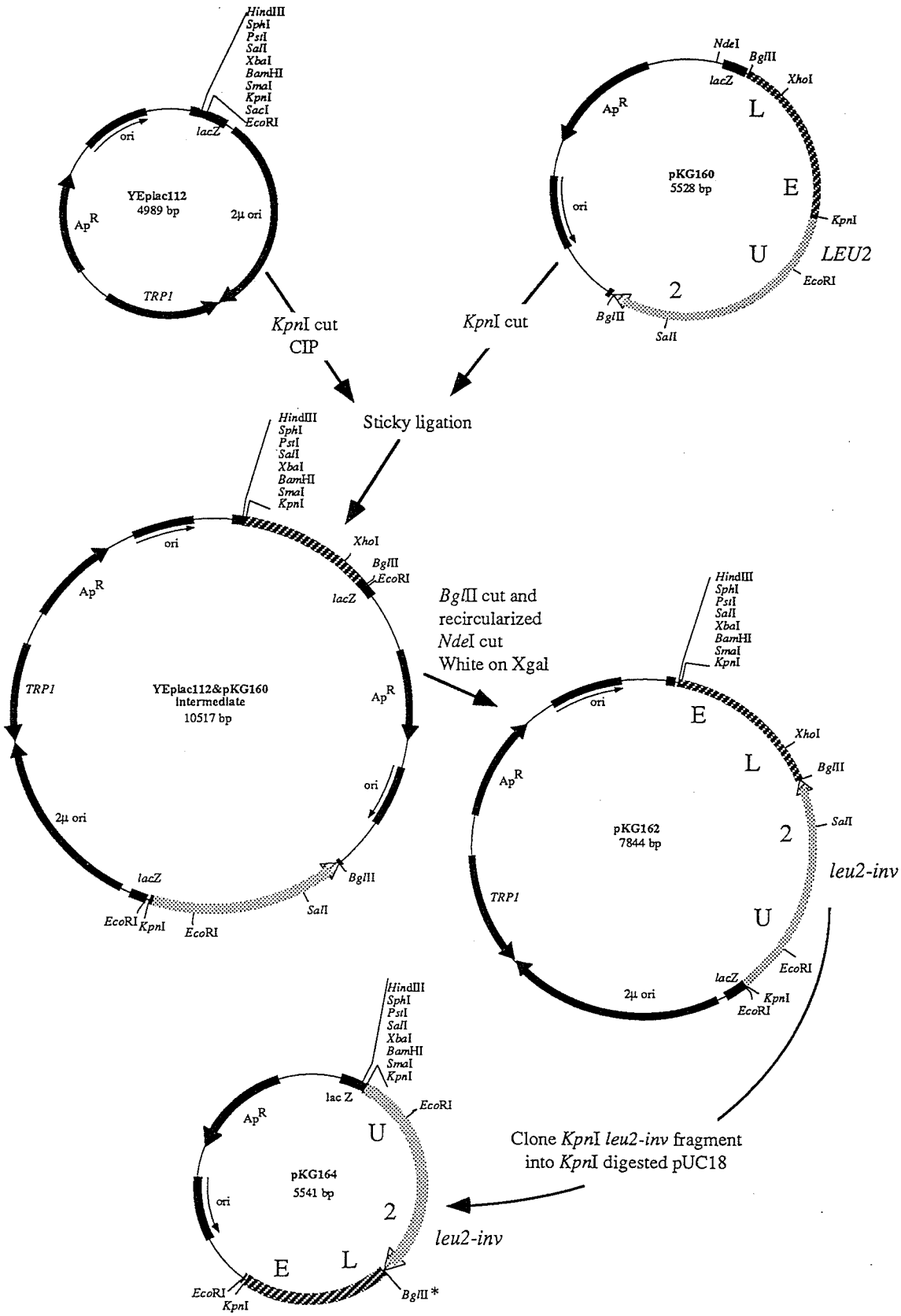

HindIII
SphI
PstI
SalI
XbaI
BamHI
SmaI
KpnI
lac Z
EcoRI
ApR
U
pKG164
5541 bp
ori
2
E
L
leu2-inv
EcoRI
KpnI
BglII
BamHI
SmaI
BamHI
hisG
URA3
hisG
BamHI fragment
BamHI cut
Sticky ligate
HindIII
SphI
PstI
SalI
XbaI
BamHI
lac Z
ApR
hisG
ori
SmaI
EcoRI
KpnI
pKG168
9391 bp
URA3
E
* HpaI
L
hisG
BglII
2
HpaI
BsrGI
U
leu2-inv
* BsrGI
EcoRI
KpnI
BamHI
SmaI

Figure 13 Construction of the *lys2-inv::URA3* selection plasmid.

The 3.8 kb BamHI fragment containing the *hisG::URA3::hisG* construct was isolated from plasmid pDG82 and cloned into *Bam*HI digested pKG164. The resulting plasmid pKG168 was digested with the restriction enzymes *Bsr*GI and *Hpa*I, sites for which allowed linearization for transformation (indicated by an asterisk).


HindIII
SphI
PstI
SalI
XbaI
BamHI
SmaI
KpnI
SacI
EcoRI
lacZ
ori
YEplac112
4989 bp
ApR
2µ ori
TRP1
NdeI
BglII
XhoI
lacZ
ApR
L
pKG160
5528 bp
E
ori
KpnI
U
LEU2
2
EcoRI
BglII
SalI
KpnI cut
CIP
KpnI cut
Sticky ligation
HindIII
SphI
PstI
SalI
XbaI
BamHI
SmaI
KpnI
XhoI
BglII
EcoRI
ori
ApR
lacZ
TRP1
YEplac112&pKG160
Intermediate
10517 bp
ApR
ori
2µ ori
lacZ
EcoRI
KpnI
EcoRI
BglII
SalI
BglII cut and
recircularized
NdeI cut
White on Xgal
HindIII
SphI
PstI
SalI
XbaI
BamHI
SmaI
KpnI
ori
E
XhoI
L
BglII
ApR
pKG162
7844 bp
2
SalI
leu2-inv
TRP1
U
EcoRI
lacZ
KpnI
EcoRI
2µ ori
Clone KpnI leu2-inv fragment
into KpnI digested pUC18
HindIII
SphI
PstI
SalI
XbaI
BamHI
SmaI
KpnI
lac Z
EcoRI
ApR
U
pKG164
5541 bp
ori
2
leu2-inv
E
L
EcoRI
KpnI
BglII *

3.3.6 Modification of *leu2-inv* for positive selection

An alternative *leu2-inv* plasmid was produced to exploit a positive selection system for identifying integrated plasmids. This system utilizes a 3.8 kb DNA fragment containing the yeast *URA3* gene flanked by identical 1.1 kb sequences of the bacterial *hisG* gene (Alani *et al.*, 1987) (see Figure 13). This fragment is cloned into the coding sequence of a gene to be disrupted, the gene is linearized from the plasmid containing it and transformed into the desired yeast strain. Selection for Ura+ transformants allows positive selection of colonies which can then be screened for the disruption by phenotype or Southern blot analysis. After the disruption is confirmed the *URA3* gene is removed by plating onto FOA medium. Reversion to *ura3* (FOA^R phenotype) occurs by homologous recombination between the *hisG* fragments and loss of the *URA3* gene (Alani *et al.*, 1987). The targeted gene remains disrupted by a single copy of *hisG* and the *URA3* gene may be used for subsequent disruptions. To utilize this selection technique, the 3.8 kb *Bam*HI fragment from pDG82 containing the *hisG:URA3:hisG* construct was cloned into the *Bam*HI site of pKG164, forming pKG168 (Figure 13).

