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Direct selection for gene replacement events in yeast

(Chromosome manipulation; cycloheximide; DNA transformation; recombinant DNA; ribosomal protein; *Saccharomyces cerevisiae*)

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SUMMARY

A method that facilitates gene replacement at the *HIS3* locus of *Saccharomyces cerevisiae* (yeast) has been developed. First, an internal region of the cloned *HIS3* gene was replaced by a DNA segment containing the wild-type ribosomal protein gene, *CYH2*. Second, by using standard yeast transformation methods, the wild-type *HIS3* locus of a cycloheximide resistant strain (*cyh2*^{r}) was replaced by this *his3-CYH2* substitution. The resulting strain is sensitive to cycloheximide because *CYH2* is dominant to *cyh2*^{r}. Third, *his3* mutations cloned into integrating or replicating vectors were introduced into this strain by selecting transformants via the vector-encoded marker. Selection for cycloheximide-resistant colonies resulted in the replacement of the *his3-CYH2* allele by newly introduced *his3* alleles. Thus, this scheme provides for the direct selection of gene replacement events at the *HIS3* locus independently of the phenotype of the cloned *his3* derivatives. In principle, it can be extended to any region of the yeast genome.

INTRODUCTION

A major attraction for studies on the yeast *S. cerevisiae* is the ability to replace normal chromosomal sequences with mutated derivatives constructed in vitro. Consequently, the phenotypes of cloned mutant DNAs can be assessed in true in vivo conditions, i.e., one copy per cell at the normal chromosomal location.

Gene replacement depends upon homologous recombination between transforming DNA sequences and their host genomic counterparts (Morse et al., 1956). The basic procedure involves two steps (see Fig. 1). First, transforming DNA is integrated at the desired chromosomal location to produce a heterogenote structure in which the transforming and host alleles are separated by vector sequences. Second, the transforming DNA is excised from the genome; this results in two classes of segregants. One of these is indistinguishable from the original strain. In the more interesting class, the transforming allele has replaced the original one.

Abbreviations: bp, base pairs; EtBr, ethidium bromide; kb, kilobase pairs; YPD medium, see MATERIALS AND METHODS, section **a**.

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In the original example using cloned DNA in yeast cells, Scherer and Davis (1979) replaced the wild-type *HIS3* gene by a deleted derivative. They identified the integration event by including the *URA3* gene in the vector DNA and then selecting for uracil-independent (Ura^+) transformants of the host $ura3^-$ strain. The excision step was identified by growing the Ura^+ transformants in non-selective medium and then screening for Ura^- segregants by replica plating. The distinction between the two classes of segregants was made initially by their *his3* phenotype (replacement events were His^-) and then confirmed by hybridization analysis.

The method described above, although simple in principle, is sufficiently cumbersome to preclude its routine use. Difficulties are encountered at each of the three stages of the procedure.

(1) Transformation by chromosomal integration is inefficient and can result from recombination with vector sequences rather than the desired ones. Although appropriate cleavage of the transforming DNA increases the transformation frequency and directs the site of integration (Hicks et al., 1978; Orr-Weaver et al., 1981), many transformants contain multiple copies of the transforming DNA. These are undesirable and distinguishing them from the desired transformants involves DNA preparations and hybridization analysis.

(2) The excision event involves replica plating to screen for rare segregants. Enrichment schemes using nystatin killing (Snow, 1966) or inositol-less death (Henry et al., 1975) are somewhat effective, but they take additional time. A selective approach has been used in which the excision of transforming DNA sequences containing suppressor tRNA genes is obtained by virtue of resistance to osmotic shock (Struhl, 1979; St. John et al., 1981). These vectors, however, have a variety of problems (see St. John et al., 1981, for a detailed account).

(3) Distinguishing between the two classes of segregants is not a simple matter unless the phenotype of the new allele is already known or suspected.

In this paper, a method is described to select directly for gene replacement. Most of the problems mentioned above are avoided, and the procedure is facile, rapid, and generally applicable.

MATERIALS AND METHODS

(a) Yeast strains and their propagation

The strains used in this paper are listed in Table I and grown either in YPD-rich medium (2% each yeast extract, peptone, glucose) or minimal medium (0.67% yeast nitrogen base without amino acids, 2% glucose) with appropriate supplements at 100 μg/ml (amino acids, uracil, adenine). Cycloheximide was added to YPD plates to 40 μM and canavanine was added to minimal plates to 100 μM.