3.4 Integration of inverted gene-based plasmids

3.4.1 Testing of disruption cassette stability

To ensure the stability of the integrated reporter genes, the three inverted cassettes were tested for their integrative ability and stability following integration. Test plasmids were constructed by first cloning the 3 cassettes into the *Eco*O109I site of pUC8. Clones containing each cassette in both orientations were isolated. To allow positive selection of transformants, one of two selectable yeast markers was cloned into each plasmid after the removal of the pUC8 *lacZ* alpha fragment and MCS by enzyme digestion. The

*Bam*HI/*Eco*RI $LEU2_{EKM}$ fragment from pDG317 was blunt end cloned into the *ura3-inv* and *lys2-inv* based plasmids and the *Hin*dIII *URA3* fragment from pRDG98 was blunt end cloned into the *leu2-inv* based plasmids. The test plasmids were digested within the inverted cassettes to linearize the plasmids and transformed into the strains DGY74 and DBY31. Transformants were selected on the appropriate drop out medium, and those that proved to be either Ura-, Lys- or Leu-were identified. Multiple colonies from each transformation were grown to saturation in nonrestrictive YPAD medium and then plated at 10^7 cells per plate onto either FOA, SC-lys, or SC-leu medium. A lack of stability would be indicated by a high amount of reversion to the original phenotype. Failure to revert at significant levels (greater than 10^{-6}) was interpreted as stable integration. All inverted cassettes were found to meet these criteria (data not shown).

3.4.2 Integration of pKG149 and pKG150

The *ura3-inv* cassette was designed to be linearized at its *Hin*dIII site, however the presence of a second *Hin*dIII site in the *GAL1-lacZ* fusion junction did not allow this site to be used to integrate pKG149 and pKG150. The restriction enzymes *Bst*BI and *Sma*I in the *ura3-inv* sequence were found to be unique within the plasmid and were used to linearize both pKG149 and pKG150 (Figure 9D) for transformation into the yeast strain DBY31.

It was not possible to detect the successful integration of pKG149 or pKG150 directly by growth on selectable medium. While FOA medium can be used to select for *ura3* mutants (Boeke et al, 1984), initial transformants plated onto FOA medium produced only FOA resistant (FOA^R) revertants, none of which contained an integrated *GAL1-lacZ* fusion. Boeke *et al.* discovered that direct selection of *URA3* disruption by this method is often not possible, perhaps because the selection conditions of FOA medium are too harsh for immediate expression of the FOA^R phenotype (Boeke *et al.*, 1987). These investigators have shown that replacement of the *URA3* gene with a smaller internal *ura3* deletion allele does not occur when selection for transformants is done on FOA. The FOA^R colonies are

found to contain the 1.1 kb *Hin*dIII *URA3* fragment, with the FOA^R phenotype apparently due to point mutations (Boeke *et al.*, 1987). A similar situation may have occurred during the initial attempt at transformation with our plasmids. To increase the probability of detecting a successful integration without using a direct FOA selection, 5μg of each linearized plasmid was co-transformed with 100 ng of the *TRP1* yeast shuttle plasmid YCplac22 (Gietz and Sugino, 1988). This was done to identify those yeast which were capable of taking up DNA during the transformation reaction. As reported by Gietz and Schiestl (1991), 30-40% of cells which can be transformed during a transformation actually take up more than one DNA molecule. Therefore, identification of YCplac22 transformed Trp+ yeast on SC-trp medium provided an initial population to screen for the *ura3* phenotype. Approximately 30,000 transformants were screened by replicating the Trp+ colonies onto FOA medium. FOA^R colonies were tested for the presence of the *GAL1-lacZ* reporter using the mating assay described below.

3.4.3 FOA^R Mating Assay

FOA resistant colonies from each transformation (pKG149 - 23 colonies, pKG150 - 32 colonies) were patched onto FOA medium and then subjected to a mating assay to verify the presence of the *GAL1-lacZ* reporter construct. The patched FOA resistant colonies (*MATa ade2 gal4 gal80 his3-Δ200 trp1-Δ901*) were replica plated onto a lawn of YPH2 cells (*MATα ade2-101(ochre) ura3-52, lys2-801 (amber), GAL+*) (Sikorski and Hieter, 1989) on YPAD medium and incubated at 30°C for 7 hours. When brought into proximity, yeast of opposite mating type (**a** and α) undergo cellular and nuclear fusion to form diploid cells (Herskowitz, 1988; Sprague, 1991). Classic mating assays select for prototrophic diploids by choosing a medium which is lacking one essential nutrient for each strain that the genotype of the opposite strain can complement. For example, YPH2 cells contain a *lys2* mutation (*lys2-801 (ochre)*) and are His+ while the transformed FOA^R cells carry the opposite alleles and are Lys+ and carry the *his3* mutation *his3-Δ200*. Neither

haploid is able to survive on SC-his-lys medium while diploid cells are prototrophic for both histidine and lysine. The mated yeast were replicated to SC-his-lys medium and incubated overnight at 30°C. The resulting colonies were replica plated to SC-his-lys galactose Xgal medium. YPH2 yeast are *GAL4+*, and growth on galactose results in the removal of Gal80 inhibition and allows Gal4p to bind to any Gal4 binding sites, such as the upstream activating sequences (UAS_G) in the *GAL1* promoter (Lohr *et al.*, 1995). All FOA resistant colonies tested were blue on the SC-his-lys galactose Xgal medium and two clones were isolated for each integrated plasmid (Figure 14). Southern blot analysis was used to verify that the integrated structure corresponded to a single copy of the plasmid (Orr-Weaver *et al.*, 1983) (Figure 15A). The strain KGY149D was chosen for further use due to its intense blue color upon *GAL4+* induction of the reporter construct.

3.4.4 Integration of the UAS_G *lys2-inv::GAL1-HIS3* constructs

The UAS_G *lys2-inv* constructs (pKG101, 102, 103, 104) were co-transformed into the strain KGY149D with the circular vector pGBD-KKQ1 (a generous gift of Dr. Phil James, University of Wisconsin Medical School). pGBD-KKQ1 is a Trp+ selectable plasmid that produces a $GAL4_{BD}$ fusion protein which is a potent self activator of Gal4 regulated promoters and *GAL1* reporter gene constructs, such as those used in the two-hybrid system (Dr. Phil James, personal communication). Therefore, when co-transformed with the linear *lys2-inv::GAL1-HIS3* plasmids into the Leu+ strain KGY149D, putative integrants were identified by their ability to grow on SC-trp, -leu, -his medium. However, the majority of the Trp+, Leu+, His+ clones were also Lys+, possibly indicating integration at other sites within the genome. Those clones that were lys minus exhibited a high amount of basal *HIS3* transcription after loss of pGBD-KKQ1, and were deemed to be unacceptable for further study (data not shown).

Figure 14 Mating Test to Determine Presence of *GAL1-lacZ* reporter.

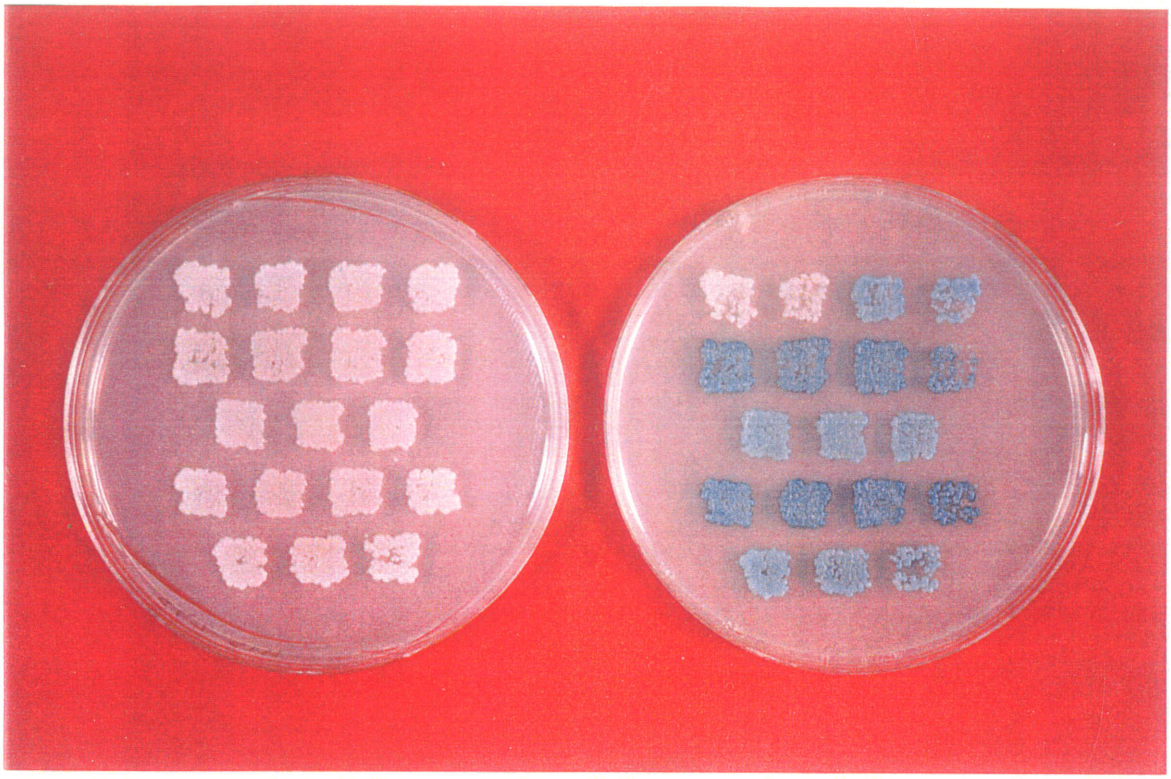
A. Cartoon depicting galactose induction of the *GAL1* promoter. In the presence of glucose, the transcription factor Gal4 is blocked by binding of Gal80 and can not activate transcription. In the presence of galactose, Gal3 binds to Gal80 and results in a conformational change of the Gal4:Gal80 complex which allows transcriptional activation by Gal4 through DNA binding at UAS_G.

B. Diploid cells produced from mating FOA^R colonies with YPH2 cells were selected on SC-his-lys medium (left plate). The His+Lys+ colonies were subsequently replica plated onto SC-his-lys galactose Xgal medium (right plate), allowing transcription of the *lacZ* reporter gene, indicated by cleavage of the chromogenic substrate Xgal and the production of blue yeast.

C. Key to photograph of yeast strains tested for galactose dependent induction of *GAL1-lacZ*. Control strain DGY63 does not harbor a reporter gene. DGY63::171 is DGY63 with plasmid pRY171 integrated at the *URA3* gene. KGY149 and KGY150 were produced by integrating pKG149 and pKG150 into yeast strain DBY31 respectively. The designations "D" and "L" refer to the relative amount of blue color produced by each strain.

A

B



SC-his -lys glucose

SC-his -lys galactose Xgal

C

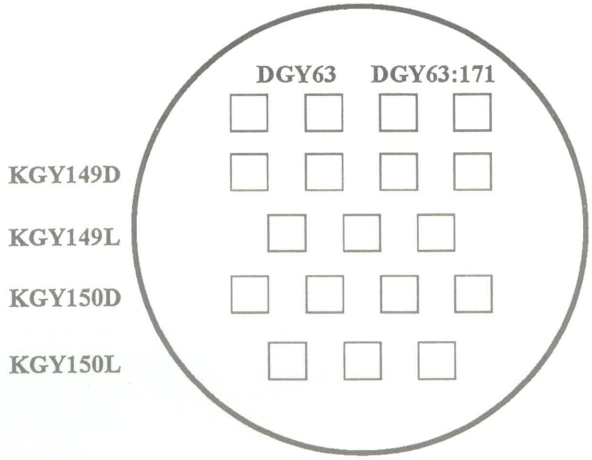
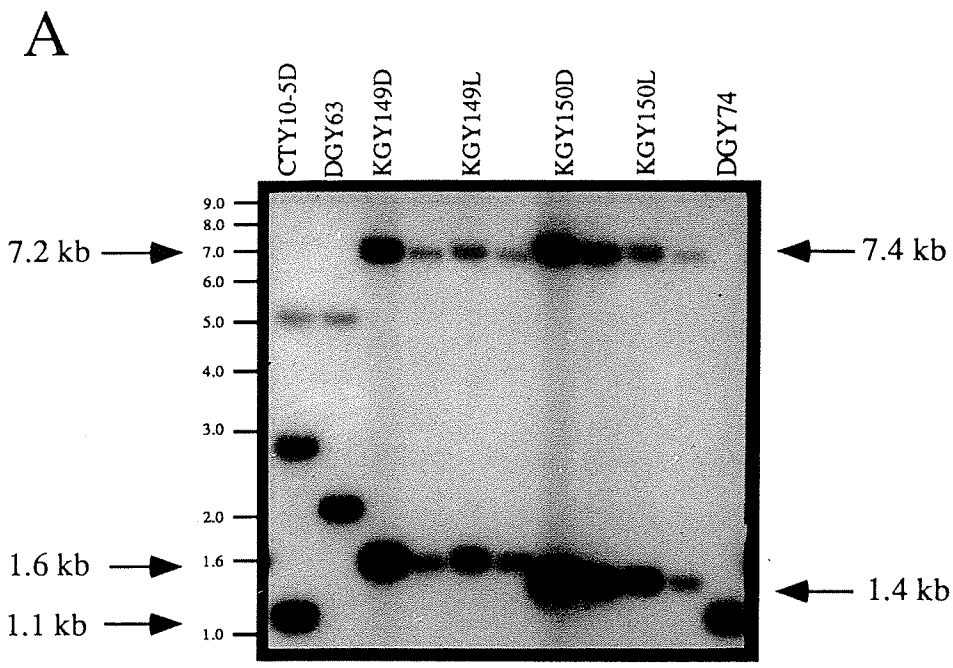


Figure 15 Southern blot verification of all integrations.