Genetic crosses were carried out as follows. Mating of haploid strains was performed by cross-streaking on YPD plates followed by replica plating

TABLE I

Strains used in the present study

YM5	**a**/α *MEL/mel GAL2/gal2 ura3*-52 *trp1*-289 *LYS1/lys1*	Mark Johnston
KY170	YM5 transformed to stable Ura^+ by YRp14-Sc3225	This work
KY171	α *ura3*-52 *trp1*-289 *his3-CYH2* (Ura^- segregant of KY170)	This work
KY172	YM5 transformed to stable Ura^+ by YRp14-Sc3100	This work
KY173	α *ura3*-52 *trp1*-289 *his3-CAN1* (Ura^- segregant of KY172)	This work
D636-3A	**a** *ura3*-52 *leu2*-2 *leu2*-112 *cyh2*^{r} *can1*^{r} *ade1 trp1*-289	P. Brown/J. Szostak
KY174	α *ura3*-52 *his3-CYH2 cyh2*^{r} *trp1*-289 (spore from KY171 × D636-3A)	This work
KY175	**a** *ura3*-52 *trp1*-289 *his3-CAN1 can1*^{r} *ade1* (spore from KY173 × D636-3A)	This work
KY176	KY174 transformed to Ura^+ by YIp5-Sc3113	This work
KY177	KY174 transformed to Ura^+ by YCp50-Sc3125	This work
KY178	KY174 transformed to Ura^+ by YCp50-Sc2888	This work
KY179	α *ura3*-52 *trp1*-289 *his3*-Δ25 *cyh2*^{r} (cycloheximide resistant colony of KY176)	This work
KY180	α *ura3*-52 *trp1*-289 *his3*-Δ28 *cyh2*^{r} (cycloheximide resistant colony of KY177)	This work
KY181	α *ura3*-52 *trp1*-289 *his3*-Δ41 *cyh2*^{r} (cycloheximide resistant colony of KY178)	This work

onto appropriate minimal plates that selected specifically for diploids. Clones of diploid cells were then incubated on sporulation plates (2% K·acetate, 0.1% yeast extract, 0.05% glucose) for 3–7 days, treated with 3% glusulase for 5 min at room temperature, and asci were dissected by micromanipulation.

DNA was introduced into yeast cells as described previously (Struhl et al., 1979).

(b) DNA mechanics

Plasmid DNAs were prepared by a rapid method that eschewed the use of CsCl. 20 ml of *Escherichia coli* cells (strain EQ82) grown to saturation in broth were harvested and resuspended in 150 μl of 20% sucrose, 50 mM Tris pH 8.5. Spheroplasts prepared by lysozyme treatment were lysed with Triton X-100 (final volume 0.5 ml), and cellular debris was removed by sedimentation for 15 min in a microcentrifuge. The lysate was transferred to a new tube, extracted once with phenol, precipitated with 2 vols. of ethanol at room temperature, washed twice with 95% ethanol, and resuspended in 100 μl of 10 mM Tris pH 7.5, 1 mM EDTA containing 1 μg pancreatic ribonuclease A.

DNA crosses were performed as follows. The appropriate plasmids were cleaved with restriction endonucleases and the DNA segments separated electrophoretically in low gelling agarose (Marine Colloids). The desired segments, visualized by EtBr staining with a long-wave ultraviolet light, were cut from the gel with a clean razor blade in as small a volume as possible. The gel slices (usually 30–50 μl in 40 mM Tris·acetate pH 8.2) were melted at 70°C. Purified DNA segments were mixed at 37°C to a final volume of 10 μl. Then, 10 μl of ice-cold, twice concentrated buffer containing DNA ligase was added, mixed quickly and incubated at 15°C for 3–24 h. Although the reaction mixture resolidified into a gel, ligation worked extremely efficiently. T4 DNA ligase was used for ligating blunt-ended fragments and T7 DNA ligase was used to join segments via cohesive ends (both enzymes were the gift of Charles Richardson). The buffer conditions for all enzyme reactions have been described previously (Struhl and Davis, 1980). To introduce the ligated products back into *E. coli* strain EQ82, the gel slice was remelted at 70°C before carrying out the standard transformation procedure. Ampicillin-resistant transformants were selected and analyzed by restriction endonuclease mapping. The construction of individual hybrid molecules is described below.

YRp14-Sc2812 was made by mutating the unique *Eco*RI site of YRp14-Sc2605 (Struhl, 1979; 1982a). The ends of *Eco*RI-cleaved YRp14-Sc2605 were filled in by DNA polymerase I in the presence of all four deoxy-triphosphates (50 μM each) and the resulting molecules separated by electrophoresis. The linear molecules were circularized by blunt-end ligation, cleaved with *Eco*RI to eliminate undesired products, and introduced into *E. coli*.

pBR322-Sc2806 was constructed by cleaving YRp14-Sc2605 DNA with *Mbo*II to an average of one cut per molecule. Full-length linear molecules were purified and treated with DNA polymerase I in the absence of deoxy-triphosphates (to render the *Mbo*II ends flush) and then with 0.3 μg of an octanucleotide *Eco*RI linker (from the Peoples Republic of China via Fred Sanger) and DNA ligase to allow blunt end ligation. The products were cleaved to completion with *Eco*RI and *Bam*HI. Following gel electrophoresis, an 0.4-kb DNA fragment was purified and ligated to *Bam*HI + *Eco*RI-cut pBR322 DNA.