A. Integration of pKG149 and pKG150. Genomic DNA was isolated from FOA^R *ura3* transformants that had turned blue on SC-his-lys galactose Xgal medium. DNA was digested with *Hin*dIII, separated on a 0.7% agarose gel at 2 volts/cm, transferred to membrane and probed with a random primed *Hin*dIII *URA3* fragment from pRDG98. The 7.2, 1.6 and 7.4, 1.4 kb bands correspond to *Hin*dIII digestion of pKG149 and pKG150 respectively and indicate correct integration. Controls include: CTY10-5D, the 1.1 kb fragment is from duplication of *URA3* during integration, the 2.9 and 5.2 kb bands are produced from digestion of the *lexAop* reporter integrated at *ura3-52* (see Figure 1); DGY63, the 2.0 and 5.2 kb bands are from digestion of *ura3-52* , the 1.1 kb fragment was lost due to homologous recombination (see text and Figure 4); DGY74, the sole 1.1 kb band indicates successful replacement of *ura3-52* with *URA3+*.

B. Integration of pKG104. Genomic DNA isolated from lysine minus transformants that are His+ upon Gal4 activation. The lanes with KGY45B DNA show the expected 7.1 kb band produced by pKG104 replacement of the *LYS2 Nco*I fragment, while controls DGY74 and DBY31 have the wild type 3.0 kb *Nco*I fragment. Genomic DNA was digested with *Nco*I, and separated at 6 volts/cm. The *LYS2 Nco*I fragment from plasmid pRDG108 which was inverted was used as the probe.

C. Integration of pKG164 (*leu2-inv*) and pKG168 (*leu2-inv::hisG:URA3:hisG*). The $LEU2_{EKM}$ *Bam*HI/*Eco*RI fragment used to replace *leu2-3,112* was used to probe *Bgl*II digested genomic DNA. DBY31 (left lane) shows the expected wild type 2.8 kb *Bgl*II fragment. KGY37, 39 and 40 were all found to be leucine minus after transformation with *leu2-inv* constructs. KGY37 is KGY149D disrupted by the insertion of the 5.5 kb plasmid pKG164 into $LEU2_{EKM}$ (far right lane). KGY39 was disrupted with plasmid pKG168, followed by loss of the *URA3* gene by homologous recombination between *hisG* repeats. The resulting band is 1.1 kb larger than the band from KGY37 due to one copy of *hisG* remaining after recombination. KGY40 was also produced by integration of pKG168, and the larger band size may be explained by the *hisG:URA:hisG* construct not recombining out of the chromosome. The FOA^R phenotype may have been caused by a sporadic mutation in the *URA3* gene.


A
CTY10-5D
DGY63
KGY149D
KGY149L
KGY150D
KGY150L
DGY74
9.0
8.0
7.0
6.0
5.0
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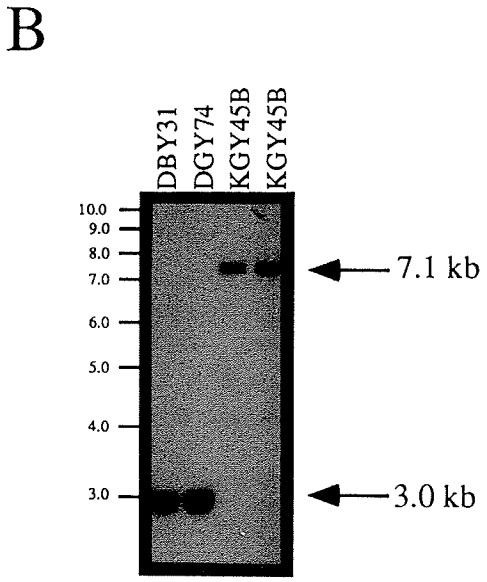
7.2 kb
1.6 kb
1.1 kb
7.4 kb
1.4 kb


B
DBY31
DGY74
KGY45B
KGY45B
10.0
9.0
8.0
7.0
6.0
5.0
4.0
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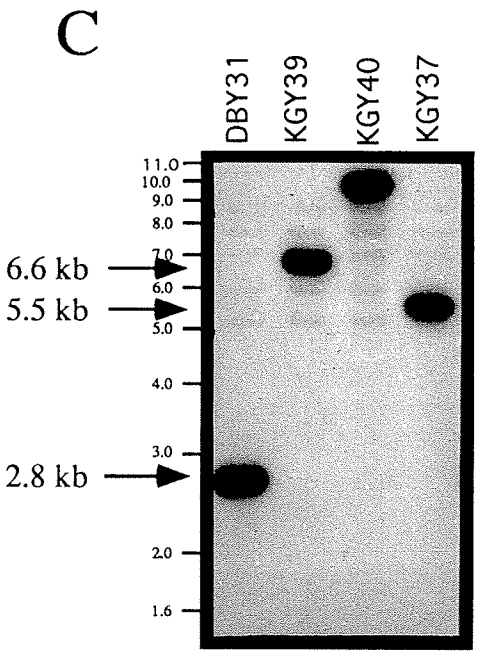
7.1 kb
3.0 kb

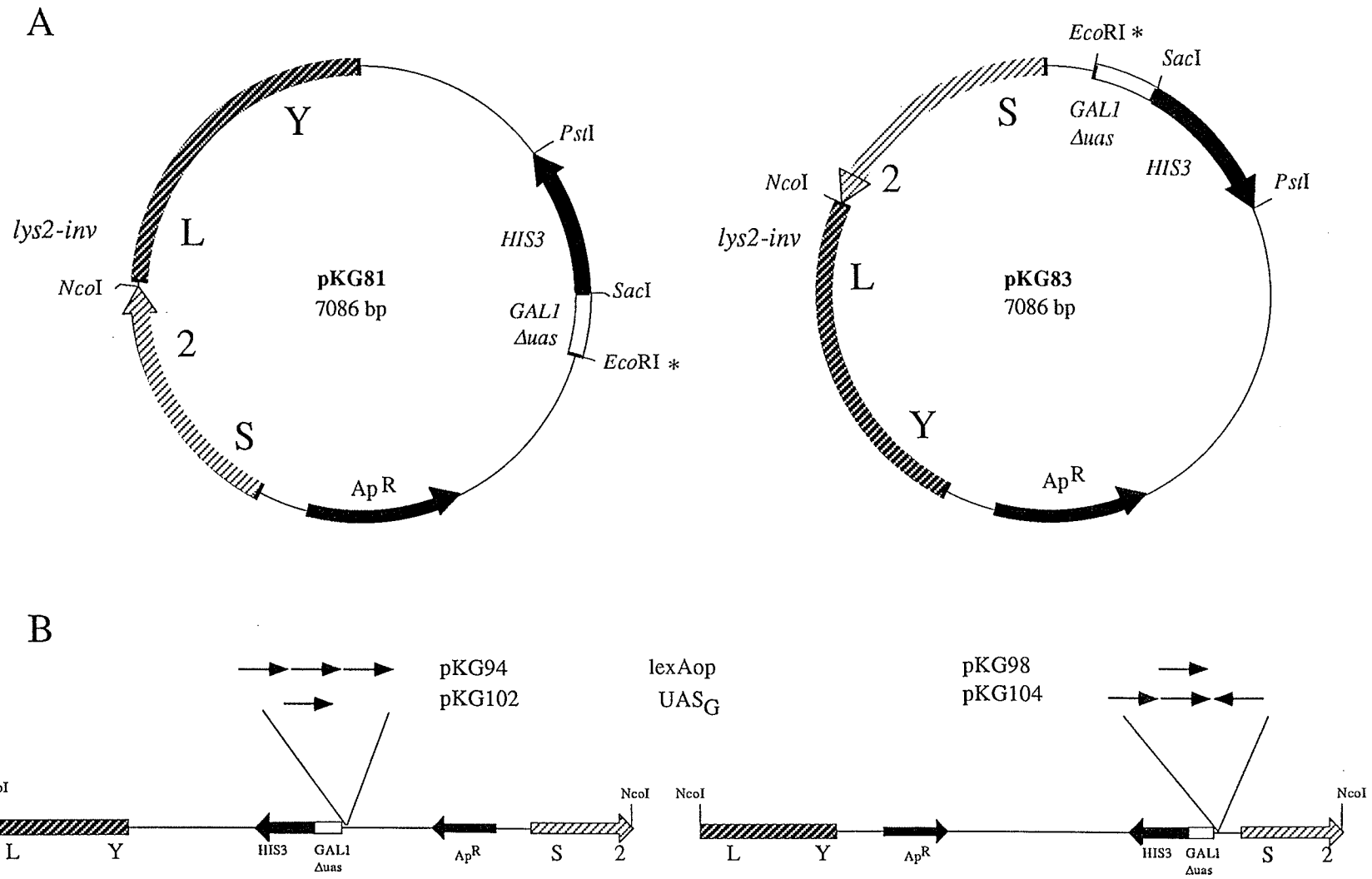

C
DBY31
KGY39
KGY40
KGY37
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.6
6.6 kb
5.5 kb
2.8 kb

The construction was attempted a second time, using the plasmids pKG102 and pKG104, both of which had the same orientation of *lys2-inv* with respect to the orientation of the *GAL1-HIS3* reporter (Figure 16). These two plasmids are derived from the same parent plasmids as pKG94 and pKG98 which had been used to successfully produce *lexAop* mediated *GAL1-HIS3* reporter strains that were auxotrophic for lysine and required small amounts of 3-AT to mask basal *HIS3* transcription (see below). The Trp+ selectable plasmid YCplac22 (Gietz and Sugino, 1988) was utilized to identify transformants as described previously. Four hundred nanograms of *Nco*I linearized pKG102 and pKG104 were co-transformed into KGY149D with 25 ng of YCplac22 (Gietz and Sugino, 1988). A total of 60,000 Trp+ transformants were replicated to SC-lys plates and 104 colonies auxotrophic for lysine identified for further study. After patching to SC-trp medium and replicating to SC-lys, nine colonies were confirmed to be actual lysine auxotrophs. The 9 clones were verified by Southern blot to contain a single integrated reporter at the *LYS2* locus (see Figure 15B). The strains were concurrently tested for plasmid dependent Gal4 activation of the *GAL1-HIS3* reporter construct. Several different plasmids were transformed into the strain and colonies were streaked onto several SC-his plates containing increasing amounts of 3-AT. Strains harboring the pKG102 plasmid exhibited high basal levels of His3 protein and could survive on SC-his medium containing high (25 mM) levels of 3-AT, even though the transformants tested were only carrying the plasmids pAS1 (a $GAL4_{BD}$ plasmid) (Durfee *et al.*, 1993) or YCplac22 (Gietz and Sugino, 1988) which by themselves should not have activated the reporter (data not shown). The single Lys- strain produced from the pKG104 plasmid transformation could not survive on low concentration (1 mm) 3-AT medium without the presence of Gal4 transcription activating plasmids which produced *GAL1* inducing hybrid proteins. This low level of basal expression of the *GAL1-HIS3* reporter is essential as it decreases the number of false positive and background colonies produced. It also lowers the amount of toxic 3-AT which has to be included in the media in order to increase the strictness of selection of histidine prototrophic

Figure 16 ***GAL1-HIS3*** **reporter constructs integrated into the** ***lys2*** **gene.**

A. The plasmids pKG81 and pKG83 were the parent plasmids of the four *lys2-inv::GAL1-HIS3* plasmids integrated with either UAS_G or *lexAop* upstream binding sites in front of the *GAL1* promoter.

B. Genomic insertion of the *lys2-inv* constructs. The arrows indicate the number and orientation of either the *lexAop* or UAS_G binding sites inserted upstream of the *GAL1* promoter.


A
Y
PstI
lys2-inv
L
HIS3
NcoI
pKG81
7086 bp
SacI
GAL1
Δuas
2
EcoRI *
S
ApR
EcoRI *
SacI
S
GAL1
Δuas
HIS3
NcoI
2
PstI
lys2-inv
L
pKG83
7086 bp
Y
ApR
B
pKG94
pKG102
lexAop
UASG
pKG98
pKG104
NcoI
NcoI
L
Y
HIS3
GAL1
Δuas
ApR
S
2
NcoI
NcoI
L
Y
ApR
HIS3
GAL1
Δuas
S
2
NcoI

positives. The strain was designated KGY45B, and was further manipulated to produce a Leu- version for use in two-hybrid and tribrid screens. Results of the Gal4 mediated activation of the *HIS3* reporter construct are presented for the Leu- version of this strain, KGY37 (see Figure 18).

3.4.5 Integration of *leu2-inv*

Four hundred nanograms of plasmid pKG164 were digested with *Bgl*II and transformed, as previously described, with 25 ng of the Trp+ selectable plasmid YCplac22 into the yeast strain KGY45B. Over 14,000 Trp+ transformants were replica plated to SC-leu medium and 23 putative Leu- colonies identified. These were patched to SC-trp medium and replica plated to SC-leu medium. One of the replicated colonies was confirmed to have a Leu- phenotype and the structure of the *leu2* gene was verified by southern blot (Figure 15C) and the strain designated KGY37.