YRp14-Sc2850 DNA was constructed by ligating the vector containing *Bam*HI-*Sal*I fragment of YRp14-Sc2812, the *Bam*HI-*Eco*RI fragment Sc2806, and the 650-bp *Eco*RI-*Sal*I fragment of pBR322 that contains the 5′ end of the tetracycline-resistance gene (Bolivar et al., 1977). Transformants were selected by virtue of resistance to both ampicillin and tetracycline (the latter gene being reformed via ligation at the *Sal*I site).

YRp14-Sc2853 was constructed by ligating the vector containing *Hin*dIII-*Sal*I fragment of YRp14-Sc2850 with the *Hin*dIII-*Sal*I fragment of Sc2605 DNA that contains the 3′ end of the *HIS3* gene.

YRp14-Sc3225 was constructed by ligating the vector containing *Hin*dIII-*Xho*I segment of YRp14-Sc2853 DNA with the 5.0-kb *CYH2*-containing *Bam*HI-*Hin*dIII fragment of pBR322-*CYH2* (obtained from Patricia Brown and Jack Szostak via Howard Fried; Fried and Warner, 1982) and the 450-bp *Bgl*II-*Xho*I fragment that includes the 3′ end of the *HIS3* gene (Struhl and Davis, 1980).

YRp14-Sc3100 was constructed analogously to YRp14-Sc3225 except that a *CAN1* DNA fragment was used instead of the *CYH2*-containing segment.

The *CAN1* fragment was produced by cleaving TLC-1 DNA (Fig. 2; Broach et al., 1979) to completion with *Bam*HI and partially with *Hin*dIII. The segment of DNA that includes the entire *CAN1* region and 275-bp of pBR322 was purified.

YIp5-Sc3113, YCp50-Sc2884, and YCp50-Sc2888 have been described elsewhere (Struhl, 1982a,b).

(c) Hybridization analysis

Yeast DNA was prepared from 5-ml YPD cultures as described previously (Struhl et al., 1979). DNAs were cleaved to completion with *Eco*RI and electrophoretically separated in a 0.7% agarose gel, or with *Hae*III and separated in a 2.0% gel and then transferred to a nitrocellulose filter. The DNA was challenged for hybridization by ^{32}P-labeled pGT2-Sc2605 DNA (for *Eco*RI cleaved DNAs) or with pBR322-Sc2676 DNA (for *Hae*III-cleaved DNAs) prepared by nick translation (Struhl and Davis, 1980).

RESULTS

(a) Rationale

The gene replacement method to be described here is based on the properties of the ribosomal gene, *CYH2* (Fig. 1). Mutant alleles of this gene (*cyh2*^{r}) confer resistance to 40 μM cycloheximide. The key fact is that cycloheximide resistance is a recessive