3.4.6 Integration of *leu2-inv::hisG:URA3:hisG*

Two micrograms of pKG168 were digested with *Bgl*II and used to transform KGY45B. Dilutions were plated onto SC-ura plates which became overgrown with Ura+ colonies. It seemed likely that this was the result of an autonomously replicating sequence (ARS) in the *Bgl*II *LEU2* fragment. Re-examination of the plasmid pKG168 suggested that digestion with *Bsr*GI and *Hpa*I would allow it to be linearized for integrative transformation (Figure 13). Transformation with 2 μg of plasmid digested with these restriction enzymes resulted in a large number of colonies on SC-ura medium but not the overgrowth previously experienced, suggesting that an ARS had been present in the large fragment. While an ARS consensus sequence was not found during preliminary analysis of the sequence of the *LEU2 Bgl*II fragment, large regions of TA repeats and TA rich sequence were found. Similar sequences have been linked with several ARS sequences on yeast chromosome III (Valle, 1993). One hundred Ura+ colonies were patched to SC-ura

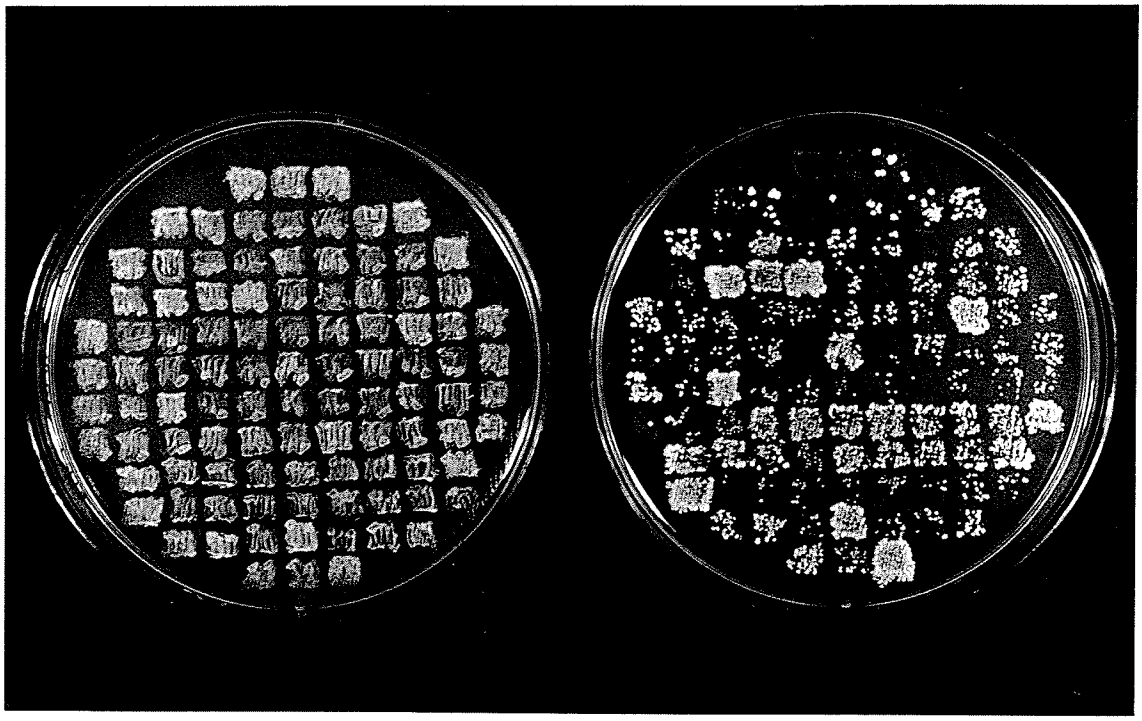
medium and subsequently replicated to SC-leu medium. As shown in Figure 17, the majority of the 100 colonies were stable leu minus integrants, with the Leu+ colonies being indicated by full growth of the patch on the SC-leu plate. Two of these clones (#1 and #2) were streaked onto FOA medium to select for loss of the *URA3* gene. The genotypes of two of the FOA^R colonies, KGY39 and KGY40, were verified by Southern blot (results Figure 15C). A surprising result is the larger band for KGY40 seen in this figure. The expected band should have been 6.6 kb, the same size as for KGY39. The FOA^R phenotype for this clone may have been caused by a spontaneous mutation in the *URA3* gene as seen by Boeke *et al.* (1987). Since it appears that the predicted loss of the *URA3* gene by homologous recombination did not occur KGY40 was not utilized further.

3.4.7 Testing of KGY37

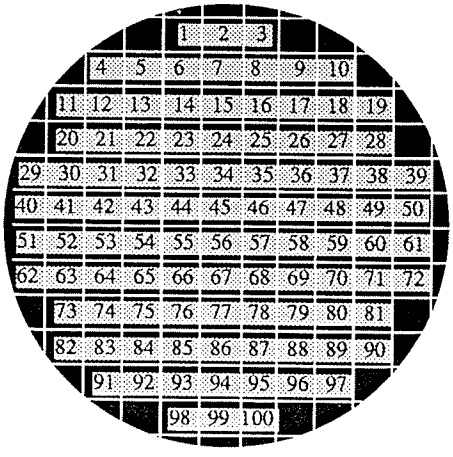
KGY37, containing both the *GAL1-lacZ* and the *GAL1-HIS3* reporter constructs, was tested for *GAL4* dependent *HIS3* and *lacZ* transcription by transformation with a number of positive and negative control plasmids. The properties of these plasmids and the activation of the *GAL1-HIS3* and *GAL1-lacZ* reporter genes are listed in Table 6. For *HIS3* activation, transformants were streaked from selective medium onto SC-his and SC-his 1 mM 3-AT medium. As seen in Figure 18, basal transcription of the *GAL1-HIS3* reporter caused KGY37 to grow on SC-his medium. Slightly decreased levels of growth are seen for the strain when harboring plasmids such as pAS1($GAL4_{BD}$) and pDG862 ($GAL4_{BD}$-*RAD7*). This may be due to the binding domain fusion proteins binding to the UAS_G sites and interfering with the assembly of the basic transcription complex. The positive control transformants, pGBD-KKQ1 and the double transformant pDG862(*RAD7*) / pJR3(*SIR3*), exhibit good growth on SC-his medium as expected. Even so, the high degree of background expression seen with the negative controls indicated that SC-his medium would be an insufficient selection for two-hybrid system use. When the identical

Figure 17 Selection of the leucine minus phenotype using a nontandem repeat construct.

One hundred Ura+ colonies produced from a transformation of KGY45B with pKG168 were patched onto SC-ura medium and incubated for two days at 30°C (right plate). Colonies were replica plated to SC-leu medium to select for leucine minus integrants (right plate). The small colonies or papillae are due to Leu+ cells being transferred along with the leucine minus Ura+ colonies during the patching process. 75% of the colonies picked were successful (leucine minus) integrants. Clones from patch 1 and 2 were streaked onto FOA and FOA^R colonies isolated for Southern analysis.



SC-ura **SC-leu**


1 2 3
4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19
20 21 22 23 24 25 26 27 28
29 30 31 32 33 34 35 36 37 38 39
40 41 42 43 44 45 46 47 48 49 50
51 52 53 54 55 56 57 58 59 60 61
62 63 64 65 66 67 68 69 70 71 72
73 74 75 76 77 78 79 80 81
82 83 84 85 86 87 88 89 90
91 92 93 94 95 96 97
98 99 100

strains were streaked on SC-his medium supplemented with 1 mM 3-AT, all background expression was inhibited. This level of 3-AT was also able to confer auxotrophy on the strain when it was harboring negative control plasmids, and as can be seen in Figure 18, only the expected positive controls pGBD-KKQ1 and the double transformant containing pDG862(*RAD7*) and pJR3(*SIR3*) grew on this medium. As mentioned previously, pGBD-KKQ1 is a self activating $GAL4_{BD}$ fusion while pDG862 and pJR3 comprise a proven two-hybrid system interaction (Paetkau *et al.*, 1994).

The *ura3*::*GAL1-lacZ* reporter was tested by filter lift and ONPG cleavage assays of selected transformants (see Materials and Methods). The plasmids pGBD-KKQ1, and the combination of pDG862 and pJR3 were again found to activate reporter gene transcription (Table 6) while the negative controls pAS1, pDG862 alone, pDG661 alone and pDG862 with pDG661 did not produce blue color during filter lift analysis. To further test the *GAL1-lacZ* reporter, the yeast strains DGY63::171, PJ69-4A and KGY37 were transformed with the plasmids pDG862 and pJR3. Transformants were used to perform ONPG assays of ß-galactosidase expression as described in Materials and Methods. The levels of ß-galactosidase produced by the *lacZ* reporter in KGY37 were the same as those produced in DGY63::171, which harbors the same *GAL1-lacZ* reporter as KGY37, and in PJ69-4A, which contains a *GAL7-lacZ* reporter gene (see Table 7).

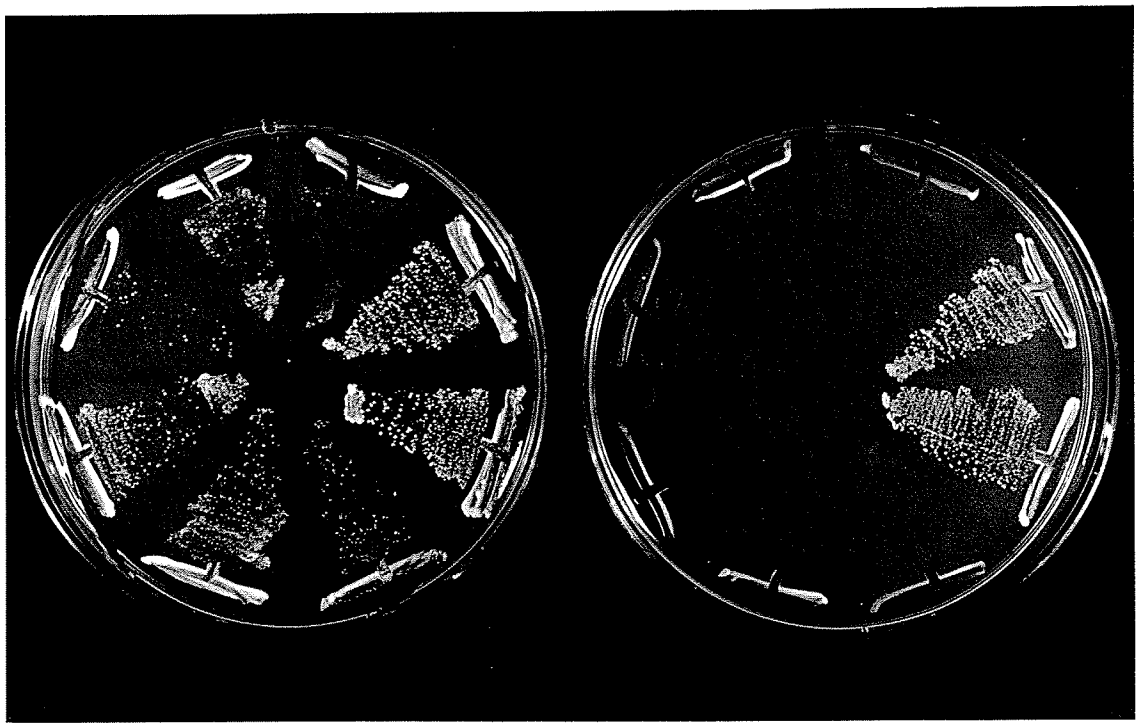
3.5 Production of KGY94, a *lexA* activated double reporter two-hybrid strain

3.5.1 Construction of lexA mediated *GAL1-HIS3* reporter plasmids

We have found that *lexA* fusion proteins are more stable and produce a stronger reporter signal than related $GAL4_{BD}$ fusions (D. Gietz, personal communication). Accordingly, the *lys2-inv::GAL1-HIS3* plasmids pKG 80, 81, 82 and 83 had *lexA* binding

Figure 18 Testing of KGY37 - *GAL4* activation of the *lys2::GAL1-HIS3* reporter.

KGY37 was transformed with several plasmids and transformants streaked onto SC-his and SC-his 1 mM 3-AT medium to test for plasmid dependent activation of the *GAL1-HIS3* reporter. On SC-his medium (left plate) basal transcription results in low levels of growth . Addition of 3-AT to 1 mM concentration masks basal transcription with only positive controls surviving (see right plate and text).



SC-his **SC-his 1mM 3AT**

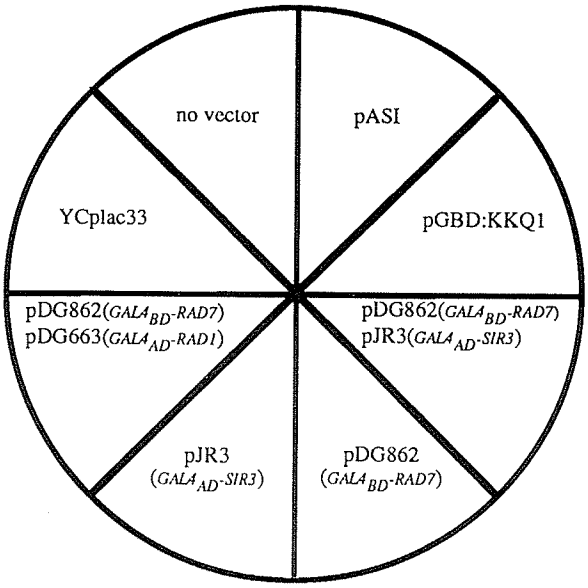


Table 6 Gal4 dependent activation of *GAL1-HIS3* and *GAL1-lacZ* reporters.

PLASMID			REPORTER		
		Expected	*GAL1-HIS3*		*GAL1-lacZ*
$GAL4_{BD}$ fusion	$GAL4_{AD}$ fusion	Activation	SC-his	1 mM 3-AT	Blue color
no vector		-	++	-	-
pAS1	- -	-	+	-	-
pGBD:KKQ1	- -	++++	++++	++++	++++
pDG862 (*RAD7*)	*SIR3*)	++++	++++	++++	++++
pDG862 (*RAD7*)	- -	-	+	-	-
- -	pJR3 (SIR3)	-	++	-	-
pDG862 (*RAD7*)	pDG663 (*RAD10*)	-	++	-	-

+, - indicate relative growth or degree of color produced

Table 7 Strain Comparative ONPG Assays of ß-galactosidase produced by different *GAL1-lacZ* reporters

$GAL4_{BD}$ fusion	$GAL4_{AD}$ fusion	STRAIN	MILLER UNITS
pDG862 (*RAD7*)	pJR3 (SIR3)	DGY63::171	115.6
pDG862 (*RAD7*)	pJR3 (SIR3)	PJ69-4A	99.2
pDG862 (*RAD7*)	pJR3 (SIR3)	KGY37	117.4

sites (*lexAop* sequences) introduced in the same manner as the UAS_G oligonucleotides (see section 3.3.4). Plasmids were digested with *Nco*I and co-transformed with YCplac22 into the yeast strain CTY10-5D, a two-hybrid strain with a *lexAop* controlled *GAL1-lacZ* reporter integrated at the *URA3* locus (Bartel *et al.*, 1988a).