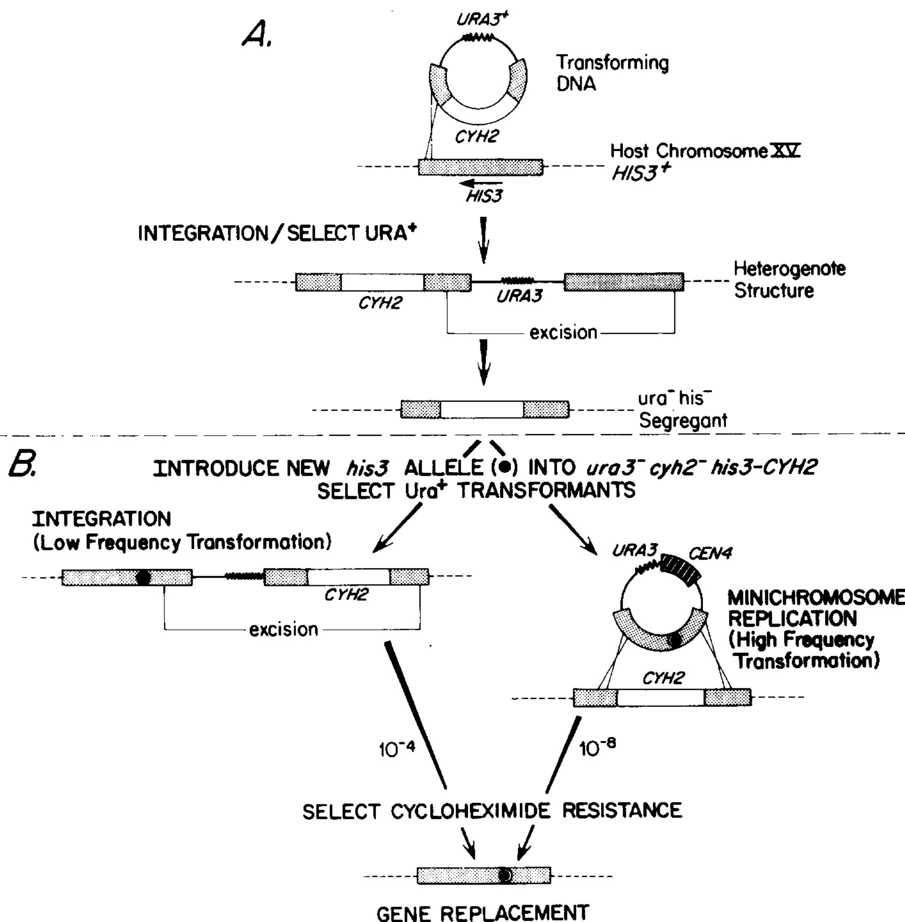

Fig. 1. Scheme for gene replacement. Panel A represents the replacement of wild-type *HIS3* sequences by *his3-CYH2*. DNA segments are represented as follows: *his3* (shaded bar), *CYH2* (open box), pBR322 (solid line), *URA3 SUP11*° (wavy line), chromosome XV (dashed lines). Only the events at chromosome XV are shown. Panel B represents the replacement of *his3-CYH2* by a cloned *his3* allele of interest (designated by a solid circle). Recombinational crossovers are indicated as X. See RESULTS, section **a–d**, for details.

trait; i.e., strains containing both the wild-type *CYH2* and the resistant *cyh2*ʳ alleles are sensitive to the drug. The general approach is to create a *CYH2*/*cyh2*ʳ merodiploid by inserting the wild-type *CYH2* gene into the middle of another locus. Gene replacement at this locus removes *CYH2*, thus uncovering the trait of cycloheximide resistance conferred by *cyh2*ʳ.

(b) In vitro construction of a *his3-CYH2* substitution allele

The wild-type *CYH2* gene was inserted into the middle of *HIS3* DNA by a series of steps whose seemingly unneccessary complexity reflects the availability of cloned DNA fragments that were isolated for other reasons (Fig. 2). The starting DNA

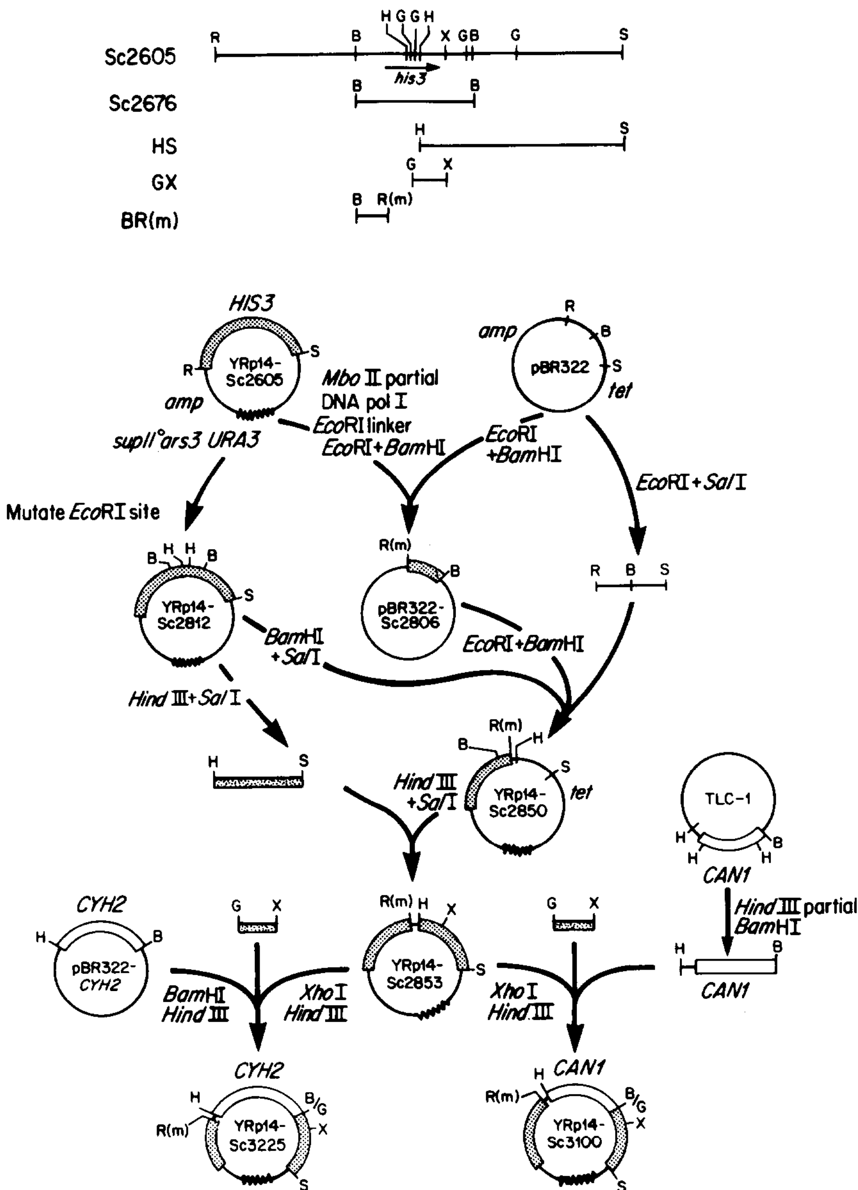

Fig. 2. Construction of hybrid DNA molecules. The top line shows the 6.1-kb *Eco*RI-*Sal*I *HIS3* DNA fragment (Sc2605). Restriction sites are indicated as R (*Eco*RI), B (*Bam*HI), H (*Hin*dIII), G (*Bgl*II), X (*Xho*I), S (*Sal*I), and the location of the *HIS3* transcript is designated by a horizontal arrow. Purified sub-fragments of Sc2605 are shown below; R(m) indicates an *Eco*RI site that was created by ligating an octanucleotide linker to the *Mbo*II site located 32 bp upstream from the site of transcription initiation (Struhl, 1982a). DNA segments are represented as described in Fig. 1. The procedural details for these constructions are described in MATERIALS AND METHODS, section **b**.

molecule, YRp14-Sc2605, contains a 6.1-kb *Eco*RI + *Sal*I-generated segment of *HIS3* DNA (Struhl, 1979; 1982a). The *HIS3* structural gene (0.7 kb in length) is centrally located within Sc2605. The YRp14 vector contains *URA3* and *SUP11°* marker genes in addition to *ARS3*, a sequence that permits autonomous replication. By various manipulations, Sc2605 was converted into Sc2853, a derivative that deletes the entire 5′ end of the *HIS3* structural gene as well as 32 non-transcribed base pairs. The *his3* deletion end points in YRp14-Sc2853 occur at unique *Eco*RI and *Hin*dIII restriction sites. The wild-type *CYH2* gene (obtained as a 5.0-kb *Bam*HI-*Hin*dIII generated DNA segment; Fried and Warner, 1982) was then inserted into the molecule. The final product, YRp14-Sc3225, substitutes the *CYH2* fragment and 29 bp of pBR322 DNA for approx. 490 bp of *HIS3* DNA. As expected, this molecule is His$^-$; it fails to complement an *E. coli* auxotroph lacking IGP dehydratase.

(c) Replacement of genomic *HIS3* sequences by *his3-CYH2*

Transformation of haploid strains with vectors containing efficient suppressor tRNA genes frequently produces homozygous (**a**/**a** or **α**/**α**) diploid colonies (Struhl, 1979; St. John et al., 1981). This is probably due to the lethality caused by multiple copies of suppressor tRNA genes per haploid genome (Cox, 1971). Accordingly, I followed the suggestion of Mark Johnston and introduced