3.5.2 Integration of *lexAop GAL1-HIS3* reporter constructs

For each transformation of the *lexAop* pKG vectors, approximately 8000 Trp+ colonies were screened by replica plating to SC-lys plates. Colonies auxotrophic for lysine were patched from the original SC-trp plate to a fresh plate, grown for 2 days and replica plated to SC-lys again. Of the 44 putative lysine auxotrophs patched, only 2 were confirmed to be Lys-, one each from the pKG94 and pKG98 transformations. These strains, KGY94 and KGY98, were grown overnight in liquid YPAD to lose the Trp plasmid YCplac22. Trp-Lys- colonies were isolated and the *lexAop:GAL1-HIS3* reporter gene tested by transforming the strains with the interacting two-hybrid fusion plasmids pDG649 ($lexA_{BD}$*:RAD7*) and pJR3 ($GAL4_{AD}$*:SIR3*) (Paetkau *et al.*, 1994). Transformants were plated on SC-trp-leu medium and then replica plated onto SC-his plates supplemented with increasing amounts of 3-AT. KGY94 was found to require low (1 mM) concentrations of 3-AT to quench basal levels of *HIS3* activity and was selected as a suitable strain for use in two-hybrid screens (data not shown).

3.6 Concluding remarks

Two improved yeast strains for use with the two-hybrid system have been produced. KGY94 (*MATa ade2 gal4 gal80 his3-Δ200 trp1-Δ901 ura3-52 leu2::pUC18 URA3::GAL1-lacZ lys2::lexAop* $_{(x3)}$*GAL1-HIS3*) is a lexA controlled two-hybrid strain which provides a second reporter gene ($lexAop_{(x3)}$*:GAL1-HIS3*) for use with lexA binding domain fusion proteins. The introduction of this nutritional reporter gene facilitates the screening of a greater number of transformants than the *GAL1-lacZ* reporter gene. The

initial positives identified using *GAL1-HIS3* can be confirmed by rescreening with the original reporter, *GAL1-lacZ*. KGY37 (*MATa ade2 gal4 gal80 his3-Δ200 trp1-Δ901 leu2::pUC18 ura3::GAL1-lacZ lys2::*$UAS_{G\ 17mers(x3)}$*GAL1-HIS3*) is auxotrophic for uracil and lysine and contains two stably integrated reporter genes (*GAL1-lacZ* and $UAS_{G\ 17mers(x3)}$*GAL1-HIS3*) subject to Gal4p induction. The rescue of the *URA3* gene as a selectable marker in KGY37 will allow introduction of a third DNA plasmid to produce a modifier protein, such as a kinase not produced in yeast, to identify a new class of two-hybrid positives. The *leu2-inv* cassette is available for introduction of a third reporter. A reporter construct consisting of the *GAL2* promoter fused to the yeast *ADE2* gene was planned for this project but was not constructed due to time constraints. The necessity of this new strain is strengthened by the recent reports of other tribrid or otherwise modified two-hybrid systems involved in detecting kinase dependent interactions (Osbourne *et al.*, 1995; Keegan and Cooper, 1996).

Another advantage offered by rescue of the *URA3* gene is the ability to use the disruption cassette of Alani *et al.* (1987) to inactivate genomic copies of genes involved in two-hybrid system interactions being studied. By deletion of the genomic copy of the gene fused to the $GAL4_{BD}$, any endogenous protein which may bind to library fusions is eliminated. In the absence of the endogenous protein, previously "hidden" interactions may be identified. The removal of an endogenous protein can also be used to test for proteins which may be mediating a two-hybrid interaction. If the X and Y protein interaction described in Figure 4 is postulated to be mediated by a third protein "Z", then the *Z* gene can be disrupted with the *hisG* disruption cassette followed by retesting to see if the Xp/Yp interaction is still detectable. Currently investigators in our laboratory (R. D. Kirkpatrick and R. Agatep, personal communication) are using KGY37 to investigate a potential interaction of this kind involving the DNA repair proteins Rad18p and Rad6p.

During the course of this project in was discovered that certain two-hybrid strains such as Y190 (Hardy *et al.*, 1993) and PJ69-4A (James *et al.*, submitted) have the *GAL1-*

HIS3 reporter integrated in the *LYS2* terminator. The *RAD16* and *LYS2* genes are neighbours on chromosome II that are convergently transcribed with the 3' ends of their respective ORFs being only 128 bp apart. The integration of the *GAL1-HIS3* reporter gene results in these strains being UV sensitive due to disruption of the *RAD16* gene. The *lys2-inv* cassette constructed here produces a *RAD16+* strain which is not UV sensitive. This will be essential when performing two-hybrid screens involving other related DNA repair proteins, which is a research area pursued in this laboratory. This result strengthens the usefulness of the inverted gene cassette system as a disruption/insertion procedure for reporter genes. It also indicates that caution must be used when choosing the site and method of insertion of exogenous DNA into the yeast genome.

Preliminary results obtained with the new strains are encouraging. A two-hybrid screen with KGY94 using a lexA-insulin receptor (IR) fusion protein as the bait has identified interactions with PI-3 kinase and Grb14. These studies are currently being pursued. As well, KGY37 was used to confirm a previously identified kinase-dependent interaction. Using strain PJ69-4A the interaction of SHPTP2 (an SH2-containing protein-tyrosine-phosphatase) and insulin receptor substrate 1 (IRS-1) has been shown to be dependent upon the presence of insulin receptor kinase (IRK). IRK was introduced using a *URA3* based plasmid. KGY37 was used to confirm the interaction with Xgal colony filter lifts and to perform ONPG assays to quantitate the production of ß-galactosidase. Due to high basal activity of the *GAL7-lacZ* reporter in PJ69-4A Xgal colony filter lifts are not possible and makes confirmation of positive interactions with *lacZ* difficult and time consuming. Present research involving KGY37 includes using the *URA3* disruption cassette of Alani *et al.* (1987) to create knock-outs of several DNA repair genes to determine their roles in protein interactions involved in DNA repair.

Further improvements to the strains include the addition of a third reporter gene (*GAL2-ADE2*) provide a second nutritional reporter. The reclamation of the *URA3* marker from the *lexA* strain will allow the use of the disruption cassette of Alani *et al.* (1987)

combined with the more stable fusion proteins produced with lexA. However, until these refinements are completed KGY37 and KGY94 will act as excellent host strains to identify a wide range of protein:protein interactions previously undetectable in the two-hybrid system.

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