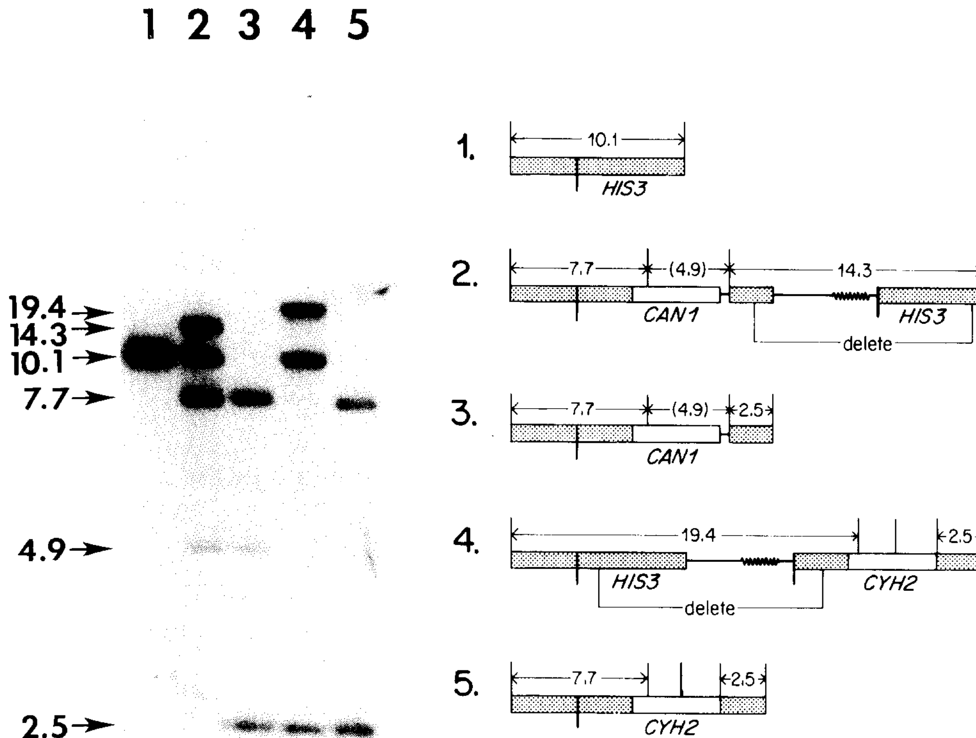

Fig. 3. Hybridization analysis of gene replacement. Lanes 1–5 in the autoradiogram (left panel) represent *Eco*RI-cleaved DNA from the following strains: 1. YM5; 2. KY172; 3. KY173; 4. KY170; 5. KY171. The hybridization probe detects sequences homologous to *HIS3* and to pBR322. The diagrams to the right of the autoradiogram schematicize the results; each line corresponds to the analogous lane in the autoradiogram and only the *HIS3* region is illustrated. The origins of DNA segments are shown as described in Fig. 1. The *Eco*RI fragment in wild-type yeast (YM5) is 10.1 kb in length (Struhl et al., 1976) (line 1). The *Sal*I site that marks the end of the cloned fragment (Sc2605) is indicated by a thick vertical line that continues beneath the DNA segments. *Eco*RI sites in the *his3* region are indicated by vertical lines continuing above the DNA segments. The lengths of fragments due to cleavage with *Eco*RI are indicated only for those fragments that hybridize to the probe. The parentheses surrounding the 4.9-kb fragment indicate faint hybridization that results from the short region of pBR322 sequence.

YRp14-Sc3225 DNA into the heterozygous (a/α) diploid strain YM5 by selecting for Ura$^+$ transformants. Because the YRp14 vector contains *ARS3*, transformants were obtained at a relatively high frequency; as expected, however, these were highly unstable for the Ura$^+$ character that was conferred by the autonomously replicating molecule (Struhl, 1979). A mitotically stable Ura$^+$ colony (KY170) was selected from this population following cycles of growth on selective and non-selective medium (Stinchcomb et al., 1979). Hybridization analysis (Fig. 3) indicated that YRp14-Sc3225 had integrated at the *his3* locus. KY170 was sporulated and then dissected in order to obtain a Ura$^+$ haploid that would be suitable for the anti-suppression selection described previously (Struhl, 1979). However, 3 of 74 haploid segregants of KY170 were Ura$^-$ His$^-$. Because the only *his*$^-$ allele present in KY170 is *his3-CYH2*, it seemed likely that these segregants represented the replacement of *HIS3* sequences by *his3-CYH2*. This was confirmed by the genomic hybridization experiment in Fig. 3. The frequency of this event (4%) seems high when compared to that reported elsewhere (Sherer and Davis, 1979; Struhl et al., 1979). However, this is not surprising because recombination during meiosis occurs at approx. 100-fold higher frequency than during mitosis.

To use the strain harboring *his3-CYH2* (KY171) for further gene replacement events, it was necessary to introduce a *cyh2*^{r} mutation at its normal location on chromosome VII. Thus, it was crossed to D636-3A and appropriate segregants were identified following dissection of tetrads. *CYH2/cyh2*^{r} merodiploids do not grow in 40 μM cycloheximide (unlike *cyh2*^{r} strains) but they do grow when the drug concentration is 5 μM (unlike *CYH2* strains).

(d) Gene replacement at the *his3* locus using *his3-CYH2*

Three experiments are described below each using cycloheximide resistance as a selection for gene replacement. The differences among them involve the state of the transforming DNA at the time of selection.

(1) Heterogenote structures

In this first experiment, the *his3* allele was integrated into the genome at the *his3* locus prior to attempting gene replacement. This corresponds to the standard replacement procedure depicted in Fig. 1A. YIp5-Sc3113 DNA was introduced into KY174 by selecting for Ura$^+$ transformants. YIp5 is the original yeast vector used for gene replacement (Scherer and Davis, 1979) and Sc3113 contains his3-Δ25, a 31-bp deletion of Sc2605 that confers a His$^+$ phenotype but is defective in regulating gene expression in response to amino acid starvation (Struhl, 1982b). Two uracil-independent colonies containing YIp5-Sc3113 at the *his3* locus were obtained and characterized (not shown). Following overnight growth in non-selective medium (5 generations), cycloheximide-resistant colonies appeared at

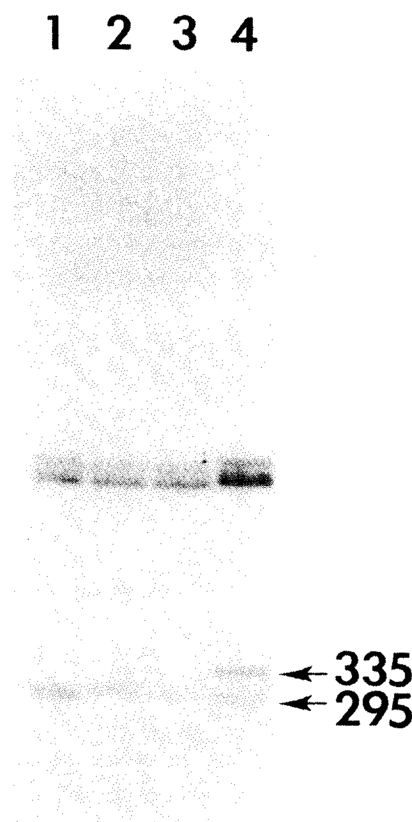

Fig. 4. Hybridization analysis of gene replacement. Each lane represents *Hae*III-cleaved genomic DNAs; lane 1 (KY180), lane 2 (KY170), lane 3(KY181), lane 4(YM5). The hybridization probe was the *HIS3*-containing 1.7-kb *Bam*HI fragment (Sc2676) (Struhl and Davis, 1980). The relevant 335-bp DNA fragment includes the entire promoter/regulatory region and the 5′ most 165 bp of the mRNA. The 1.4-kb fragment includes the remainder of the *HIS3* structural gene, and the 295-bp fragment maps upstream of the gene. See MATERIALS AND METHODS, section c.

a frequency of 10^{-4}. Out of 20 such colonies 17 remained Ura$^+$; the other were Ura$^-$. The Ura$^-$ colonies (for example KY179) arise from excision of the transforming DNA such that only *his3-Δ25* remains in the strain (Fig. 4). The Ura$^+$ class presumably represents gene conversion of *his3-CYH2* by *his3-Δ25*; these should contain two copies of *his3-Δ25*, in addition to the entire YIp5 vector.

(2) Replicating molecules

Here, replacement of *his3-CYH2* was performed without the intermediate step involving chromosomal integration of the transforming DNA. Stable integration was avoided by introducing cloned *his3* alleles on autonomously replicating minichromosomes. Because these hybrid molecules contain a functional centromere (*CEN4*), genomic integration would produce a highly unstable dicentric chromosome (Mann and Davis, 1983). In this situation, gene replacement could arise from two independent, reciprocal, recombination events, or from gene conversion. Either possibility involves recombination between non-homologous chromosomes, an event that occurs in yeast albeit at low frequency (Scherer and Davis, 1980).

Two *his3* alleles were cloned into YCp50, a vector containing *URA3* as a marker gene, *ARS1* for autonomous replication, and *CEN4* for proper chromosome segregation (S. Scherer, personal communication). The *his3-Δ41* DNA (deleted for nucleotides −106 to −8) lacks the TATA box promoter element and hence confers a His$^-$ phenotype, while *his3-Δ28* (deleted between −106 and −53) retains the TATA box and is His$^+$ (Struhl, 1982a). The YCp50 hybrid DNAs were introduced into KY174 by selecting for Ura$^+$ transformants; as expected these were very stable for the vector marker. The transformants (KY177 and KY178) were grown in non-selective medium for 10 generations and then plated on medium containing cycloheximide. After 3–4 days, resistant colonies were observed at a frequency of 10^{-8}. In similar experiments with other YCp50-*his3* hybrids, an occasional culture produced resistant colonies at considerably higher frequency (between 10^{-6} and 10^{-7}). Probably, the event leading to cycloheximide resistance occurred at an early stage of the culture (Luria and Delbruck, 1943). After 7–14 days, more resistant colonies appeared; however, upon restreaking, they grew slowly in the presence of 40 μM cycloheximide and hence were discarded. These colonies may contain modifiers (possibly extra copies of *cyh2*^{r}) that allow the partially resistant *CYH2*/*cyh2*^{r} merodiploids to survive higher concentrations of cycloheximide.

Since *CEN4* molecules are relatively stable during mitotic growth, the fast growing cycloheximide resistant colonies were almost always Ura$^+$. To more easily analyze these colonies, Ura$^-$ segregants that have lost the autonomously replicating molecules were identified by replica plating. In principle, cycloheximide resistance could occur by gene replacement of *his3-CYH2*, by mutation of *CYH2*, or by gene conversion of *CYH2* by *cyh2*^{r}. Gene replacement can be distinguished from the other possibilities by genetic means. For Ura$^-$ segregants of KY177, those resulting from replacement (e.g., KY180) are His$^+$ in contrast to those due to the other mechanisms. For KY178, the desired Ura$^-$ colonies (such as KY181), while being His$^-$, can recombine with *his3-Δ1* (Scherer and Davis, 1979) to produce His$^+$ colonies; *his3-Δ1* and *his3-CYH2* cannot recombine in such a manner. The results from a number of experiments indicate that 30–70% of the cycloheximide-resistant colonies arise from replacement of *his3-CYH2* by the *his3* allele introduced on the transforming DNA molecule.

(3) Direct selection during the transformation procedure

During the transformation procedure, the apparent frequency of chromosomal integration is relatively high, a result that may reflect the ability of individual yeast cells to take up many DNA molecules (Hicks et al., 1979; Stinchcomb et al., 1979). Thus, it seemed likely that direct selection of cycloheximide-resistant transformants would not only save time, but would increase the relative frequency of replacement as compared to mutation or gene conversion of *CYH2* (see previous section).

Initial attempts to select directly for cycloheximide-resistant colonies were unsuccessful because the recipient cells were growth-inhibited before the desired recombination event occurred. To circumvent this, the mixture of competent cells and YCp50-Sc3125 DNA was plated on uracil selective medium and incubated for 48 h. Cycloheximide was then spread on the plate to a final concentration of 40 μM. Colonies appeared 3–6 days later at a frequency of roughly one per μg of transforming DNA.

Unfortunately, all but one of these colonies grew slowly when restreaked in the presence of cycloheximide. The exceptional colony, when cured of the autonomously replicating molecule, was His$^+$, which strongly suggested that gene replacement occurred. This was confirmed by hybridization analysis (Fig. 4).

(e) Construction of strains containing a *his3-CAN1* substitution allele

CAN1 encodes arginine permease. Mutations *can1*^{r} cause resistance to canavanine, but like *cyh2*r mutations, are recessive to the wild-type, drug-sensitive allele. Therefore, the gene replacement scheme described in this paper can be applied with canavanine resistance.

The *CAN1* gene was purified as a 5.3-kb *Bam*HI-*Hin*dIII DNA segment of TLC1 (see MATERIALS AND METHODS, section **b**). This segment was inserted into the middle of the *HIS3* gene in exactly the same manner as performed for the 5.0-kb *Bam*HI-*Hin*dIII *CYH2* fragment. The resulting plasmid, YRp14-Sc3100, being analogous to the *his3-CYH2* plasmid YRp14-Sc3225, was used to replace the wild-type *HIS3* allele of YM5 with *his3-CAN1* as described before; analysis of strains KY172 and KY173 confirms that both events occurred as expected (Fig. 3). The replaced *his3-CAN1* allele was then crossed into a *can1*^{r} background. The resulting merodiploid (KY175) was sensitive to canavanine, although resistant colonies were obtained at a frequency of 10^{-7}.

DISCUSSION

Gene replacement as described here has several advantages over other schemes that are used currently. Principally, the desired recombination event can be identified by a direct genetic selection without regard to the phenotype produced (appropriate diploids can be used for haplo-lethal phenotypes). Three protocols have been employed, each with their own advantages. In practice, the choice among them is made on the basis of available vectors and temperament.

The chief virtue of the pre-integration protocol is that cycloheximide-resistant colonies occur relatively frequently. This is due to the fact that the two necessary crossovers are selected separately and to the proximity of the recombining alleles in the heterogenote structure on chromosome XV. However, this method has some of the same problems encountered in standard procedures. As mentioned in the introduction, the initial integration step occurs at low frequency and sometimes produces undesirable structures. Moreover, many cycloheximide-resistant colonies arise from gene conversion instead of replacement, although these are easily screened out in a subsequent step.

The inconveniences of the low-frequency integration step are avoided by using replicating vectors. This consideration becomes important when a large number of mutated derivatives are being analyzed or when the phenotypes on replicating molecules are of interest. The disadvantage of this method is that replacement must occur by gene conversion between sequences on nonhomologous chromosomes, a rare event. Presumably, the recombination frequency can be increased by low doses of X-rays or by the nonmutagenic method of thymidineless death (Kurz et al., 1980).

The direct selection method during transformation is quick, but unfortunately very inefficient. Thus, this approach offers a straight win/lose proposition, which is generally unsuccessful unless extremely high transformation efficiencies are obtained.

Recently, Rothstein (1983) has also described direct selection for gene replacement. In this method, a selectable marker gene is inserted into a region of cloned DNA. The resulting molecule is cleaved with appropriate restriction enzymes to produce recombinogenic ends (Orr-Weaver et al., 1981) on both sides of the insertion. The transformants (selected via the marker gene) arise from the repair of two double-stranded breaks. The result is that a chromosomal sequence is replaced by a derivative produced in vitro. When compared to the method described here, the technique developed by Rothstein (1983) is easier and more rapid. Its disadvantage is that the final strains still contain the marker gene at the locus of interest. Thus, it is particularly useful for disrupting genes, but less suitable for phenotypic studies involving local mutations of a given gene or genetic element.

This paper describes genetic selections that are based on the recessive nature of canavanine and

cycloheximide-resistant alleles. Nevertheless, the mechanisms that lead to drug resistance differ in these two cases, and these differences are relevant to gene replacement strategies. For instance, resistance to canavanine (due to loss of arginine permease activity) occurs spontaneously at much higher frequency than cycloheximide resistance (which requires a specific change in a ribosomal protein). Thus, experimental protocols involving autonomously replicating DNA molecules are inadvisable for canavanine selections because mutations of the *his3-CAN1* allele occur much more frequently than the desired gene replacement events.

Another issue to consider is that the replacement method generates strains that are all drug resistant. For the experiments described here, analysis of *his3* phenotypes would be performed in strains with altered ribosomes caused by the *cyh2*^{r} mutation.

A final point concerns the in vitro construction of the substitution allele. Many experiments are designed to examine the phenotypes of mutations in a small defined region of DNA (the promoter regulatory sequences at the 5′ end of a gene for instance). It is useful if this region is deleted in the substitution allele. In this way, the formation of wild-type recombinants between the substitution allele and the transforming allele is precluded.

In summary, I have developed a gene replacement scheme that alleviates many of the problems and much of the tedium that is usually encountered. Although the methodology is applied here exclusively to the *his3* locus, it should be possible to adapt the basic principle to essentially any other region of the genome.

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